Differential Inflammasome Activation Between Major Mouse Models of Silicosis: C57BL/6 vs. Balb/c

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Abstract

Prolonged silica exposure can result in silicosis, characterized by chronic inflammation and fibrotic lesions. The pathology is widely variable in the human condition, ranging from acute silicosis with almost immediate onset following exposure, to chronic silicosis that can take years to develop. This is due in part to differences in exposure and genetic variability, which is addressed in research by using genetically identical mouse models in controlled exposures. This study sought to compare two common mouse strains, C57BL/6 and Balb/c, and their activation of the Nalp3 inflammasome, a caspase-1 activation platform that cleaves pro-inflammatory cytokines and is known to play a role in silicosis. C57BL/6 and Balb/c mice were instilled with silica and samples were collected at 1 or 3 days post-instillation. Interleukin (IL)-1ß mRNA, caspase-1 activation and IL-1ß levels in lavage fluid were compared. Additionally, bone marrow-derived macrophages (BMdM) were cultured and IL-1ß mRNA, precursor protein, and secreted mature protein levels were compared following silica treatment. In both alveolar macrophages and BMdM, C57BL/6 expressed a higher level of IL-1ß mRNA. This trend was not seen in the translation product, as Balb/c produced higher levels of intracellular pro-IL-1ß. In addition, Balb/c mice secreted higher levels of mature IL-1ß in all of the cell populations studied. Balb/c showed higher caspase-1 activation at 1 day, though this was surpassed by C57BL/6 at 3 days. These data suggest that there are differences in the translation and processing capabilities of the two strains that allow for more efficient IL-1ß production in the Balb/c strain.

Introduction

Silica is a naturally occurring and abundant component of the environment. Exposure to silica, prevalent in the dusty trades (i.e. farming, mining), can result in chronic inflammation and fibrosis, known as silicosis. Inhalation of silica particles leads to their deposition in the lungs, where they are interact with alveolar macrophages. This elicits an inflammatory response characterized by inflammatory cytokines such as interleukin (IL)-1ß and tumor necrosis factor (TNF)-α[1]. The initial acute inflammation is followed by chronic inflammation, fibrotic lesions, and increased susceptibility to secondary infection that characterize silicosis.

The progression from silica inhalation to fibrosis is inconsistent in the human condition, varying in both rapidity of onset and the symptoms experienced by patients. This variability is due in part to the silica exposure profile and confounding factors such as genetics, diet, exercise, and smoking. In a research setting, the hereditary variability is eliminated by using inbred mouse strains. Two of the most common mouse models used to study this and other inflammatory diseases are the C57BL/6 and Balb/c strains, considered to be Th1- and Th2-prone strains, respectively. In support of this strain paradigm, our lab and others have found Th2 immunity to be necessary for the development of silicosis in the Balb/c model[2]. On the other hand, other labs using the C57BL/6 mouse strain have reported silicosis to be Th2-independent[3]. Thus, these two strains exhibit distinct fibrosis pathologies and may represent/model two points on the continuum of the human condition.

Recent studies have implicated a cytoplasmic receptor called Nalp3 (nacht LRR protein 3; also known as NLRP3 or cryopyrin) as an integral part of the silicosis pathology[4]. Upon activation, Nalp3 forms a complex with adaptor proteins called the inflammasome. The inflammasome activates caspase-1, which can then cleave pro-IL-1ß into its biologically active form. IL-1ß is known to act in concert with other cytokines to illicit an inflammatory response, but it was recently shown to be necessary for the formation of fibrosis as well[1]. Additionally, fibrosis has been shown to be Nalp3-dependent, further emphasizing the role of both Nalp3 and IL-1ß in fibrotic development[4].

While the activation and downstream effects of the inflammasome have been and continue to be characterized by this and other labs, this study sought to compare its activation in the C57BL/6 and Balb/c models. Differences in inflammasome activation between these mouse strains could indicate areas for further study in the human condition. Similarly, processes that are conserved in these two distinct pathologies could indicate processes conserved in the human condition and possibly therapeutic targets.

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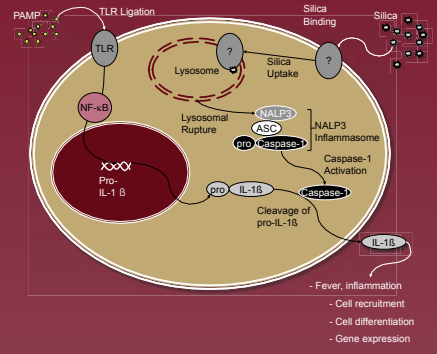
3. Misson P, *et al.* (2007).Type 2 immune response associated with silicosis is not instrumental in the development of the disease. *Am J Physiol Lung Cell Mol Physiol* 292: L107-13.
4. Cassel SL, *et al.* (2008). The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci USA* 105:9035-40.

Results

Figure 1. Model of Inflammasome activation.



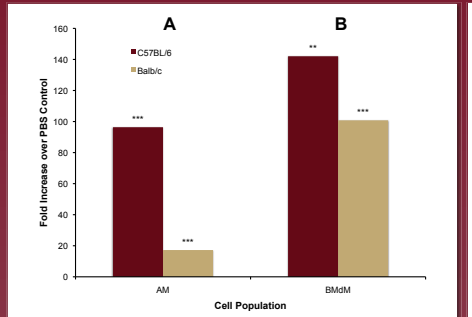


Figure 2. Comparative analysis of IL-1ß mRNA following silica exposure *in vivo* and *in vitro*. (**A**) C75BL/6 and Balb/c mice were instilled with Silica (1 mg in PBS) or vehicle and sacrificed 24 hours after instillation. Alveolar macrophages (AM) were collected through lavage. (**B**) BMdM from each strain were plated and treated with silica (200 µg/ml in RPMI) or vehicle control and co-cultured with or without macrophage stimulation (OVA). RT-PCR was used to quantify mRNA levels for both cell populations. Both populations showed significant increases in IL-1ß mRNA with C57BL/6 expressing higher levels than Balb/c in both populations. Results are the mean fold increase over PBS controls (***P<0.01*, ****P<0.001*).

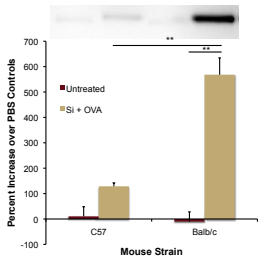


Figure 3. Comparative analysis of intracellular pro-IL-1ß levels following *in vitro* silica exposure. BMdM from C57BL/6 and Balb/c mice were treated with silica (200 µg/ml in RPMI) or vehicle control and co-cultured with or without macrophage stimulation (OVA). Cells were lysed and SDS-PAGE was used to separate the proteins. Western blot was probed for 31 kDa pro-IL-1ß. Densitometry was used to calculate protein concentration. Balb/c BMdM treated with silica and OVA had significantly higher levels of pro-IL-1ß than the control cells. Additionally, the treated cells of Balb/c strain were significantly higher than the C57BL/6 counterparts. Results are the mean percent increase over PBS controls ± SEM (***P<0.01*).

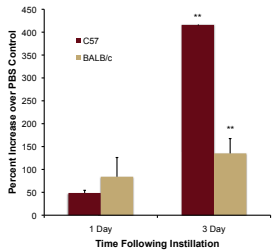


Figure 4. Caspase-1 activation in alveolar macrophages post silica instillation. C57BL/6 and Balb/c mice were instilled with silica (1 mg in PBS) or vehicle control and sacrificed at 1 or 3 days post-instillation. Alveolar macrophages (AM) were stained with anti-CD11b and anti-CD11c and incubated with a fluorescent caspase-1 substrate. CD11b and CD11c expression was used to identify macrophages from the total lung population and median fluorescent intensity from capsase-1 activation was measured using flow cytometry. Both strains showed increased caspase-1 activation at 1 and 3 days, with the increase being significant at the 3 day time point. C57BL/6 caspase-1 activation was lower than Balb/c at 1 day, but higher at 3 days post-instillation. Results are the mean percent increase over PBS controls ± SEM (***P<0.01*).

Assay	Exposure Time (Days)	Strain	Interleukin (IL)-1ß (pg/ml) PBS	Interleukin (IL)-1ß (pg/ml) Silica[1]	Silica Treatment Percent Increase (Silica over PBS)	Silica Treatment *P* value	Strain Difference Percent Difference (Silica Balb/c over C57BL/6)	Strain Difference *P* value
AM *In vitro*	1	C57BL/6	7.25	28.17	288.83	N/A	390.07	N/A
		Balb/c	2.32	138.06	5850.82			
BMdM *In vitro*	1	C57BL/6	3.17	1903.19	59907.31	<0.001	40.81	0.1053
		Balb/c	2.90	2679.95	92350.39	<0.001		
AM *In vivo*	1	C57BL/6	1.71	31.59	1744.10	<0.001	60.50	0.0138
		Balb/c	4.21	50.71	1104.90	<0.001		
	3	C57BL/6	1.33	5.29	298.61	0.091	420.91	0.0663
		Balb/c	12.76	27.58	116.19	0.399		

Figure 5. Comparative analysis of secreted IL-1ß in both *in vivo* and *in vitro* systems. C57BL/6 and Balb/c mice were lavaged and the alveolar macrophages (AM) plated in 96-well plates. AM and BMdM were treated with silica (200 µg/ml in RPMI) or vehicle control or co-cultured with macrophage stimulation (OVA). Alternatively, mice from each strain were instilled with silica (1 mg in PBS) or vehicle control and sacrificed and lavaged at 1 or 3 days post-instillation. Secreted 17 kDa mature IL-1ß in lavage fluid or cell culture supernatant was measured with enzyme-linked immunosorbant assay (ELISA). Results are the mean and are considered significant when *P<0.05*. [1]*In vitro* silica groups were stimulated with OVA to stimulate the NF-κB signaling pathway.

Materials and Methods

***In vitro* studies**

- Bone marrow was isolated from C57BL/6 and Balb/c mice and cultured with macrophage colony stimulating factor to induce differentiation into BMdM.
- Alveolar macrophages (AM) were collected through whole lung lavage of untreated C57BL/6 and Balb/c mice.
- AM and BMdM were treated with silica (200 µg/ml) for 1 hour and plated 100 µl/well at 10^6 cells/ml in 96-well plates.
- AM and BMdM were co-cultured with or without ovalbumen (OVA) to stimulate the NF-κB pathway and incubated for 24 hours.
- Supernatants were collected for cytokine analysis and cells were processed for Western Blot or real time polymerase chain reaction (RT-PCR).

***In vivo* studies**

- Age-matched Balb/c and C57BL/6 mice (6-8 weeks old) were instilled trans-orally with 25 µl silica (40 mg/ml in PBS) or vehicle.
- Mice were sacrificed 1- or 3-days post-instillation and whole lung lavage fluid (4 x 1.0 ml PBS) collected for cellular and cytokine analysis.

Messenger RNA (mRNA) Analysis

- AM collected from lavage fluid and BMdM collected from culture were treated with Trizol to extract RNA.
- RT-PCR was used with pro-IL-1ß-specific primers to amplify and quantify the amount of pro-IL-1ß mRNA.

Protein Analysis

- BMdM collected from culture were lysed and protein was separated by SDS-PAGE and transferred for western blot analysis.
- Anti- IL-1ß mAb was used to probe for the 31 kDa intracellular pro-IL-1ß.
- Densitometry was performed and a regression model calculated to quantify the amount of pro-IL-1ß.
- Enzyme-Linked Immunosorbent Assay (ELISA, R&D Systems, Minneapolis, MN) was used to measure the concentration of the 17 kDa secreted IL-1ß in lavage and cell culture supernatants.

Caspase-1 Activation

- Cells were co-stained with anti-CD11b and anti-CD11c fluorescently labeled mAbs to identify neutrophil and macrophage populations.
- Cleavage of Caspalux®1-E_1D_2 (OncoImmunin Inc., Gaithersburg, MD), a fluorescent caspase-1 substrate (YVHDAP) was used to measure caspase-1 activation in alveolar macrophages.

Summary and Conclusions

- C57BL/6 mice expressed higher levels of IL-1ß mRNA.
- Balb/c expressed higher levels of the translation product and the mature protein.
- There was no significant difference between C57BL/6 and Balb/c caspase-1 activation.
- It is possible that the two mouse strains differ in translation and modification machinery that results in higher levels of secreted IL-1ß in the Balb/c strain, even with lower levels of mRNA.

Future Directions

- Instill C57BL/6 mice with IL-1ß and IL-1ß to see if the higher level of inflammatory cytokines is pushing towards the Th2 phenotype.
- Further characterize translation of IL-1ß mRNA in the two strains to elucidate the reason for the difference in protein levels.
- Compare events leading to inflammasome activation in the two strains.

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