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Suppression of NLRP3 inflammasome pathway improves acute lung injury

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ABSTRACT

NLRP3 inflammasome is a pathway stimulated by a different cellular insults to activate innate immune defenses via the activation of Caspase-1 and several of pro-inflammatory cytokines, such as IL-1β and IL-18. NLRP3 expression is increased in numerous human inflammatory diseases, including pulmonary diseases. Pleural injection of carrageenan caused an acute inflammatory response, characterized by tissue damage, leukocyte infiltration, inflammatory exudates and increased myeloperoxidase activity. The aim of this study was to investigate the effect of the administration of two different inflammasome blocking agents BAY 11-7082 (30 mg/kg, i.p.) and Brilliant Blue G (BBG) (45.5 mg/kg, i.p.) in a mouse model of carrageenan-induced pleurisy. BAY 11-7082 or BBG administration 1 h after carrageenan injection reduced pulmonary membrane thickening and polymorphonuclear leukocyte infiltration, decreased NF-kB translocation in the nucleus the assembly of the NLRP3 inflammasome complex. Moreover ,they down-regulated iNOS, nitrotyrosine, and poly-ADP-ribosyl polymerase expression and repressed carrageenan-induced apoptosis. The results demonstrate that inflammasome-blocking agents administration can reduce the development of acute carrageenan-induced lung injury

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ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
BBG	Brilliant Blue G
CAR	Carrageenan
CARD	Caspase activation and recruitment domain
DAMP	Danger Associated Molecular Patterns
FIIND	Function-to-find domain
IL-	Interleukin-
LRRs	Leucine-rich repeat
iNOS	Inducible nitric oxide synthase
MPO	Myeloperoxidase
NACHT	Nucleotide-binding oligomerization
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	NF-E2–related factor 2
NLR	Nucleotide-binding domain leucine rich repeat containing receptor (NLR)
P2X7	Purinergic receptor
PAMP	Pathogen Associated Molecular Patters

PKC	Protein kinase C
PYD	Pyrin
PLC	Phospholipase C
PMN	Polymorphonuclear leukocyte
PRR	Pattern Recognition Receptors
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF	Tumor necrosis factor

1. INTRODUCTION

1.1 Lung Diseases

Lung diseases are responsible for one-sixth of deaths worldwide and represent one of the world's major health problems. The incidence of lung diseases has remained unchanged since the beginning of the century and probably the situation will not change for several decades (Talley *et al.*, 2004). They cause disability and premature death, resulting in a significant cost related to treatment, hospital care and loss of productivity of patients who are unable to work (Fragoso, 2017; McCallum *et al.*, 2017). Infections, genetics and smoking are responsible for most of this diseases (Schwartz, 2012). The lungs are part of a composite apparatus, expanding and relaxing each day to bring in oxygen and eject carbon dioxide. Problems in any part of this system cause lung diseases. They can affect: airways, alveoli, interstitium, blood vessels, chest wall or pleura. The trachea branches into two tubes called bronchi, which in turn divide to become progressively smaller throughout the lungs. Diseases affecting the airways are: asthma, chronic obstructive pulmonary diseases, chronic bronchitis, emphysema, acute bronchitis and cystic fibrosis (Willen *et al.*, 2018). The airways divide into tiny tubes, called bronchioles, which dead-end into air sacs (alveoli). Alveoli constitute the most of the lung tissue. Diseases that affect the alveoli are: pneumonia, tuberculosis, pulmonary edema, lung cancer, acute respiratory distress syndrome and pneumoconiosis (Adjemian *et al.*, 2017). The interstitium is the thin lining between the alveoli. Blood vessels go through the interstitium and let gas exchange between the blood and alveoli. Lung diseases affecting the interstitium include: interstitial lung disease, pneumonias and pulmonary edemas (Cheng *et al.*, 2018). The right side of the heart gets blood from the veins and pumps it into pulmonary arteries that takes to it the lungs. Diseases that affects this blood vessels are pulmonary embolism and pulmonary hypertention (Klinger, 2016). The chest wall also has a key role in breathing. Muscles take

the ribs together and help the chest to expand. Obesity hypoventilation syndrome and neuromuscular disorders are classified as lung diseases that can affect the chest wall (Robinson, 2016). The pleura is a lining that surrounds the lung. Diseases that affect the pleura are: mesothelioma, pneumothorax and pleurisy (Boutin *et al.*, 1999).

1.2 Pleurisy

Pleurisy is a disabling and severe disease characterized by a progressive decline in lung function. It affects men more often than women. Pleurisy is on the rise worldwide: every year there are 30-35,000 new diagnoses in Europe, while in the United States the disease affects about 200 thousand people. Pleurisy (PLOOR-ih-see) is an acute or chronic inflammation of the pleura. It is an anatomical structure that consisting of two very thin layers of tissue that cover the outer surface of the lungs (visceral pleura) and which continue without interruption to cover the inner surface of the thoracic cavity (parietal pleura). In normal condition, this two layers of tissue are very close and slip on each other during normal breathing (English *et al.*, 2006). They are wet by a very small amount of liquid (pleural fluid) that acts as a lubricant to allow the movement of the lungs into the thoracic cavity. Pleurisy occurs when the pleura is inflamed and irritated. In this case, the two layers of the membrane rub against each other as two pieces of sandpaper, causing pain when you exhale and inhale (English *et al.*, 2006). Pleurisy is also characterized by a fluid build-up, called pleural effusion, that increases the space between this two layer putting pressure on the lungs and produces them to stop working properly. At the beginning, this fluid causes sharp chest pain (pleuritic pain), but after a while it may act like a cushion, leading to chest pain disappear. However, as soon as the fluid increases a patient with a pleural effusion will experience shortness of breath. Eventually also an

infection in the fluid can occur causing chills, fever and a dry cough. It is called empyema. Several underlying conditions can induce pleurisy:

- Bacterial infection (pneumonia);
- Viral infection (influenza);
- Fungal infection;
- Autoimmune disorders (rheumatoid arthritis);
- Medications;
- Rib fracture;
- Lung cancer close to the pleural surface.

Treatment of pleurisy includes treating the underlying condition and pain control. Instillation of carrageenan (CAR) into the pleural cavity caused local inflammation, lung injury, and recruitment of polymorphonuclear leukocytes (PMNs) (Oliveira *et al.*, 2010). This experimental model has been usually employed to analyse the pathophysiology of acute lung inflammation and to investigate the mediators involved in cellular injury. The acute response stimulated by CAR is characterized by production of pleural exudate; accumulation of inflammatory mediators, such as cytokines and chemokines, proteases and complement activation products; and high expression of reactive oxygen species (ROS) (Ward, 2010). Several evidences suggest that one of the main intracellular pathway elicited during this disease is the NLRP3 inflammasome. Expression of inflammasome proteins can be detected in a lot of immune and non-immune cell types such as T cells (Lalor *et al.*, 2011), monocytes/macrophages (Shalhoub *et al.*, 2011), myofibroblast/fibroblast (Artlett *et al.*, 2011), epithelial cells (Feldmeyer *et al.*, 2010; Mortaz *et al.*, 2011), keratinocytes (Feldmeyer *et al.*, 2010; Dai *et al.*, 2011) and hepatic

stellate cells (Watanabe *et al.*, 2009) underlying the importance of this pathway in the immune response.

1.3 Inflammasomes

Inflammasomes are the main inflammatory pathway of the innate immune system. They are pattern recognition receptors able to recognize a wide range of conserved molecular motifs unique to microbes. Additionally, they can identify chemical alarm signals created by activated cells that are involved in tissue damage and host infection (Martinon *et al.*, 2005). They are constituted by multiprotein oligomers that answer to inflammatory stimuli by starting an intracellular inflammatory cascade (Schroder *et al.*, 2010). At the end of this cascade there is the activation and up-regulation of the innate immune system. We can distinguish two different kind of inflammasomes: canonical inflammasomes and non-canonical inflammasomes. The first one converts pro-Caspase-1 into the catalytically active enzyme, while the second promotes the activation of pro-Caspase-11. Both Caspase-1 and 11 induce pyroptosis but only Caspase-1 induces the secretion of the interleukins IL-1β and IL-18 (Martinon *et al.*, 2005) (Figure 1).

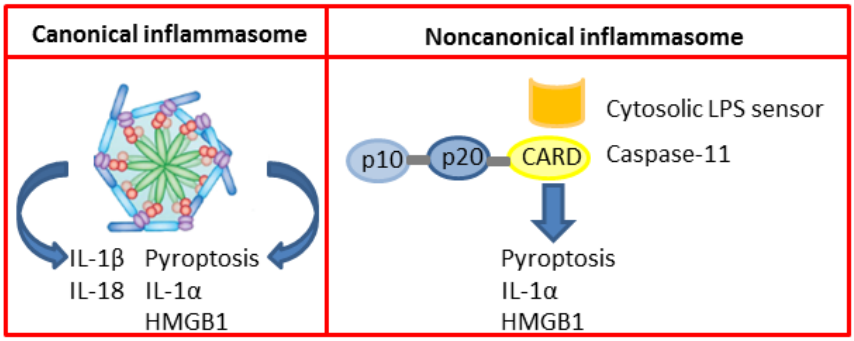

Figure 1. Mechanisms of the Inflammasomes

Canonical inflammasome complexes induces autoactivation of Caspase-1. It subsequently converts proIL-1β and proIL-18 into the bioactive cytokines and triggers pyroptosis. Non-

canonical inflammasome can be activated in by the detection of cyotosolic LPS triggers activation of Caspase-11 in macrophages infected with Citrobacter rodentium, Escherichia coli and other Gram-negative bacteria the cytosol. Caspase-11 induces pyroptosis and release of IL-1α and HMGB1 directly and mediates secretion of IL-1β and IL-18 indirectly through canonical Nlrp3 inflammasome.

There are several classes of pattern recognition receptors, e.g. leucine rich repeats, toll like receptors, NOD-like receptors (NLRs), and all of them are evolutionary conserved. It has been recently shown that leucine rich repeats included within NLRs are a new class of pattern recognition receptor that are responsible for the three dimensional curved structures of the proteins (Bella *et al.*, 2008; Hindle *et al.*, 2009). These structures can bind specific ligands present on pathogens (Bell *et al.*, 2005). NLRs are constituted by an N terminal effector domain, a central NACHT or NOD (a domain showed on CIITA, NAIP, TP-1 and HET-E proteins) and a C terminal leucine rich repeat domain (Inohara *et al.*, 2001). There have been identified twenty-three NLRs holding NOD and leucine rich repeat domain, all classified by their N terminal region; NAIPs contain a BIR protein; NLRP 1-14 contain pyrin and NLRC 1-5 contain a caspase recruitment domain (CARD) protein (Kanneganti *et al.*, 2007). The NLR receptor is the sensor of the inflammasome, it detects the alarm signal. The mechanism by which it happens still need to be clarified, but once triggered, the sensor starts the formation of the inflammasome complex. This sensor enables the recruitment of Caspase-1 either directly, through a CARD, or indirectly, through a PYRIN domain, which can link the PYRIN-CARD containing adaptor ASC. The resulting interaction of these proteins in turn leads to the cleavage and activation of Caspase-1. Additionally, recent studies have shown other families of genes that can assembly the inflammasomes: the RIG-I-like receptor (RLR) family (Ireton *et al.*, 2011) and the AIM2-like receptor (ALR) family, which has a HIN200 DNA binding domain instead of an LRR

(Schattgen *et al.*, 2011). Among all this different inflammasomes families the most characterized are NLRP1, NLRP6, NLRP12, NLRC4, AIM2 and NLRP3 (Figure 2).

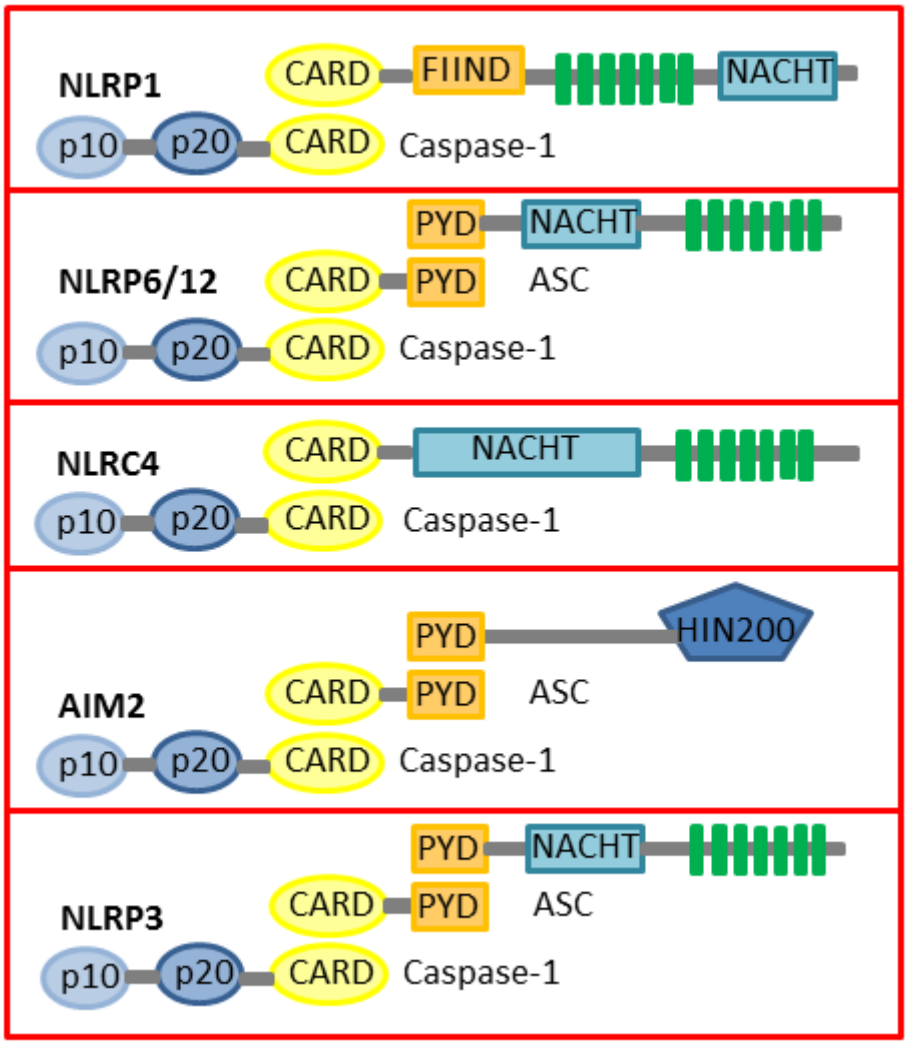

Figure 2. Composition of NLRP1, NLRP6, NLRP12, NLRC4, AIM2 and NLRP3

NLRs (Nod-like receptor) are composed by CARD (caspase recruitment domain) or PYD (pyrin) motif, NACHT (nucleotide binding and oligomerization domain) and a number of LRRs (leucine-rich repeat). NLRP1 lacks the amino-terminal PYD motif and is autocatalytically cleaved FIIND (domain with function to find) domain. NLRP1 and NLRC4 recruit Caspase-1 through their CARD motifs. AIM2 is characterized by an amino-terminal PYD and a carboxy-terminal DNA-binding HIN200 domain. The PYD-CARD adaptor protein ASC stabilizes is required for assembly of the AIM2 and NLRP3 inflammasomes.

The first inflammasome discovered was the NLRP1 (Martinon *et al.*, 2002). It has two natural ligands: the Bacillus anthracis lethal toxin and the muramyl dipeptide (MDP), a peptidoglycan fragment from Gram positive and negative bacteria (Boyden *et al.*, 2006; Faustin *et al.*, 2007). They can only activate human NLRP1(Faustin *et al.*, 2007; Kovarova *et al.*, 2012), but not mouse because it displays species-specific differences at the genetic level. Mouse NLRP1 can be activated by anthrax toxin. Humans have a single NLRP1 gene with both a CARD and a PYRIN domain, while mice possess three homologous genes Nlrp1 a, b and c containing CARD domain.

NLRP6 is extremely expressed in goblet and epithelial cells in the intestine (Elinav *et al.*, 2011; Wlodarska *et al.*, 2014), and in hematopoietic cells (Chen *et al.*, 2011). The most important role of this inflammasome pathway is maintaining the intestinal homeostasis (Chen *et al.*, 2011; Elinav *et al.*, 2011; Normand *et al.*, 2011; Hu *et al.*, 2013).

NLRP6 has several characteristics that resemble NLRP12. As NLRP6, NLRP12 has a key role in protecting against AOM/DSS-induced colon cancer and DSS-induced colitis (Zaki *et al.*, 2011; Allen *et al.*, 2012). Moreover, it maintains intestinal homeostasis by negatively modulating several inflammatory signalling pathways such as MAPK and NF-kB (Zaki *et al.*, 2011; Allen *et al.*, 2012).

NLRC4 can be activated by many different bacterial pathogens such as Pseudomonas aeruginosa, Legionella pneumophila, Shigella flexneri and Salmonella typhimurium (Mariathasan *et al.*, 2004; Amer *et al.*, 2006; Franchi *et al.*, 2006; Miao *et al.*, 2006; Lamkanfi *et al.*, 2007; Sutterwala *et al.*, 2007; Suzuki *et al.*, 2007; Miao *et al.*, 2008). NLRC4 interacts with bacterial flagellin or structural components of the bacterial type III secretion system, that are leaked or injected into the host cell (Miao *et al.*, 2010). These bacterial proteins directly bind by NLR-family apoptosis-inhibiting proteins (NAIPs) in the

cytosol. NAIPs in turn interact with NLRC4 and starts the assembly of the NLRC4 inflammasome complex, leading to the activation of Caspase-1, release of IL-1β, IL-18 and pyroptosis.

AIM2 is a member of the PYHIN family, proteins containing a PYRIN domain and the conserved DNA-binding domain hematopoietic IFN-inducible nuclear protein (Schattgen *et al.*, 2011). Theoretically, these proteins can bind nucleic acids and recruit ASC to start the formation of an inflammasome. AIM2 can assembly an inflammasome by recognition of cytosolic DNA of viral or bacterial origin (Fernandes-Alnemri *et al.*, 2010; Jones *et al.*, 2010; Rathinam *et al.*, 2010; Sauer *et al.*, 2010), or self-DNA from apoptotic cells (Choubey, 2012; Zhang *et al.*, 2013).

The most important and studied cytosolic inflammasome is the NLRP3 inflammasome. It contains the NOD-like receptor NLRP3, the adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro-Caspase-1, who cleaves into its active form. Caspase-1 in turn activates IL-18 and IL-1β, which contributes to the inflammation.

1.4 The NLRP3 inflammasome

Activation and prime of the NLRP3 inflammasome pathway needs different exogenous (bacterial hemolysins, pneumolysin, etc.) and endogenous (ATP, uric acid crystals, etc.) stimuli (Franchi *et al.*, 2012). The precise mechanism still needs to be clarified, but new evidences indicate that a 2-step mechanism is required to activate NLRP3 (Juliana *et al.*, 2010) (Figure 3).

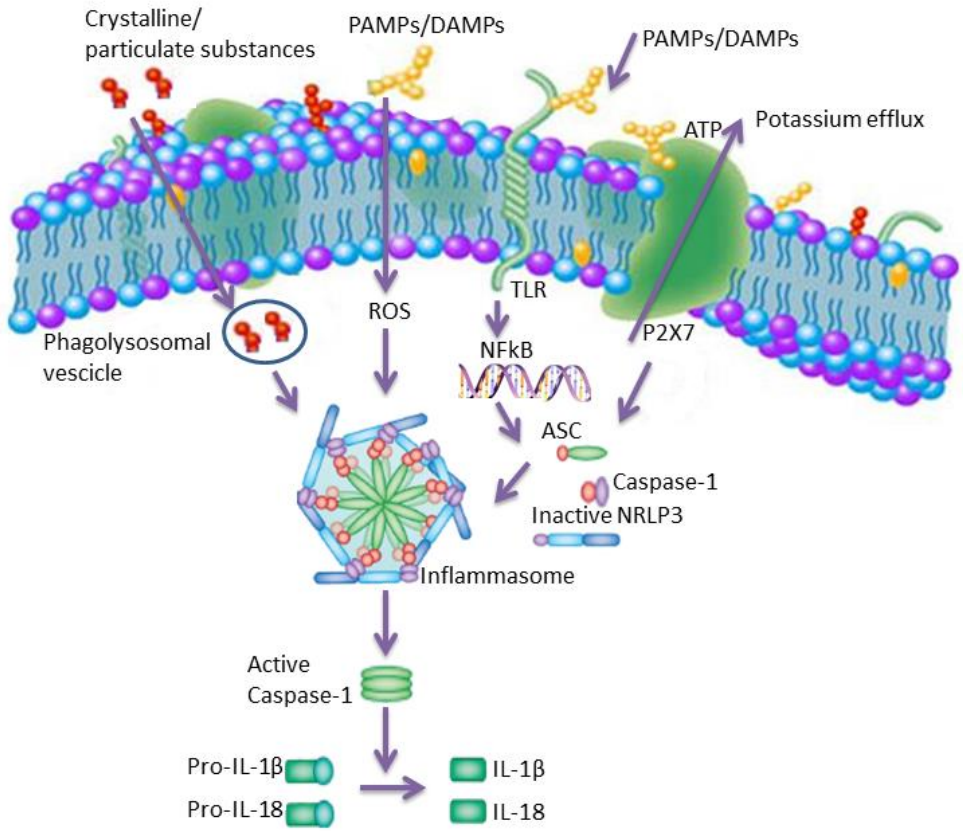

Figure 3. NLRP3 inflammasome activation

Upon exposure to DAMPs or PAMPs, TRLs are phosphorylated and activate NF-kB. NF-kB promotes the priming event. Extracellular ATP can induce K^+/potassium efflux through a purinergic P2X7-dependent pore. These stimuli activate the NLRP3 inflammasome by facilitating the oligomerization of inactive NLRP3, ASC and pro-Caspase-1. The formation of this complex led to the activation of Caspase-1, which contributes to the production of active IL-1β and IL-18.

At the beginning, NLRP3 transcription is activated by NF-kB–inducing factors (Bauernfeind *et al.*, 2009). Protein expression is not enough for activation of the full pathway: another transcription-independent step is required. It can be triggered by stimuli such as ATP, phagocytosis of monosodium urate crystals, or pore-forming toxins. The induction of this pathway leads to mitochondrial and lysosome damage, production of ROS and dysregulated ionic balance (K+ efflux, elevated intracellular Ca2+). Multiple publications indicates that the purinergic receptor takes part in lung inflammatory events

(Dostert *et al.*, 2008). In particular, the purinergic receptor P2X7 participates to the release of IL-1β and has been proposed to be upstream of the NLRP3 inflammasome oligomerization (Ferrari *et al.*, 2006). It is an extracellular ATP-gated plasma membrane ion channel receptor exposed on a variety of immune cells and has many biologic functions (Ferrari *et al.*, 2006). P2X7 can be activated by extracellular ATP at the site of inflammation and tissue injury (Dostert *et al.*, 2008), leading to cation flow across the plasma membrane (Burnstock *et al.*, 2011). This receptor is involved in immune responses induced by extracellular ATP, including lung injuries (Riteau *et al.*, 2010; Belete *et al.*, 2011), through its implication in immune processes such as apoptosis (Woods *et al.*, 2012) and inflammasome activation. P2X7 joins in lung disease, such as pulmonary fibrosis and pleurisy (Moncao-Ribeiro *et al.*, 2011), asthma and chronic obstructive disease (Denlinger *et al.*, 2009; Eltom *et al.*, 2011). Upon activation, NLRP3 recruit the PYD- and CARD-containing bipartite adapter ASC (apoptosis-associated speck-like protein containing a CARD) via PYD/PYD interactions (Masumoto *et al.*, 1999). ASC in turn recruits Caspase-1 via CARD/CARD interactions. The penultimate step for the assembly of the inflammasome is the cleavage and activation of Caspase-1. Caspase-1 (also defined as IL-1β converting enzyme, ICE) is first present as a 45 kDa inactive precursor (Walker *et al.*, 1994), which is constituted by a N-terminal subunit of 15 kDa, a central subunit of 20 kDa, ASC proteins together resulting in the cleavage of Caspase-1 (Yamin *et al.*, 1996). The active Caspase-1 is composed by a tetramer consisting of two fragments of 20 kDa and two fragments of 10 kDa (Wilson *et al.*, 1994). Once activated, Caspase-1 can cleave other protein precursors (Carta *et al.*, 2006; Ogura *et al.*, 2006). Many of these proteins have a role in glycolysis (Shao *et al.*, 2007), in the cytoskeleton of the cell (Shao *et al.*, 2007; Keller *et al.*, 2008), and inflammation and mitochondria function (Keller *et al.*, 2008). Moreover, once activated, Caspase-1 causes its own secretion (Keller *et al.*, 2008).

The two most important proteins cleaved by Caspase-1 are IL-1β and IL-18. They are secreted from the cells containing an activated inflammasome. The inactive IL-1β is a protein of 30.7 kDa, while inactive IL-18 is 22.3 kDa. They are respectively cleaved by Caspase-1 to the active 17.5 kDa and 17.3 kDa active forms. Once activated and secreted by the cells, IL-1β and IL-18 can be involved in paracrine and autocrine signalling. They are structurally similar and are in particular for the β-pleated sheets (Dinarello, 1999). IL-1β is a pleiotropic cytokine that can be found in localised inflammation targeting bacterial, parasitic or viral infections; or in systemic inflammation. It is produced by different cell types such as fibroblast, ephitelial cells and T cells; in response to damaged tissues or pathogens. IL-1β can also modulate its own mRNA expression through a signalling pathway involved p38 MAPK phosphorylation and NF-kB activity (Dinarello, 1998). IL-1β secretion requires two signals: the first one is commonly obtained through toll-like receptor signalling or IL-1β receptor self up-regulation of IL-1β gene transcription, the second one is through the inflammasome activation. The release of IL-1β into the peripheral blood leads to increase expression of other pro-inflammatory mediators, up-regulate cortisol levels and induce fever acting on the hypothalamus. IL-18 secretion, previously known as interferon-γ inducing factor, is also induced by inflammasome activation by the effect of this cytokine still needs to be completely elucidated. It is constitutively expressed in its inactive form in several cell types and only necessitates Caspase-1 cleavage for its activation and secretion. Like IL-1β, IL-18 can increase its own mRNA expression and its signalling pathway involves NF-kB activity (Dinarello, 1999). It activates T cells leading to increase expression of IL-2, TNF-α and GM-CSF (Dai *et al.*, 2011).

1.5 BAY 11-7082

Small molecules are able to inhibit the pro-apoptotic and pro-inflammatory effects of NLRP3 inflammasome. Among these, one of the most studied is Bay 11-7082 (Figure 4). It is an NF-kB inhibitor, which is able to selectively block the Ikb kinase activity and to inhibit the activation of the NLRP3 inflammasome by blocking its ATPase activity required to recruit ASC. Its efficacy has been shown in endothelin-1 induced lung edema and oxidative stress (Piechota *et al.*, 2011), burn-induced remote acute lung injury (Sio *et al.*, 2010; Han *et al.*, 2015) and postnatal lung inflammation (Hou *et al.*, 2015).

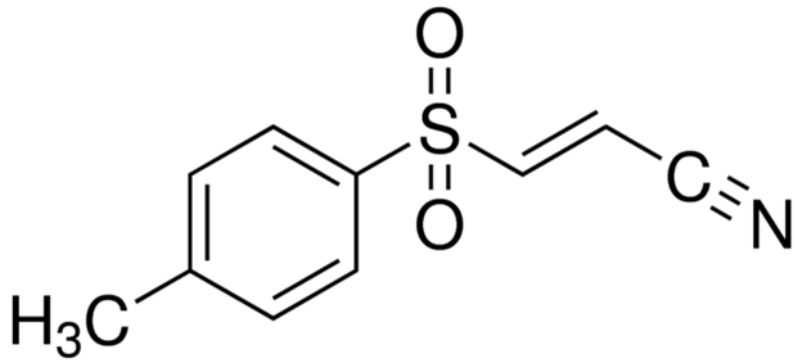

Figure 4. BAY 11-7082

1.6 Brilliant Blue G

Another way to avoid the activation of the NLRP3 inflammasome is targeting its ATPase activity (Uberti *et al.*, 2004). Brilliant Blue G (BBG) blocks the membrane-bound purinergic P2X7 receptor (Díaz-Hernández *et al.*, 2009) (Figure 5). This receptor is expressed on multiple lung cell types such as pulmonary endothelia, type I alveolar epithelial cells and resident cells of the immune system. BBG can down-regulate the expression of ASC and avoid NLRP3 inflammasome activation (Zhao *et al.*, 2013). It has been tested in silica-induced lung changes (Monçao-Ribeiro *et al.*, 2014), acute lung injury

(Grailer *et al.*, 2014) and pulmonary edema after subarachnoid hemorrhage (Chen *et al.*, 2014).

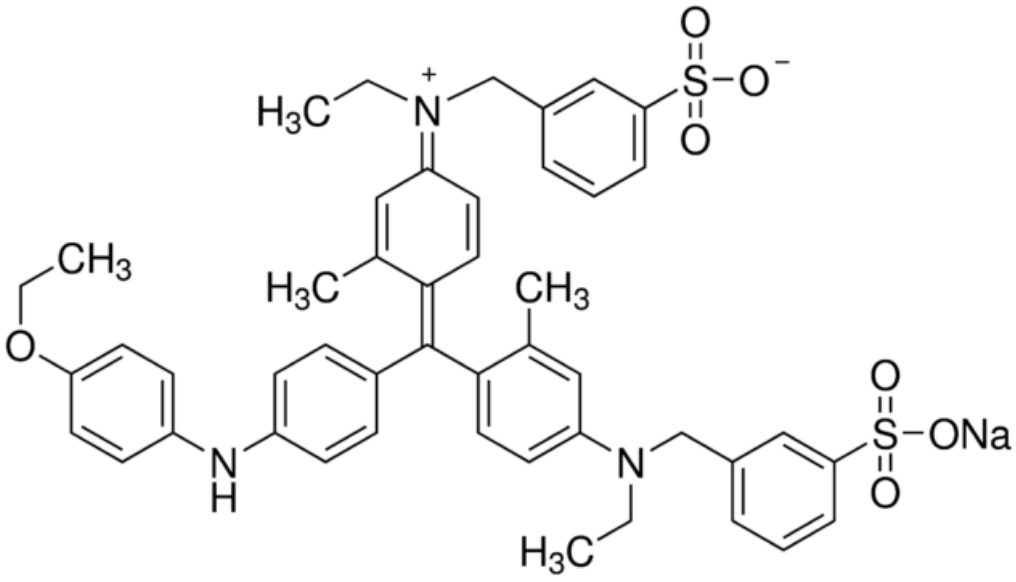

Figure 5. Brilliant Blue G

2. AIM OF THE STUDY

The aim of this project was to clarify the role of the NLRP3 inflammasome in the pathogenesis of respiratory diseases, in particular pleurisy, testing two inflammasome-blocking agents, BAY 11-7082 and BBG, in a murine model of carrageenan-induced pleurisy. The main focuses were: pulmonary membrane thickening, polymorphonuclear leukocyte infiltration, NF-kB translocation in the nucleus, assembly of the NLRP3/ASC/caspase-1 complex, iNOS, nitrotyrosine, poly-ADP-ribosyl polymerase expression and CAR-induced apoptosis.

3. MATERIALS AND METHODS

3.1 Animals

CD1 male mice weighing 25–30 g (Harlan Nossan,Milan, Italy) were kept in a controlled location with standard rodent water and chow. Mice were kept in stainless cages at 22±1°Cunder a 12h light/dark cycle. The study was approved by the University of Messina Review Board for the care of animals. All animal experiments obeyed regulations in the United States (Animal Welfare Assurance A5594-01, Department of Health and Human Services), Europe (O.J. of E.C. L 358/1, 12/18/1986), and Italy (DM 116192).

3.2 Carrageenan-induced pleurisy

Pleurisy was induced by Carrageenan (CAR) as previously described (Cuzzocrea *et al.*, 2000). Animals were anesthetized with isoflurane and cut at the sixth intercostal space. Saline containing 2% l-CAR (0.1 ml) or saline alone (0.1ml) was injected into the pleural cavity. The incision was sutured, and animals were allowed to recover. Mice were sacrificed by inhalation of CO_2 at 4 h after CAR injection. The pleural cavity was injected with 2ml of saline solution containing heparin (5 U/ml) and indomethacin (10 mg/ml). This solution and exudate were aspirated, and the total volume was measured. Exudate having blood was eliminated from the study. The volume of exudate was determined from the total volume recovered by subtracting the injected one (2ml). The leukocytes enclosed in the exudate were suspended in PBS and counted with a Burker's chamber after vital Trypan Blue staining.

3.3 Experimental group

Mice were randomly separated into the following groups (n = 10 for each):

- CAR + saline group: mice were subjected to CAR induced pleurisy.
- CAR + BBG group: same as the CAR + saline group, but BBG (45.5 mg/kg, i.p.) was administered 1 h after CAR injection.
- CAR + BAY 11-7082 group: same as the CAR + saline group, but BAY 11-7082 (30 mg/kg, i.p.) was administered 1 h after CAR injection.
- Sham + saline group: sham-surgery group in which the same surgical procedures as in the CAR group were performed, except that saline was injected instead of CAR.
- Sham + BBG group: same as the sham + saline group, but BBG (45.5 mg/kg, i.p.) was administered 1 h after saline injection.
- Sham + BAY 11-7082 group: same as the sham + saline group, but BAY 11-7082 (30 mg/kg, i.p.) was administered 1 h after saline injection.

The used dose for BBG and for BAY 11-7082 was chosen in agreement with Minutoli et al. (Minutoli *et al.*, 2015). The minimum number of animals for each group was found using the statistical test a priori power analysis of G-power software. This statistical test provides an efficient method for choosing the sample size necessary to perform the experiment.

3.4 Histological examination

Lung tissues were collected 4 h after CAR injection. Samples were fixed for 24 h in PBS-buffered formaldehyde solution (10% w/v) at room temperature, dehydrated using graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Seven μm

sections were deparaffinized with xylene and stained with hematoxylin and eosin. All sections were analysed using an Axiovision microscope (Zeiss, Milan, Italy). The following morphologic criteria were employed for scoring: 0, normal lung; grade 1, minimal edema or infiltration of bronchiolar or alveolar walls; grade 3, moderate edema and inflammatory cell infiltration without obvious damage to lung architecture; grade 4, severe inflammatory cell infiltration with obvious damage to lung architecture. All histologic studies were performed in a blinded fashion.

3.5 Immunohistochemical localization of nitrotyrosine, PAR, NLRP3, ASC, Caspase-1, IL-18, IL-1β, TNF-α, Bax, Bcl-2, myeloperoxidase, iNOS, manganese superoxide dismutase and NRF2

Four hours after CAR injection, lung samples were fixed in PBS-buffered formaldehyde (10% w/v) and embedded in paraffin, and 7-μm sections were used from samples. After deparaffinization, endogenous peroxidase was quenched with hydrogen peroxide (0.3% v/v) in methanol (60% v/v) for 30 min. Samples were permeabilized with Triton X-100 in PBS (0.1% w/v) for 20 min. Nonspecific adsorption was reduced by incubating the sections in normal goat serum in PBS (2% v/v) for 20 min. Endogenous avidin or biotin binding sites were stopped by incubation for 15 min with avidin and biotin (Vector Laboratories, Burlingame, CA, USA), respectively. Sections were probed overnight with purified goat polyclonal antibody directed toward anti-nitrotyrosine antibody (06-284; Millipore, Billerica, MA, USA), anti-PAR antibody (H-250: sc-7150, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti-NLRP3 (sc-66846, 1:400 in PBS, v/v; Santa Cruz Biotechnology), anti-ASC antibody (N-15: sc-22514-R, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–Caspase-1 p20 (G-19: sc-1597, 1:500 in PBS, v/v; Santa Cruz

Biotechnology), anti–IL-18 antibody (H-173: sc-7954, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–IL-1β antibody (H-153: sc-7884, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–TNF-α antibody (N-19: sc-1350, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti-Bax (P-19: sc-526, 1:555 in PBS, v/v; Santa Cruz Biotechnology), anti–Bcl-2 (N-19: sc-492, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti-myeloperoxidase (MPO) antibody (2C7: sc-59600, 1:325 in PBS, v/v; Santa Cruz Biotechnology), anti-iNOS antibody (610432, 1:500 in PBS, v/v; Transduction Laboratories, Lexington, KY, USA), anti-MnSOD antibody (06-984, 1:500 in PBS, v/v; Millipore), or anti-NRF2 antibody (C-20: sc-722, 1:550 in PBS, v/v; Santa Cruz Biotechnology). Samples were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Immunohistochemistry photographs were evaluated by densitometry by using Optilab Graftek software (Ljubljana, Slovenia).

3.6 MPO

MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was quantified as previously described (Mullane *et al.*, 1985). Lung tissue from each animal was removed and weighed. Each sample was homogenized in a solution containing 0.5% hexa-decyl-trimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged at 20,000 g at 4°C for 30 min. A solution of tetra-methylbenzidine (1.6 mM) and 0.1 mMH2O2 was then allowed to react with an aliquot of the supernatant. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was

quantified in units per gram weight of wet tissue and was calculated as the quantity of enzyme degrading 1 mmol of peroxide per minute at 37°C.

3.7 Western blot analysis for IkB-α, NF-kB, NLRP3, ASC, Caspase-1, IL-18, IL-1β, iNOS, manganese superoxide dismutase and NRF2

Lung tissues from each mouse were suspended in extraction Buffer A containing 0.15 mM pepstatin A, 0.2 mM PMSF, 1 mM sodium orthovanadate, and 20 mM leupeptin; homogenized at the highest setting for 2min; and centrifuged at 1000 g for 10min at 4°C. Cytosolic fraction was represented by the supernatants. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 150mM NaCl, 1% TritonX-100, 1mM EGTA, 10 mM Tris-HCl (pH 7.4), 0.2 mM PMSF, 1 mM EDTA, 0.2 mM sodium orthovanadate, and 20 mm leupeptin. After centrifugation for 30min at 15,000 g at 4°C, the nuclear protein contained in the supernatants was stored at 280°C for further analysis. The levels of IkB-α, NLRP3, ASC, Caspase-1, IL-18, IL-1β, iNOS, and manganese superoxide dismutase (MnSOD) were quantified in the cytosolic fraction, whereas NF-kB p65 and NRF2 levels were quantified in the nuclear fraction. The filters were blocked with 1X PBS and 5% (w/v) nonfat dried milk for 40 min at room temperature and probed with specific IkB-α antibody (C-21: sc-371, 1:550 in PBS, v/v; Santa Cruz Biotechnology), anti–NF-kB p65 (F-6: sc-8008, 1:400 in PBS, v/v; Santa Cruz Biotechnology), anti-NLRP3 (sc-66846, 1:400 in PBS, v/v; Santa Cruz Biotechnology), anti-ASC antibody (N-15: sc-22514-R, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–Caspase-1 p20 (G-19: sc-1597, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–IL-18 antibody (H-173: sc-7954, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti–IL-1β antibody (H-153: sc-7884, 1:500 in PBS, v/v; Santa Cruz Biotechnology), anti-iNOS antibody (610432, 1:500

in PBS, v/v; Transduction Laboratories), anti-MnSOD antibody (06-984, 1:500 in PBS, v/v; Millipore), or anti-NRF2 antibody (C-20: sc-722, 1:550 in PBS, v/v; Santa Cruz Biotechnology) in1X PBS (5%w/v) non fat dried milk, and 0.1% Tween-20 (PMT) at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To establish that blots were loaded with equal amounts of proteins, membranes were also incubated in the presence of the antibody against β-actin protein (1:10,000; Sigma-Aldrich, St. Louis, MO, USA). The relative expression of the protein bands of IkB-α (37 kDa), NF-kB p65 (65 kDa), NLRP3 (106 kDa), ASC (24 kDa), Caspase-1 (20 kDa), IL-18 (24 kDa), IL-1β (33 kDa), iNOS (130 kDa), MnSOD (24 kDa), and NFR2 (57kDa) was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Milan, Italy) and a computer program (MolecularAnalyst; IBM,Armonk,NY, USA) and standardized for densitometric analysis to β-actin levels.

3.8 Materials

All materials were acquired from Sigma-Aldrich. BAY 11-7082 was obtained from Adipogen (Liestal, Switzerland), and Brilliant Blue G (BBG) was obtained from Sigma-Aldrich. All stock solutions were set in nonpyrogenic saline (0.9% NaCl) (Baxter, Thetford, United Kingdom).

3.9 Statistical evaluation

All parameters in the text and figures are expressed as means ± SEM of n evaluations. For the in vivo studies, n symbolizes the number of animals used. In the experiments including histology or immunohistochemistry, the figures shown are demonstrative of at least 3 experiments (histologic or immunohistochemistry coloration) performed on different experimental days on the tissue collected from all the animals in each group. The results were analyzed by 1-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. Data are expressed as means ± SEM from 10 mice per group.

4. RESULTS

4.1 Effects of BAY 11-7082 or BBG treatment on carrageenan-induced pleurisy and histologic examination

When compared with lung tissues taken from sham animals (data not shown), histologic examination of lung samples from carrageenan-treated mice showed significant edema and tissue injury (Fig. 6A; see histologic score in Fig. 6D). Treatment with BAY 11-7082 reduced the degree of lung damage (Fig. 6B; see histologic score in Fig. 6D). Also, BBG administration decreased carrageenan-induced lung injury (Fig. 6C; see histologic score Fig. 6D). Moreover, when compared with the sham mice, intrapleural injection of carrageenan led to the development of acute pleurisy, generating turbid exudates with large amount of PMNs (Fig. 6E and F). Administration of BAY 11-7082 or BBG 1 h after carrageenan injection caused a reduction of pleural exudates and inflammatory cells recruitment in the pleural cavity (Fig. 6E and F). There was no significant difference between BAY 11-7082 and BBG treatment.

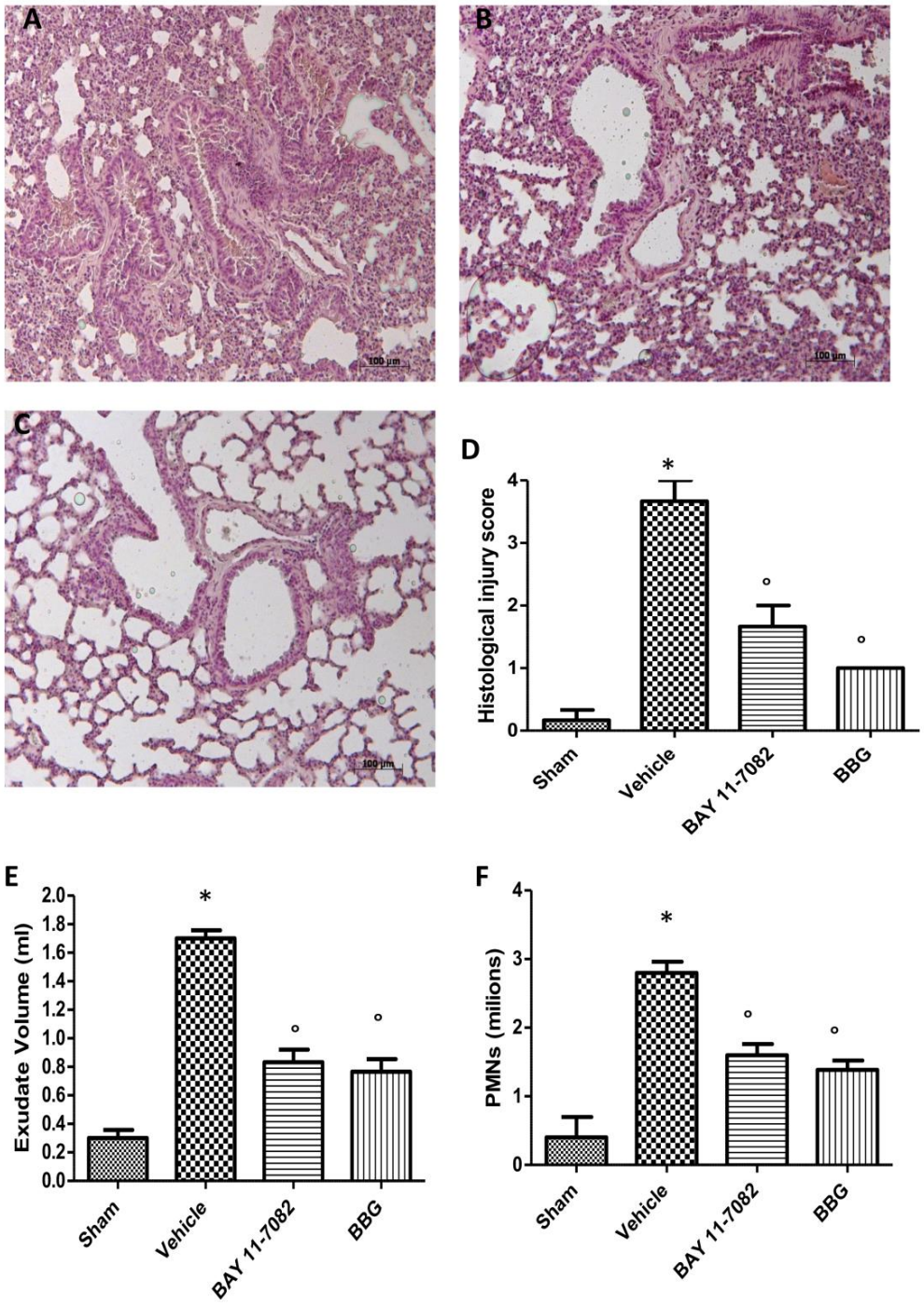

Figure 6. Effect of BAY 1-7082 or BBG administration on histologic alterations, exudate volume, and PMN infiltration in the lung

Lung tissues from CAR-treated mice displayed edema and tissue injury (A, D), and infiltration of the tissue with polymorphonuclear cell (E, F). BAY 11-7082 (B) or BBG (C) administration significantly decreased lung injury (D) and neutrophil infiltration (E, F). Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.2 Effects of BAY 11-7082 or BBG treatment on MPO expression and release of proinflammatory cytokines induced by carrageenan

Analysis of lung samples taken from carrageenan-treated mice showed an increased infiltration of neutrophils as assessed by MPO expression and activity (Fig. 7A, G and H), an indicator of polymorphonuclear cell infiltration, compared with sham-treated mice (data not shown). BAY 11-7082 or BBG administration reduced in the same way MPO expression in tissue obtained from treated mice (Fig. 7B, C and G). Carrageenan injection also led to an up-regulated release of pro-inflammatory cytokines such as TNF-α (Fig. 7D and G) compared with sham- treated animals (data not shown). Treatments equally reduced TNF-α production release (Fig. 7E and G).

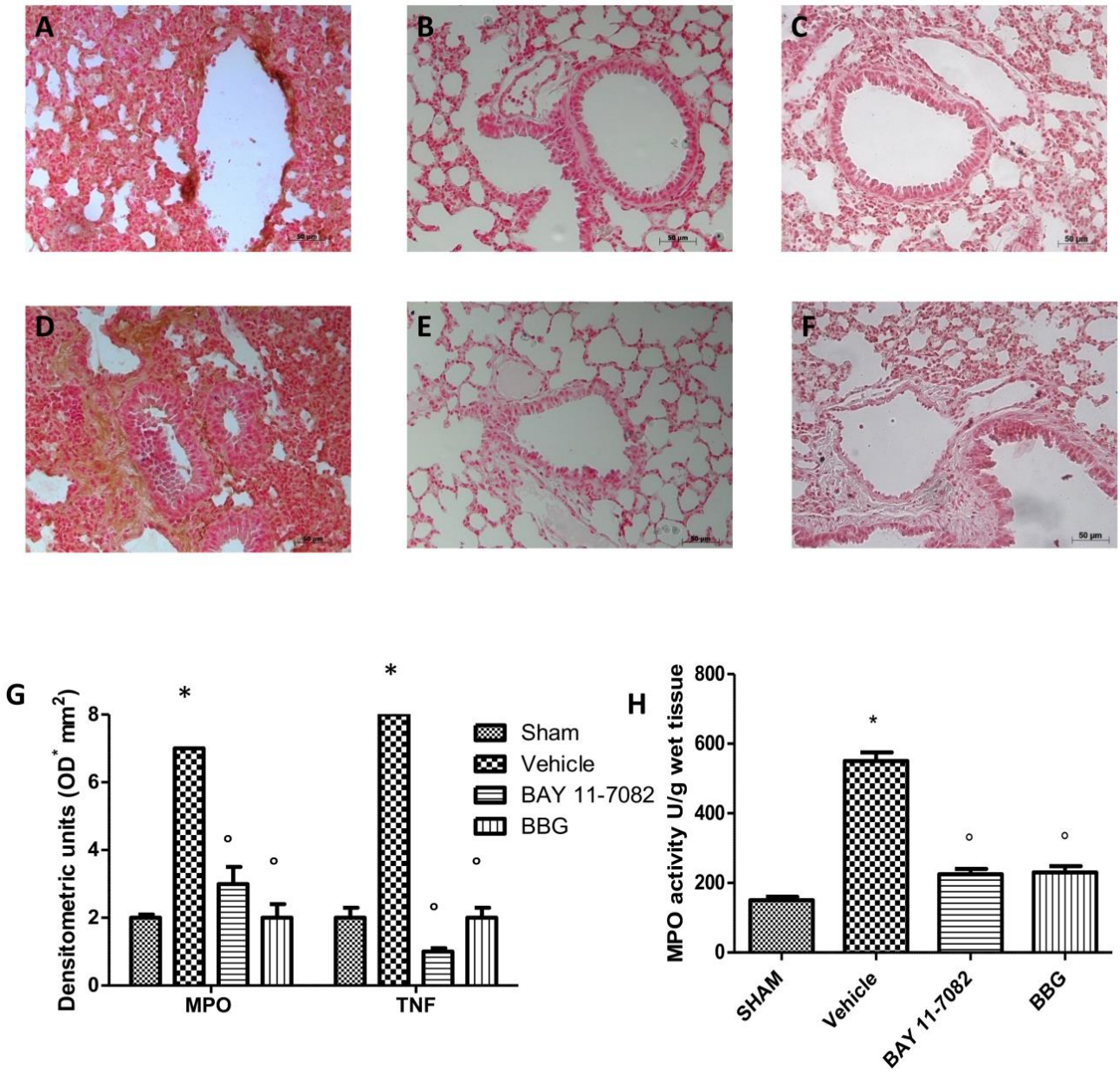

Figure 7. Effect of BAY 1-7082 or BBG administration on the immunohistochemical localization of MPO and TNF-α

After four hours carrageenan injection, MPO activity was elevated in vehicle-treated animals (A, G, H), whereas BAY 11-7082 (B) or BBG (C) administration significantly down-regulated MPO activity (G, H). Lungs from carrageenan-treated mice displayed positive staining for TNF-α (D, G). There was a clear reduction in the immunostaining for TNF-α in tissues from mice administrated with BAY 11-7082 (E, G) or BBG (F, G). Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.3 Effects of BAY 11-7082 or BBG treatment on IkB-α and NF-kB expression induced by carrageenan

Basal expression of IkB-α was detected in samples from sham-treated mice, whereas IkB-α levels were notably decreased in tissues from carrageenan-treated mice (Fig. 8A). BAY 11-7082 administration prevented carragenan-induced IkB-α degradation (Fig. 8A). Additionally, carrageenan-injection up-regulated NF-kB levels in the nuclear fractions compared with the sham-treated animals (Fig. 8B). Treatment with BAY 11-7082 significantly decreased the levels of NF-kB (Fig. 8B). Moreover, BBG administration reduced IkB-α degradation (Fig. 8C) and increased NF-kB expression (Fig. 8D). There was no significant difference between BAY 11-7082 and BBG.

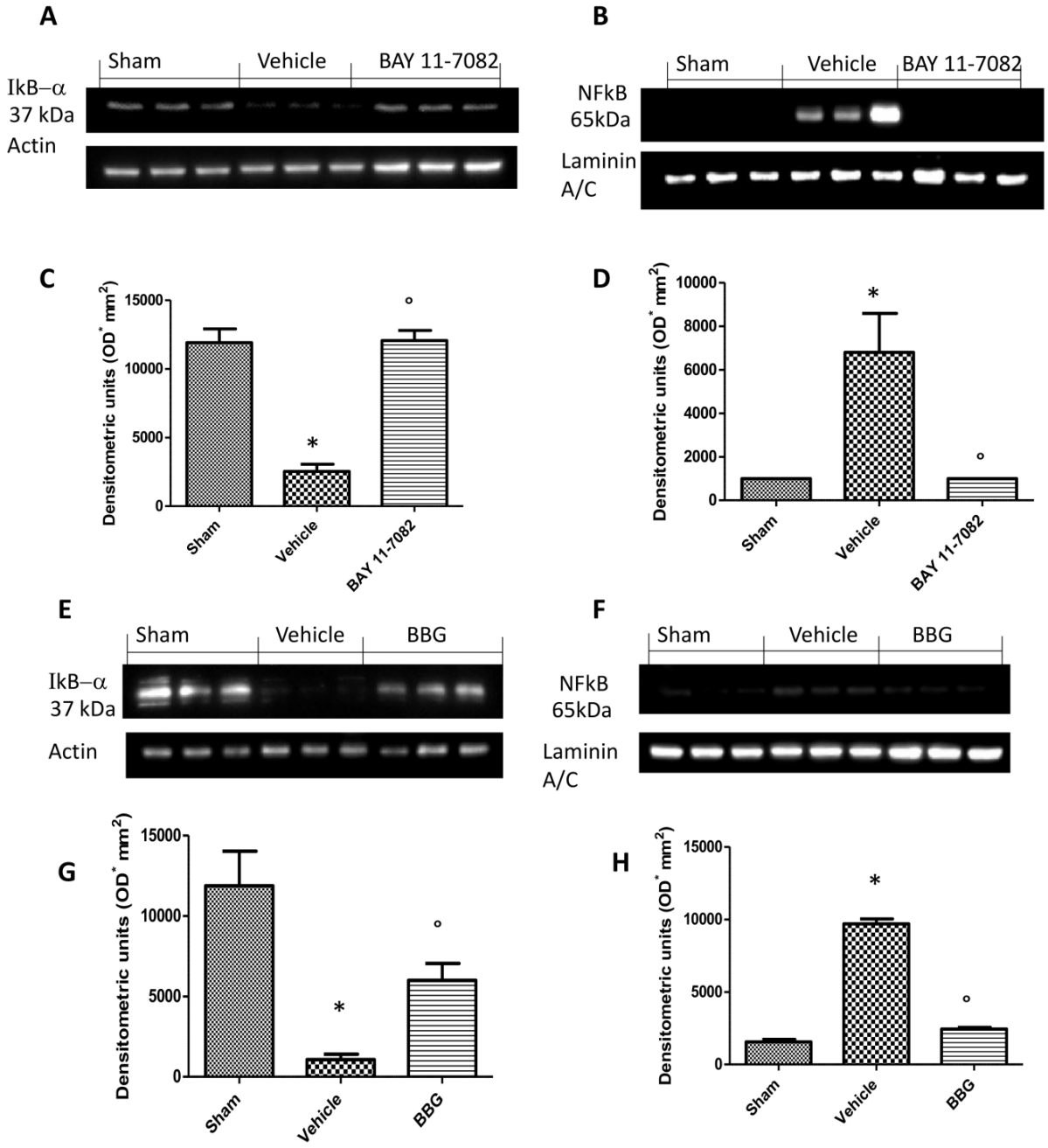

Figure 8. Effect of BAY 1-7082 or BBG administration on IkB-α degradation and nuclear NF-kB quantification

Basal levels of IkB-α were found in tissues from sham animals (A) and were significantly down-regulated in samples from carrageenan-injected mice (C). BAY 11-7082 (A) or BBG (C) administration significantly reduced IkB-α degradation. Four hours after carrageenan instillation, NF-kB levels were markedly increased in the nuclear fraction (B) compared with the sham-animals (D). BAY 11-7082 (B) or BBG (D) significantly reduced NF-kB in the nucleus. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.4 Effects of BAY 11-7082 or BBG treatment on NLRP3 expression induced by carrageenan

In order to evaluate the effect of BAY 11-7082 and BBG in the inflammasome activation, we investigated NLRP3 expression. Lung samples from carrageenan-treated mice displayed positive staining for NLRP3 (Fig. 9A and D) compared with sham-treated animals (data not shown). BAY 11-7082 or BBG both reduced this staining (Fig. 9B and D). Western blot analysis also confirmed these data. We found up-regulated NLRP3 expression in the carrageenan-treated group compared with the sham animals and a reduction of this expression in samples obtained from mice administrated with BAY 11-7082 or BBG (Fig. 9E and G).

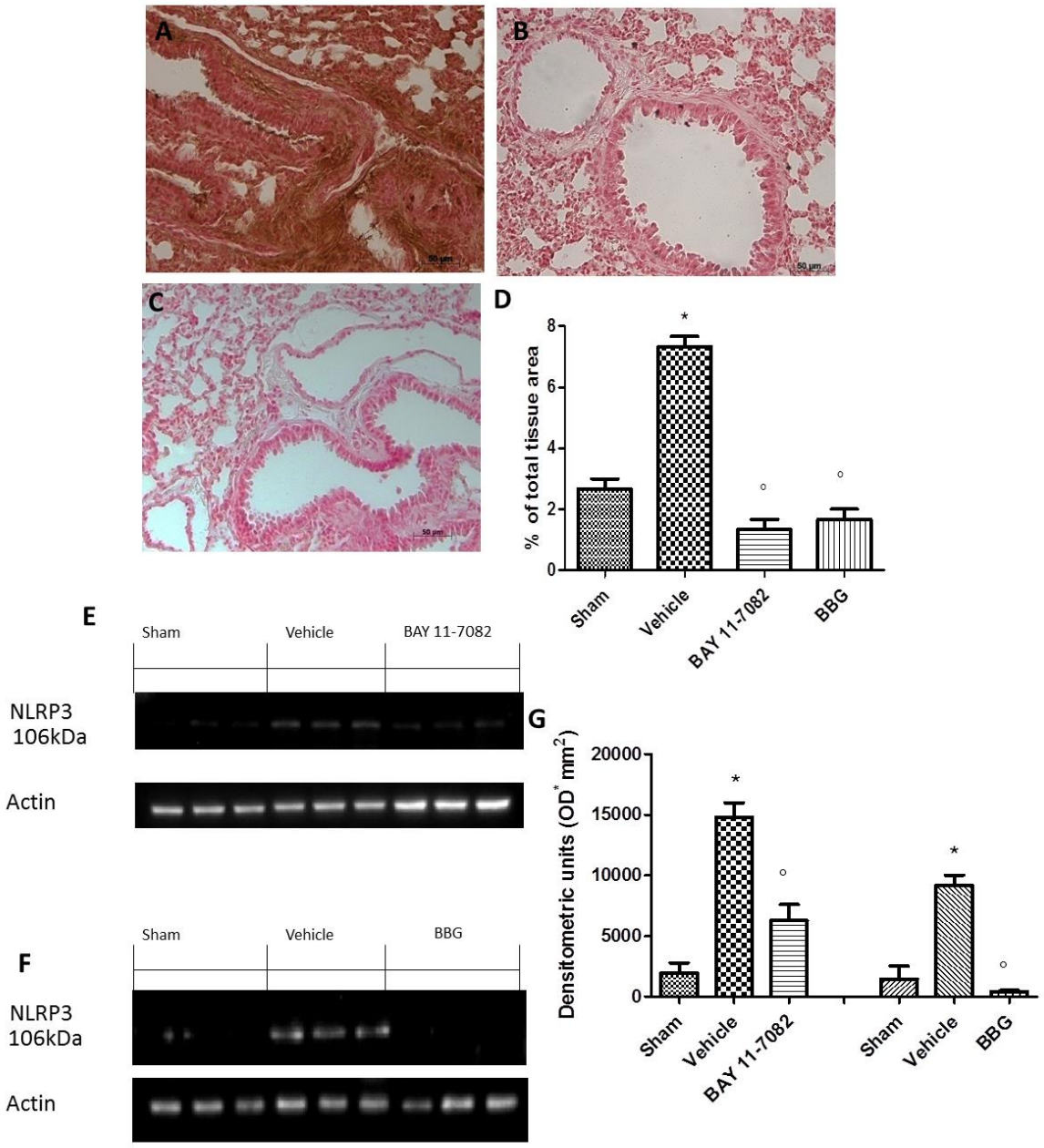

Figure 9. Effect of BAY 1-7082 or BBG administration on NLRP3 expression

Lung tissues taken from carrageenan-treated animals showed positive immunostaining for NLRP3 (A, D). BAY 11-7082 (B, D) or BBG (C, D) reduced this staining. Western blot analysis confirmed up-regulated levels of NLRP3 expression in samples collected from carrageenan-injected animals compared with the sham mice (E–G). BAY 11-7082 (E, G) or BBG (F, G) administration decreased NLRP3 expression. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.5 Effects of BAY 11-7082 or BBG treatment on ASC and Caspase-1 expression induced by carrageenan

Lung samples obtained from carrageenan-treated animals showed positive staining for ASC (Fig. 10A and D). No positive staining was observed in samples from sham-treated mice (data not shown). BAY 11- 7082 or BBG administration reduced this staining (Fig. 10B and D). Lung sections from sham-treated animals did not indicate staining for Caspase-1 (data not shown), whereas samples from carrageenan-treated mice exhibited positive staining for Caspase-1 (Fig. 10D and H). Treatment with BAY 11-7082 or BBG reduced the staining for Caspase-1 (Fig. 10F and H). Western blot analysis exposed an increased expression of ASC and Caspase-1 in tissue collected from carrageenan-treated mice (Fig. 10I and L) compared with sham-treated animals. BAY 11-7082 or BBG administration reduced ASC and Caspase-1 expression (Fig. 10I and L). There was no significant difference between the inhibitors.

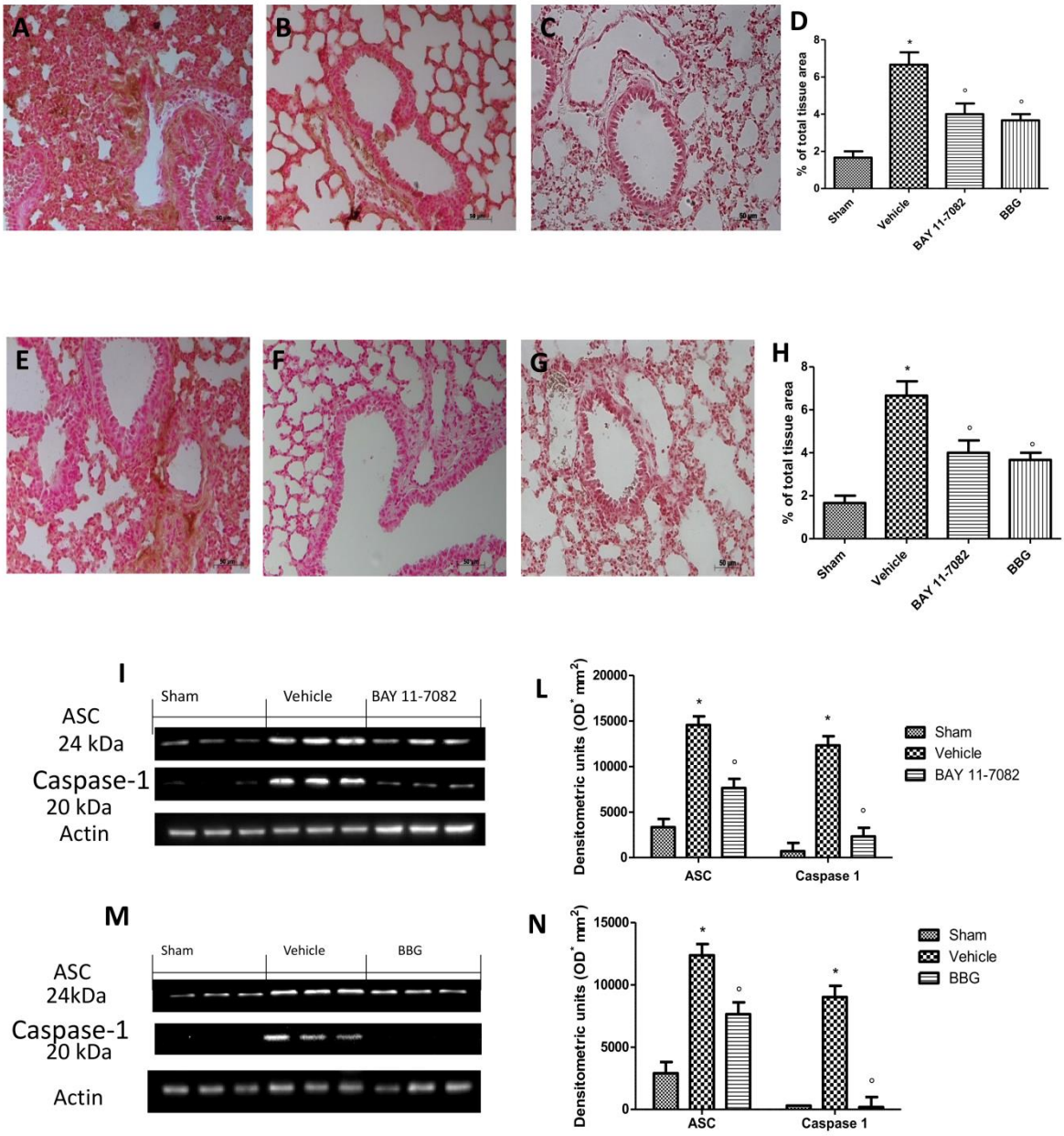

Figure 10. Effect of BAY 1-7082 or BBG administration on ASC and Caspase-1 expression

Four hours after carrageenan injection, positive ASC immunostaining in lungs collected from vehicle-treated animals was found (A, D). BAY 11-7082 (B, D) or BBG (C, D) administration reduced this staining. This treatment also decreased Caspase-1 positive staining (F, H) compared with vehicle-treated animals (E, H). Western blot analysis exposed increased expression of ASC and Caspase-1 in tissues collected from CAR-treated mice (I, J) compared with the sham-treated animals (K, L). BAY 11-7082 (I, J) or BBG (K, L) significantly inhibited this up-regulation. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.6 Effects of BAY 11-7082 or BBG treatment on IL-1β and IL-18 expression induced by carrageenan

Tissues obtained from carrageenan-treated animals showed positive staining for IL-1β (Fig. 11A and D). No positive staining was found in samples from sham-treated mice (data not shown). BAY 11-7082 or BBG treatment equally reduced IL-1β-positive staining (Fig. 11B and D). Tissues from sham-treated mice did not expose staining for IL-18 (data not shown), whereas lung samples from carrageenan-treated animals displayed positive staining for IL-18 (Fig. 11D and H). BAY 11-7082 or BBG decreased the degree of positive staining for IL-18 (Fig. 11F and H). Western blot analysis confirmed an improved expression of IL-1β and IL-18 in samples taken from carrageenan-treated mice (Fig. 11I and L) compared with sham-treated animals. BAY 11-7082 and BBG decreased IL-1β and IL-18 expression in the same way.

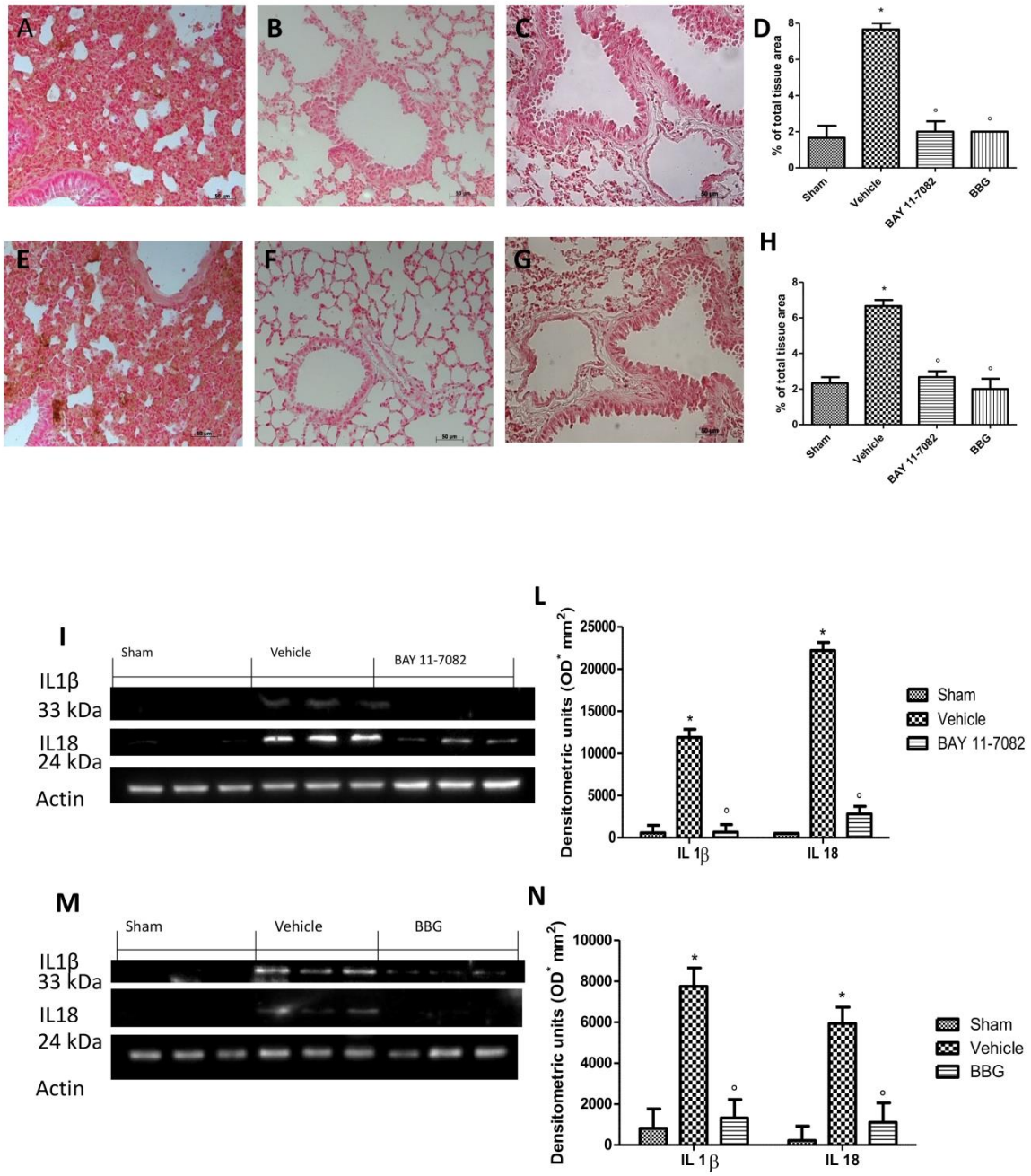

Figure 11. Effect of BAY 1-7082 or BBG administration on IL-1β and IL-18 expression

Lung sections obtained from CAR-treated mice exposed positive staining for IL-1β (A, D). Immunostaining for IL-1β showed a clear reduction in the lungs of mice administrated with BAY 11-7082 (B, D) or BBG (C, D). Positive IL-18 immunostaining in lung tissues from vehicle-treated animals was found (E, H). Administration with BAY 11-7082 (F, H) or BBG (G, H) reduced this staining. Western blot analysis of lung samples obtained from CAR-injected animals showed increased expression of IL-1β and IL-18 compared with the sham-treated animals (I, L). BAY 11-7082 (I, J) or BBG (K, L) reduced this expression. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.7 Effects of BAY 11-7082 or BBG treatment on MnSOD and NRF2 expression induced by carrageenan

Whether, to investigate carrageenan modulates the oxidative process, we tested the lung expression of the antioxidant enzyme MnSOD. Staining of samples from sham-treated mice displayed a constitutive expression of MnSOD (data not shown), while carrageenan significantly decreased lung MnSOD staining (Fig. 12A and D). BAY 11-7082 or BBG administration significantly increased MnSOD staining (Fig. 12B and D). Tissues from sham-treated mice showed basal staining for NRF2, carrageenan injection notably reduced this staining (Fig. 12E and H). Treatment with BAY 11-7082 or BBG improved NRF2 staining (Fig. 12F and H). Western blot analysis showed the up-regulation of MnSOD and NRF2 expression by BAY11-7082 or BBG administration compared with carrageenan-treated mice (Fig. 12I and N). No statistical difference was found between the inhibitors.

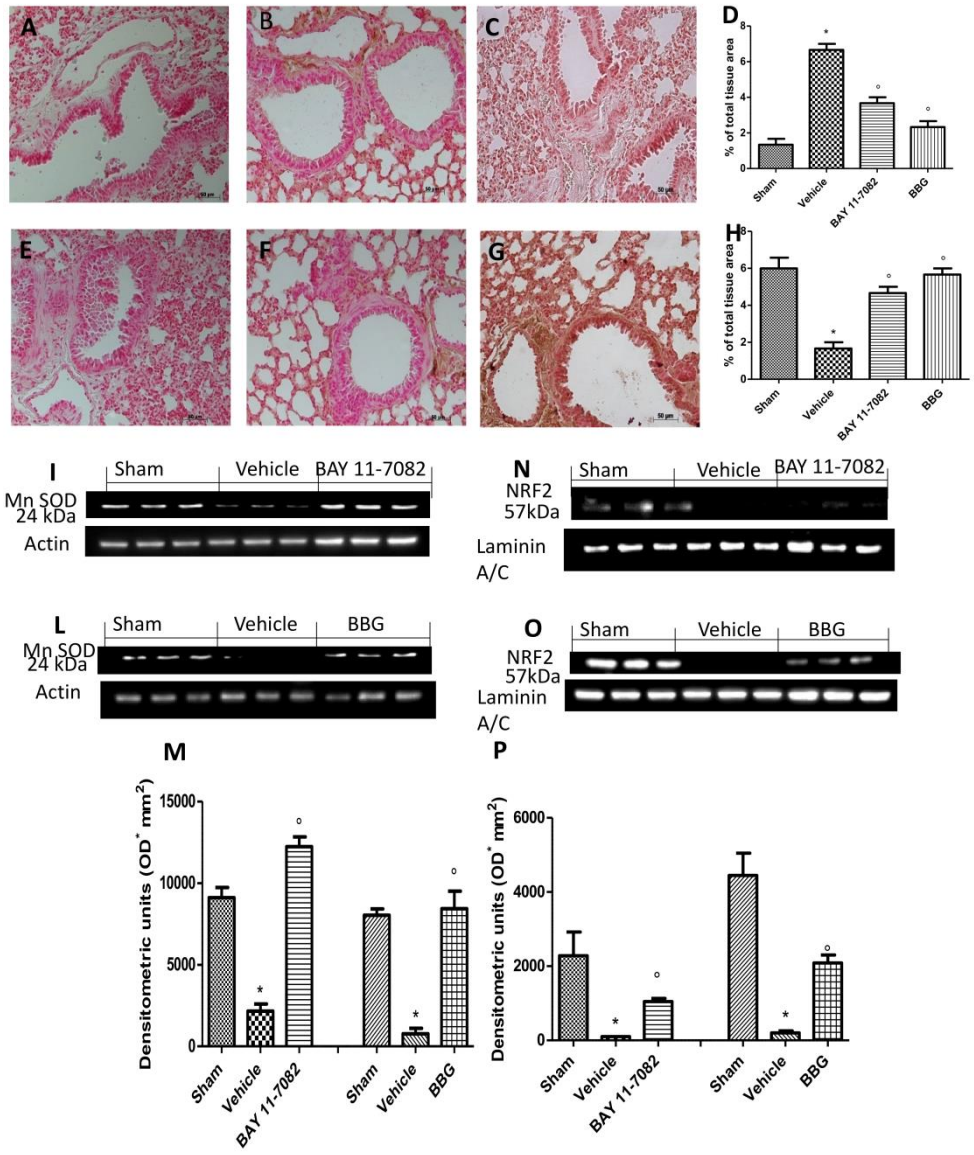

Figure 12. Effect of BAY 1-7082 or BBG administration on MnSOD and NRF2 expression

No positive staining for MnSOD was found in tissues from CAR-treated mice four hours after the injection (A, D). BAY 11-7082 (B, D) or BBG (C, D) administration increased this staining. Also no positive staining for NRF2 was detected in tissues from CAR-treated mice (E, H). BAY 11-7082 (F, H) or BBG (G, H) increased NRF2 staining. Western blot analysis confirmed a decrease of MnSOD expression in the cytosolic fraction of lung samples taken from CAR-treated animals compared with the tissues from sham-treated mice (I, K). Treatment with BAY 11-7082 (I, K) or BBG (J, K) restored MnSOD level. Western blot analysis displayed a reduction of NRF2 expression in the nuclear fraction of samples collected from CAR-treated mice compared with sham-treated animals (L, N). BAY 11-7082 (L, N) or BBG (M, N) administration increased NRF2 levels. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.8 Effects of BAY 11-7082 or BBG treatment on iNOS expression induced by carrageenan

Four hours after carrageenan injection, immunohistochemical analysis of tissues taken from the vehicle-treated group showed positive staining for iNOS (Fig. 13A and D) compared with samples from the sham-treated mice (data not shown). Treatment with BAY 11-7082 or BBG significantly attenuated this staining (Fig. 13B and D). iNOS expression in samples from carrageenan-treated mice was also found amplified in Western blot analysis. Western blot analysis also confirmed that treatment with BAY 11-7082 (Fig. 12E and G) or BBG (Fig. 12F and G) decreased iNOS expression. There was no significant difference between BAY 11-7082 or BBG administration.

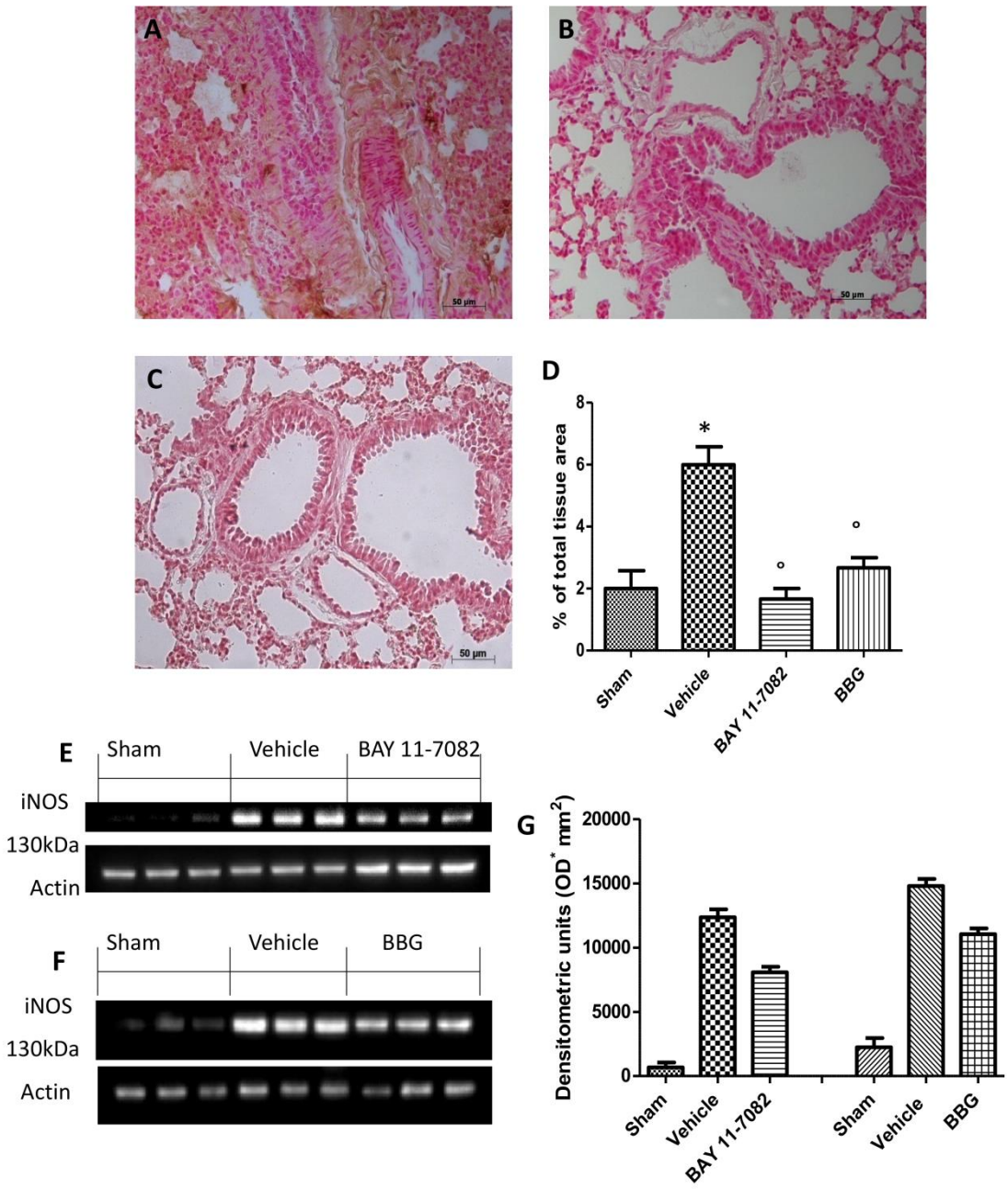

Figure 13. Effect of BAY 1-7082 or BBG administration on iNOS expression

Lung tissues taken from CAR-treated animals displayed positive staining for iNOS (A, D). iNOS-positive staining was notably decreased in tissue sections from mice administrated with BAY 11-7082 (B, D) or BBG (C, D). An increase in iNOS quantification, measured by Western blot analysis, was detected in lungs obtained from mice subjected to CAR-injection compared with tissues from sham-treated animals (E, G). BAY 11-7082 (E, G) or BBG (F, G) significantly reduced this protein quantification. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.9 Effects of BAY 11-7082 or BBG treatment on nitrotyrosine and PAR expression induced by carrageenan

Immunohistochemical analysis of lungs from carrageenan-injected animals displayed positive staining for nitrotyrosine (Fig. 14A and G). No positive staining for nitrotyrosine was found in the samples of carrageenan-injected mice that received BAY 11-7082 or BBG (Fig. 14B, C and G). Four hours after carrageenan injection, lungs were collected, and immunohistological staining for poly-ADP–ribosylated proteins was performed. In carrageenan treated mice, positive staining for the PAR was detected (Fig. 14D and G). The two inhibitors equally reduced this staining (Fig. 14E, F and G).

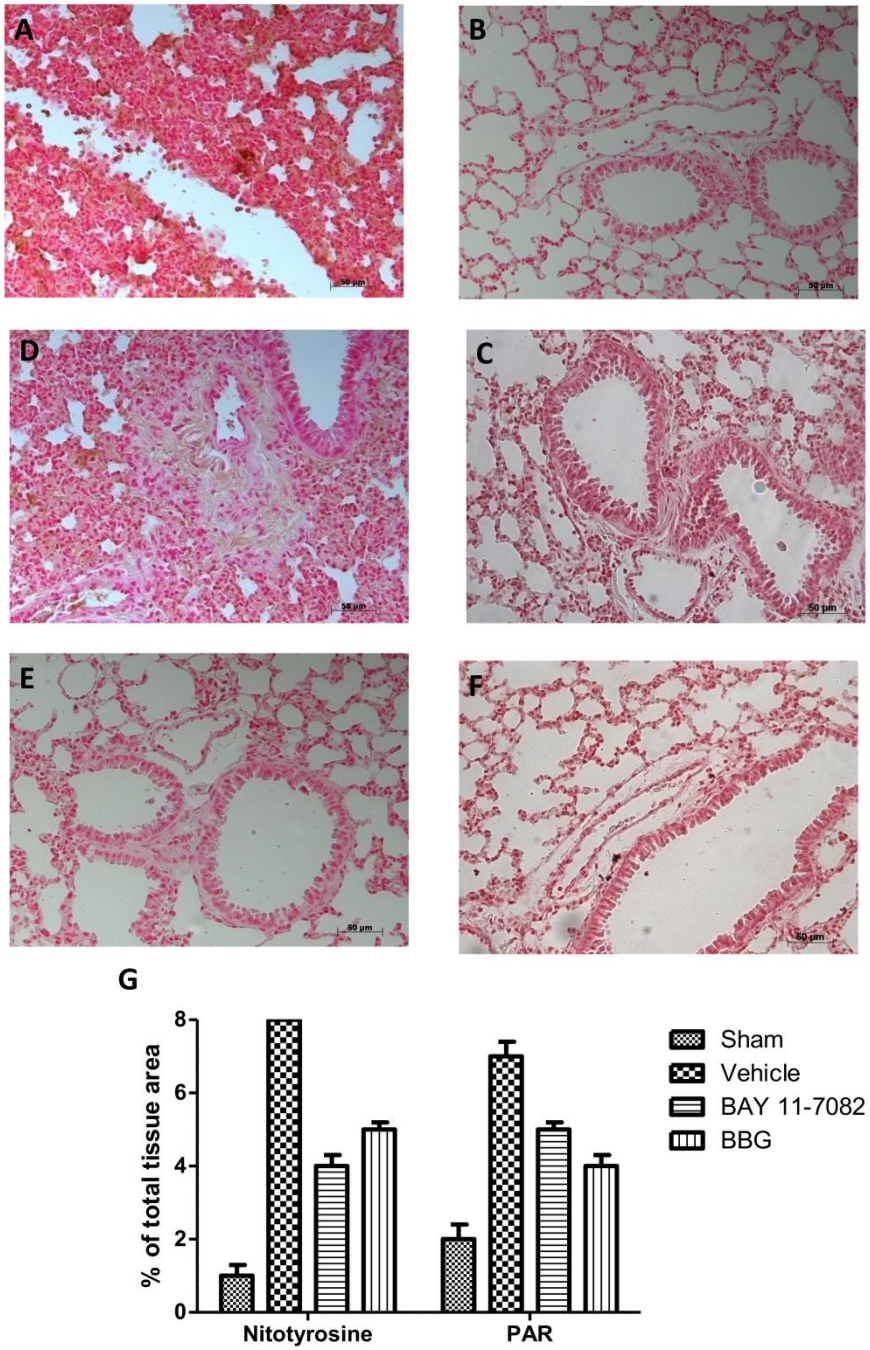

Figure 14. Effect of BAY 1-7082 or BBG administration on nitrotyrosine and PAR expression

Lungs from CAR-treated mice exposed intense positive staining for nitrotyrosine (A, G). This staining was notably reduced in animals treated with BAY 11-7082 (B, G) or BBG (C, G) Also, positive PAR immunostaining was found in tissues collected from vehicle-treated animals (D, G). BAY 11-7082 (E, G) or BBG (F, G) administration decreased this staining. Values are means ± SEM (n = 10 mice per each group). *P<0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

4.10 Effects of BAY 11-7082 or BBG treatment on Bax and Bcl-2 expression induced by carrageenan

Tissues obtained from sham-treated mice did not express any staining for Bax (data not shown), whereas lungs from carrageenan-treated animals displayed positive staining (Fig. 15A and G). BAY 11-7082 or BBG administration reduced Bax staining (Fig. 15B, C and G). Moreover, tissues from sham-treated animals revealed positive staining for Bcl-2 (data not shown), whereas in the carrageenan-treated group Bcl-2 staining was notably reduced (Fig. 15D and G). Treatment with BAY11-7082 and BBG significantly down-regulated the staining for Bcl-2 in mice subjected to carrageenan-induced pleurisy (Fig. 15E and G). There was no significant difference between them.

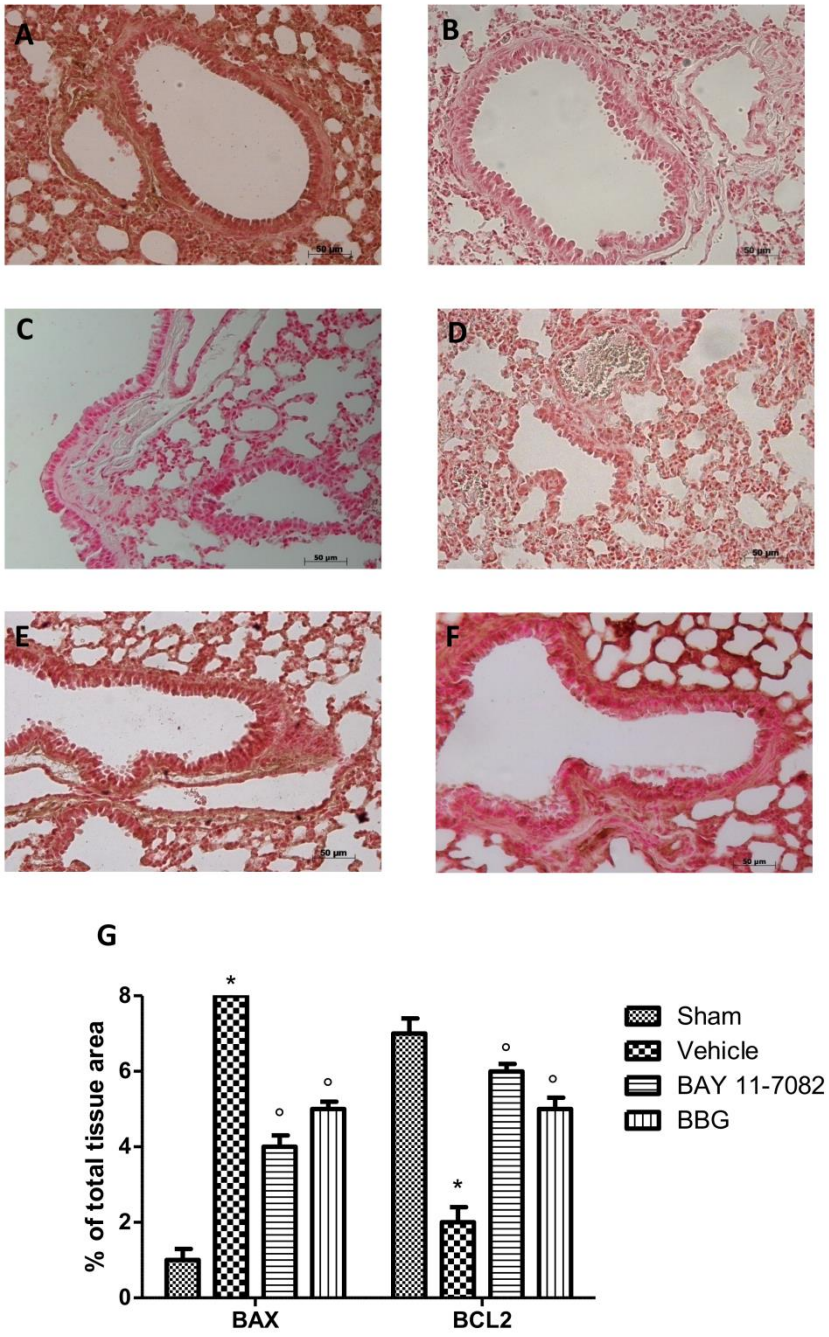

Figure 15. Effect of BAY 1-7082 or BBG administration on Bax and Bcl-2 expression

Lungs from CAR-treated mice showed positive staining for Bax (A, G). There was a clear reduction of this staining in samples from mice administrated with BAY 11-7082 (B, G) or BBG (C, G). Four hours after CAR injection, no positive Bcl- 2 immunostaining was detected in lung tissues obtained from CAR-treated mice (D, G). BAY 11-7082 (E, G) or BBG (F, G) increased Bcl-2 staining. Values are means ± SEM (n = 10 mice per each group). *P <0.05 vs. sham-treatment group, °P <0.05 vs. CAR.

5. DISCUSSION

Carrageenan-induced pleurisy is a usually employed model of acute inflammation (Di Rosa *et al.*, 1971; Velo *et al.*, 1973). This injection into the pleural cavity causes leukocyte infiltration in the lungs and inflammatory exudates in the chest (Impellizzeri *et al.*, 2011a). The animal model lends itself to analysis of the principal mediators involved in inflammation.. New data related NLRP3 inflammasome activation to acute injury induced by carrageenan administration (Wang *et al.*, 2015). It has also been described that two blocking agents BAY 11-7082 and Brilliant Blue G (BBG) attenuated the inflammatory pathway by inhibiting the activation of the NLRP3 inflammasome (Minutoli *et al.*, 2015). In particular, BAY 11-7082 inhibits the IkB kinase activity (Uberti *et al.*, 2004) and in turn the translocation of NF-kB into the nucleus carrageenan-induced, whereas BBG blocks the membrane-bound purinergic P2X7 receptor (Díaz-Hernández *et al.*, 2009), leading to the suppression of ASC expression and inhibiting the NLRP3 inflammasome activation. This study showed that both the administrations of the inflammasome blocking agents BAY 11-7082 and BBG significantly attenuated the histologic signs and biochemical markers of pleurisy. The two treatments reduced the exudate volume and the infiltration of the PMNs into the pulmonary parenchyma. Moreover, the reduction of the infiltrating cells well correlated with the reduced MPO expression found in tissues collected from BAY 11-7082 and BBG administrated animals. MPO release in the early phase of the inflammatory reaction to carrageenan injection may be related with the events that triggered the late mononuclear cell migration and numerous pro-inflammatory cytokines secretion, such as TNF-α (Kon *et al.*, 2001). BAY 11-7082 and BBG were able to reduce TNF-α over-expression induced by carrageenan. As already mentioned, several studies underlined that these stimuli led to the activation of the NF-kB pathway (Impellizzeri *et al.*, 2011b). In normal condition, NF-kB is located in the cytosol, in its inactive state, bound to an

inhibitor IkB protein (in this case, IkB-α). This inhibitor can be phosphorylated by a kinase enzyme (in this case IkB kinase) allowing it to release NF-kB. During inflammation a variety of extracellular signals, also through the intermediation of membrane integral receptors, can activate the IkB kinase enzyme. Once IkB-α is phosphorylated, it goes to ubiquitation and degradation in the proteasome, whereas NF-kB goes to the nucleus. It regulates the expression of genes influencing a broad range of biological processes including inflammation, innate and adaptive immunity and stress responses. Treatment with BAY 11-7082 or BBG significantly inhibited NF-kB translocation in the nucleus in two different way (Uberti *et al.*, 2004; Díaz-Hernández *et al.*, 2009). Among the transcription of the genes activated by NF-kB (Impellizzeri *et al.*, 2011b) there is also the NLRP3 inflammasome (Stutz *et al.*, 2013). NLRP3 is an ATPase and its activity is required to trigger the formation of the NLRP3 inflammasome complex, leading to ASC oligomerization an Caspase-1 activation (Fernandes-Alnemri *et al.*, 2007; Proell *et al.*, 2013). Once activated Caspase-1 proteolytically activates pro–IL-1β and pro–IL-18 (Thornberry *et al.*, 1992; Gu *et al.*, 1997) and induces their release (Dinarello, 2009). Treatment with BAY 11-7082 and BBG reduced NLRP3 expression, in turn ASC pyroptosome formation and pro-Caspase-1 self-cleavage in active Caspase-1. This inhibitors also decreased IL-1β and IL-18 expression in lungs. Several studies underlines that carrageenan administration led to a large increase of ATP release in lung epithelial cells (Saïd-Sadier *et al.*, 2012). It is an endogenous DAMP and one of the first described NLRP3 inflammasome activators. High ATP extracellular concentrations led to P2X7R activation which in turn supports a rapid production of ROS (Cruz *et al.*, 2007). Generation of ROS resulted in oxidative stress, which produced cell damage directly or by altering signalling pathways (Salvemini *et al.*, 1996; Cuzzocrea *et al.*, 2006). Several redox-sensitive proteins are up-regulated or down-regulated from the oxidative stress (Bowie *et*

al., 2000). This study demonstrated that treatment with BAY 11-7082 or BBG was able to stabilize NF-E2–related factor 2 (NRF2) and up-regulate the levels of the antioxidant enzyme MnSOD. The experiments also confirmed the increase in iNOS expression in lung tissues from carrageenan-treated animals. Treatments with the tested inflammasome inhibitors attenuated iNOS expression. Among the stimuli induced by the generation of ROS, that characterized the pleurisy induced by carrageenan, there are the increased formation of nitrotyrosine expression (Impellizzeri *et al.*, 2011b) and poly-ADP-ribosyl polymerase clivation after single DNA strand breakage (Cuzzocrea *et al.*, 1998). Animals treated with BAY 11- 7082 or BBG showed reduced nitrotyrosine and PAR staining. It has been demonstrated that animals may respond to stress induced by carrageenan injection in a number of ways including pathways that induce apoptosis (programmed cell death) (Elmore, 2007). By inhibiting the inflammasome activation, BAY 11-7082 and BBG treatments were able to reduce the expression of the pro-apoptotic protein BAX and restore the expression of the anti-apoptotic protein Bcl-2 to the sham levels.

Taken together, the data showed in this study demonstrate the activation of the NLRP3 inflammasome pathway induced by carrageenan injection and its modulation by BAY 11-7082 and BBG. This two inflammasome blocking agents may be an useful strategy to attenuate the symptoms of pleurisy.

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