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14. ABSTRACT

We discovered that germline BAP1 mutations cause a novel cancer syndrome characterized by a very high incidence of malignant mesothelioma (MM). In order to study the mechanism(s), we have conducted a number of in vitro and in vivo experiments and obtained very good results. We found that BAP1 silenced HM cells (and macrophages) release more HMGB1 into the extra cellular space. These in vitro findings suggested that germline BAP1 mutations by increasing the release of HMGB1 create an environment favorable to malignant transformation. Moreover, we found that BAP1 silenced HM cells are much less sensitive to asbestos induced cytotoxicity compared to cells with wild type BAP1, and a larger pool of cells survives asbestos exposure increasing the probability of malignant transformation. Accordingly we found that BAP1 silenced HM cells exposed to asbestos form significantly more foci in tissue culture compared to cells containing wild type BAP1. Together these in vitro studies suggested that germline BAP1 mutations would increase susceptibility to asbestos carcinogenesis, an hypothesis that we tested in Aim 3 in mice and that was proven correct. We found that $BAP1^{+/-}$ mice develop more MMs and had shorter survival (probably related to earlier tumor development) compared to wild type littermates. Moreover, BAP1 loss increased the susceptibility to low doses of asbestos that rarely cause MM in animals carrying wild type BAP1. Mechanistically, we linked the increased susceptibility of BAP1+/- mice to asbestos carcinogenicity to differences in the chronic inflammatory response, and to the release of specific cytokines and chemokines that follows asbestos exposure in BAP1 +/- mice.

15. SUBJECT TERMS
mesothelioma, BAP1, asbestos, mechanisms

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Introduction

We have previously reported that germline BAP1 mutations cause a novel cancer syndrome characterized by high incidence of malignant mesothelioma (MM), uveal melanoma, cutaneous melanoma and other cancers. During the Year-1, we found that BAP1 can regulate NF-kB activity. Since NF-κB activation is a critical signaling pathway for mesothelial cell transformation and MM development as we have previously demonstrated (Yang H et al, PNAS 2006), the new findings on BAP1's influence on NF-κB activity contributed our understanding regarding how BAP1 mutation predispose people to cancer.

During the Year-2, we further assessed the impact of BAP1 in the process of asbestos-induced cell transformation in vitro and also assessed the impact of BAP1 in the process of mesothelioma development and progression in vivo. We studied the influence of BAP1 expression on HMGB1 levels as well as cytokine changes induced by BAP1. In brief, we found that BAP1 silenced HM cells (and macrophages) release more HMGB1 into the extra cellular space. These in vitro findings suggested that germline BAP1 mutations by increasing the release of HMGB1 create an environment favorable to malignant transformation. Moreover, we found that BAP1 silenced HM cells are much less sensitive to asbestos induced cytotoxicity compared to cells with wild type BAP1, and a larger pool of cells survives asbestos exposure increasing the probability of malignant transformation. Accordingly we found that BAP1 silenced HM cells exposed to asbestos form significantly more foci in tissue culture compared to cells containing wild type BAP1. Together these in vitro studies suggested that germline BAP1 mutations would increase susceptibility to asbestos carcinogenesis, an hypothesis that we tested in Aim 3 in mice and that was proven correct. we found that $BAP1^{+/-}$ mice develop more MMs and had shorter survival (probably related to earlier tumor development) compared to wild type littermates. Moreover, BAP1 loss increased the susceptibility to low doses of asbestos that rarely cause MM in animals carrying wild type BAP1. Mechanistically, we linked the increased susceptibility of BAP1+/- mice to asbestos carcinogenicity to differences in the chronic inflammatory response, and to the release of specific cytokines and chemokines that follows asbestos exposure in BAP1 +/- mice. We are reporting our findings in detail below.

Body:

(1) To assess the impact of BAP1 in the process of asbestos-induced human mesothelial cells (HM) transformation *in vitro*, we performed in vitro HM transformation assay using our established tissue culture system. We found that by knocking down BAP1 expression using siRNA, HM underwent morphological transformation and developed anchorage independent growth, as well as formed a higher number of tridimensional foci compared to control HM containing wild type BAP1 (Figure 1).

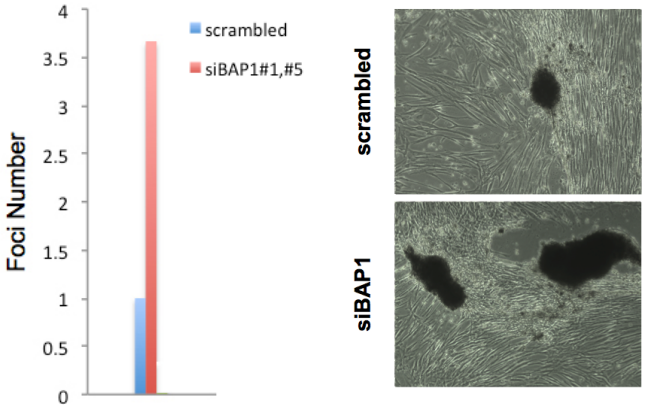

Figure 1. Increased foci formation was observed in HM following BAP1 silencing. HM transfected with mock- (scrambled) or siRNA-BAP1 (siRNA#1 and siBAP1#5) were exposed to asbestos at 5 μg/cm^2 and were cultured for up to 2 months for observation of foci formation.
Left panel, foci number; right panel, foci images.

(2) We studied cell growth and cell death in asbestos-exposed HM with or without BAP1 expression. Moreover, we measured HMGB1 release in HM in which we knocked down BAP1 compared to control HM containing wild type BAP1. We found that knocking down BAP1 using siRNA led to increased HMGB1 release into the cell culture media (Figure 2). We further tested macrophages and found that macrophages in which we knocked down BAP1 using siRNA also released more HMGB1 into extra cellular space compared to macrophages containing wild type BAP1 (Figure 3).

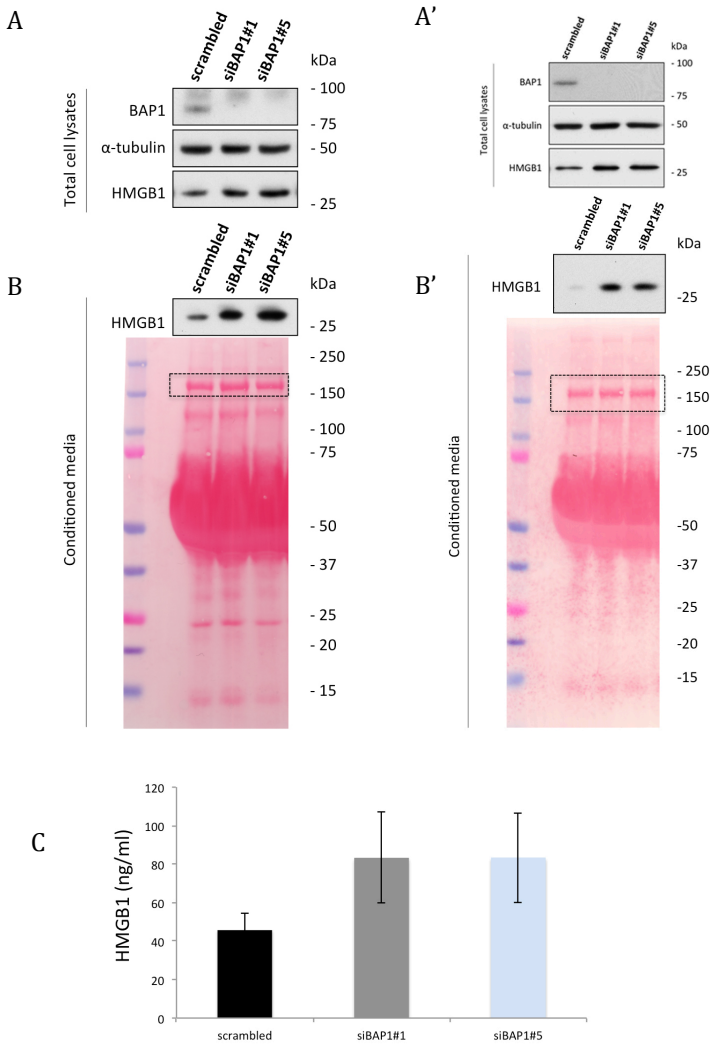

Figure 2. HM cells release more HMGB1 into extra cellular space after BAP1 silencing.
(A and A' represent two separate experiments). Total protein was extracted from HM transfected with mock- (scrambled) or siRNA-BAP1 (siRNA#1 and siBAP1#5) and were analyzed by Western blot. (B and B') Conditional cell culture media were concentrated with Amicon centrifugal filter (Millipore) and then were analyzed by Western blot. (lower panel is the Ponceau staining of the gel showing equal loading). (C) HMGB1 concentrations in the concentrated cell culture media were analyzed by ELISA assay. Experiments were done in triplicate.

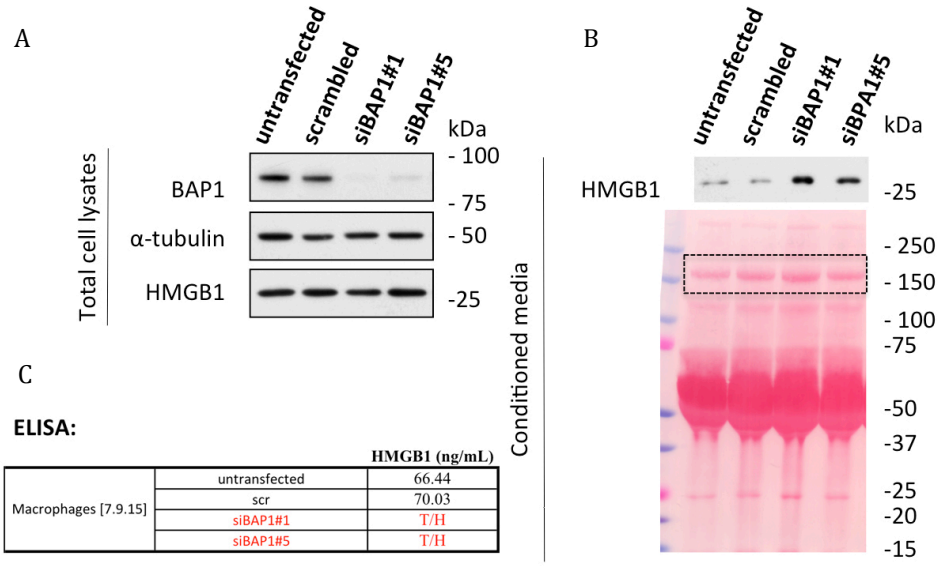

Figure 3. Macrophages release more HMGB1 into the extra cellular space after BAP1 silencing.
(A) Total protein was extracted from macrophages transfected with mock- (scrambled) or siRNA-BAP1 (siRNA#1 and siBAP1#5) and were analyzed by Western blot. (B) Cell culture media were concentrated and analyzed by Western blot. (C) HMGB1 concentrations in the concentrated cell culture media were analyzed by ELISA assay. (Note: The concentrations of HMGB1 in the concentrated media of macrophages transfected with siBAP1#1 and siBAP1#5 were very high and exceeded the detection range of the ELISA kit.

(3) We observed that when we silenced BAP1 in HM, these cells were more resistant to asbestos induced cytotoxicity (Figure 4 A and B), and, accordingly we found that more HM survived after asbestos exposure when BAP1 was silenced. Moreover, although BAP1 silencing induced more HMGB1 release in normal culture condition- as we show above (Figs 2 and 3), HM with silenced BAP1 exposed to asbestos released less HMGB1 compared to control HM containing wild type BAP1 (Figure 4C).

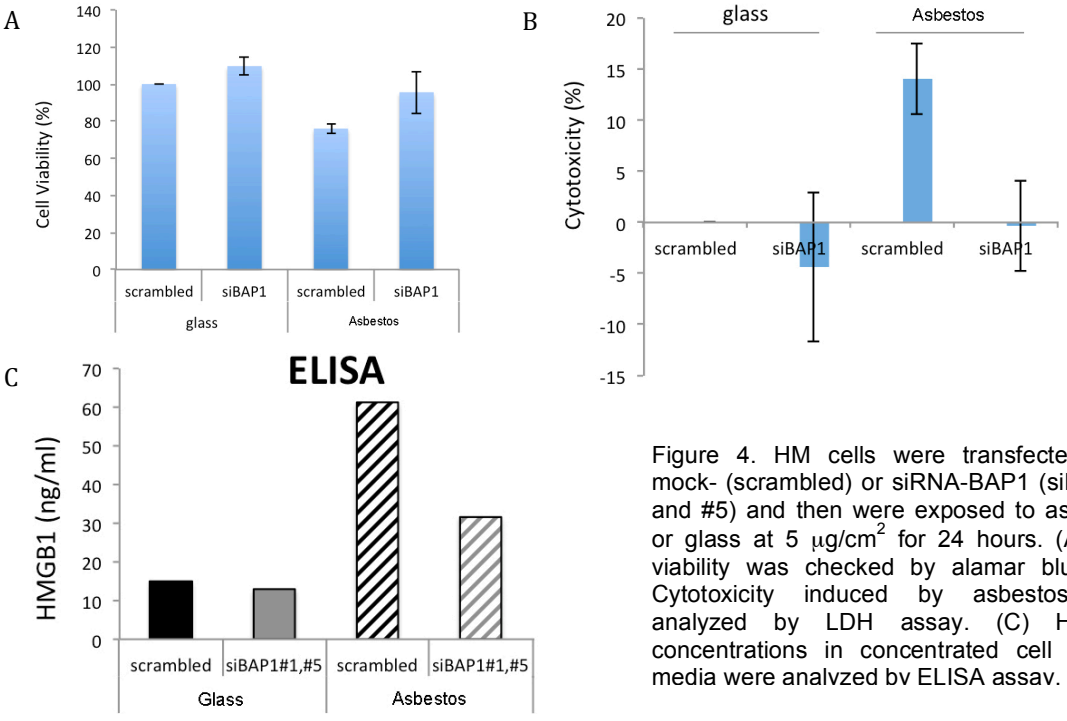

Figure 4. HM cells were transfected with mock- (scrambled) or siRNA-BAP1 (siRNA#1 and #5) and then were exposed to asbestos or glass at 5 μg/cm^2 for 24 hours. (A) Cell viability was checked by alamar blue. (B) Cytotoxicity induced by asbestos was analyzed by LDH assay. (C) HMGB1 concentrations in concentrated cell culture media were analyzed by ELISA assay.

(4) To assess the impact of BAP1 in the process of MM development and progression *in vivo*, we performed animal experiment using transgenic mice with heterozygous BAP1 knockout (BAP1+/- mice). We found that the incidence of MM was significantly higher in BAP1+/- mice exposed to asbestos compared to BAP1+/+ wild type mice and that mesotheliomas in BAP1+/- mice developed with a reduced latency. Moreover, we observed dramatic difference in MM incidence in mice receiving low doses of asbestos (36% in BAP1 +/- mice vs. 8% in BAP1 +/+ wild type mice) (Figure 5).
The results are very significant and are exactly as we had anticipated in our grant proposal. We performed histological evaluation of tumors. The results are reported in Figures 5-7 below and also in our recently published manuscript (Napolitano A, et al. Oncogene 2015).

(5) We also compared the profiles of cytokines and chemokines present in peritoneal lavages of BAP1 +/- and wild type mice injected with asbestos. Compared to wild type littermates, the levels of monocyte chemoattractant protein-1 (MCP-1) were significantly lower in BAP1+/- mice exposed to glass (2.5 pg/mL [2.3-5.2] vs 33.6 pg/mL [6.5-51.7], $P < 0.01$) and in $BAP1^{+/-}$ mice exposed to asbestos (52.4 pg/mL [4.7-113.4] vs 178.5 pg/mL [102.9-373.2], $P < 0.05$). Analogously, compared to wild type littermates, the levels of leukemia inhibitory factor (LIF) were significantly lower in the BAP1+/- mice exposed to glass (0.9 pg/mL [0.9-1.0] vs 6.9 pg/mL [1.1-13.5], $P < 0.01$), and in the BAP1+/- mice exposed to asbestos (78.2 pg/mL [41.0-134.4] vs 201.9 pg/mL [116.9-274.8], $P < 0.05$). Moreover, lavages from BAP1+/- mice exposed to asbestos contained significantly lower amounts of keratinocyte-derived chemokine (KC) compared to wild type littermates (253.4 pg/mL [19.5-557.1] vs 675.3 pg/mL [469.8-1741.5], $P < 0.05$). We also observed that eotaxin levels were significantly lower in BAP1+/- mice compared to wild type littermates in the glass exposed control group (1.73 ng/mL [1.11-2.06] vs 3.27 ng/mL [1.94-3.92], $P < 0.05$); the same trend, although non-significant, was retained following asbestos exposure (3.33 ng/mL [2.56-4.33] vs 4.70 ng/mL [3.13-6.30], $P = 0.28$). Levels of IL-6 also differed between genotypes upon asbestos exposure, though this difference did not reach nominal significance ($P = 0.08$). Both IL-6 and LIF belong to the IL-6 family of cytokines, and in our samples their levels significantly correlated ($R^2 = 0.62$, $P < 0.0001$) (Figure 6). Finally, levels of G-CSF, IL-5, IP-10, and VEGF significantly increased after asbestos exposure, independently of the genotype (Figure 7). Levels of several other cytokines were below the lower limit of detection of our assay. Together, these results indicated that germline BAP1 heterozygosity significantly influenced the peritoneal inflammatory response upon asbestos exposure.

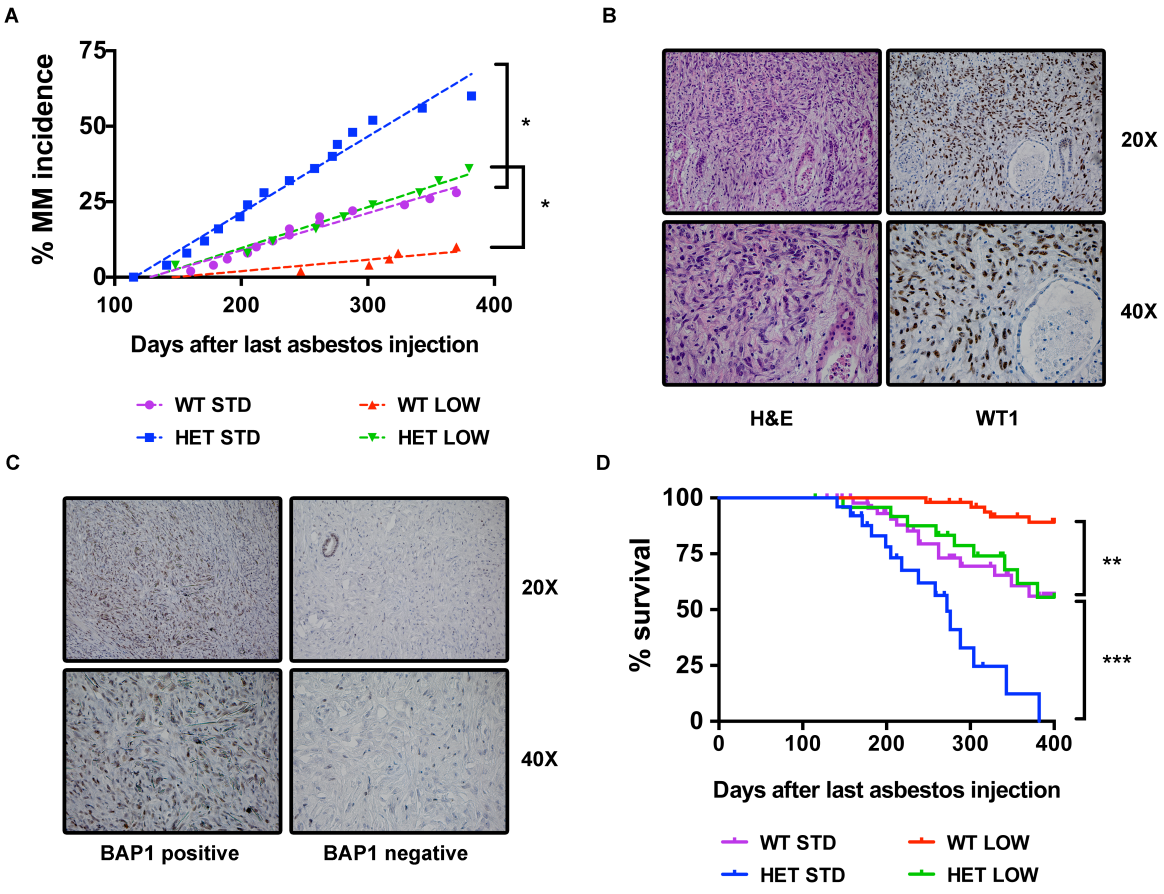

Figure 5. BAP1+/- mice develop more MMs and have shorter survival compared to wild type littermates. Briefly, BAP1+/+ mice (WT) (n = 50 per group) and BAP1+/- mice (HET) (n = 25 per group) were injected intraperitoneally every week for ten weeks with 0.05 mg (low dose, LOW) or 0.5 mg (standard dose, STD) of asbestos. 0.5 mg of glass beads were injected at the same schedule as control. (a) MM incidence in BAP1+/- mice and wild type littermates after long-term exposure to glass beads or asbestos fibers (standard and low dose) was compared using Fisher's exact test. * ($P < 0.05$) (b) Formalin-fixed/paraffin-embedded samples were stained with Hematoxylin and Eosin (H&E) according to standard procedure. The pathological diagnosis of mesothelioma was based on H&E staining and supported by WT1 nuclear staining in tumor cells. H&E and immunostainings were blindly interpreted by two US board specialized pathologists with expertise in human and animal mesotheliomas (c) Tumors were also stained with a rabbit polyclonal anti-BAP1 antibody to evaluate presence and localization of BAP1. (**d**) Survival curves of BAP1+/- mice and wild type littermates after long-term exposure to asbestos fibers (standard and low dose) were compared using log-rank (Mantel-Cox) test. . ** ($P < 0.01$), *** ($P < 0.001$).

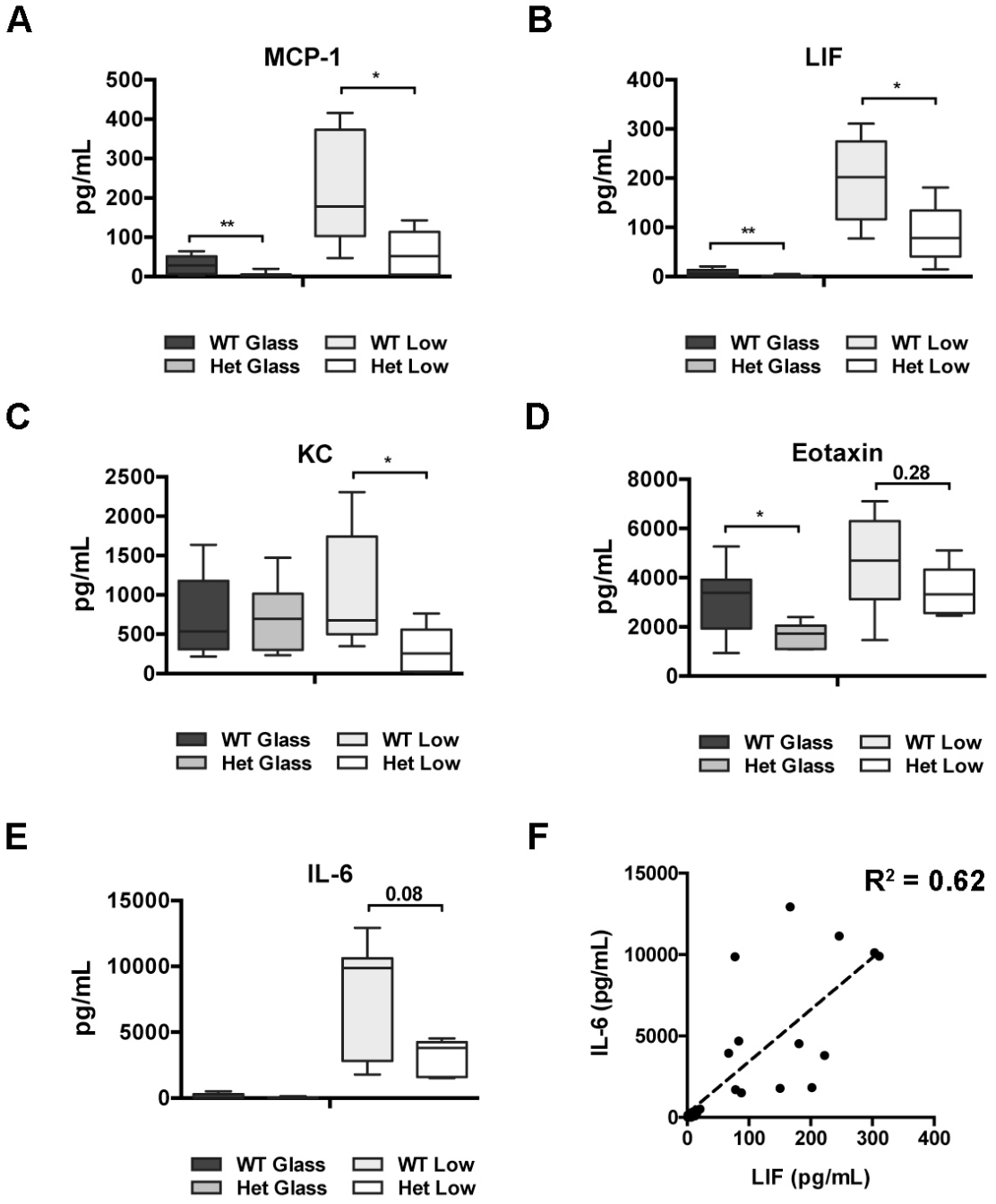

Figure 6. Several cytokines and chemokines are differentially expressed in lavage from BAP1+/- mice following asbestos exposure. The supernatants recovered from the peritoneal lavages were concentrated 45-60 times using Amicon Ultra Centrifuge Filters with a 3,000 Dalton cutoff. Levels of 32 cytokines and chemokines were detected in concentrated lavages using human cytokine multiplex kits (EMD Millipore Corporation, Billerica, MA). Levels of MCP-1 (A), LIF (B), KC (C), eotaxin (D) and IL-6 (E) in lavages from BAP1 wild type and heterozygous mice after short-term exposure to glass beads or asbestos fibers. Comparisons between heterozygous and wild type groups were calculated using Mann-Whitney U test for rank comparisons. * ($P < 0.05$), ** ($P < 0.01$) (F) Correlation of IL-6 and LIF levels (both belonging to the IL-6 family of cytokines) calculated using linear regression. The experiment was replicated two times.

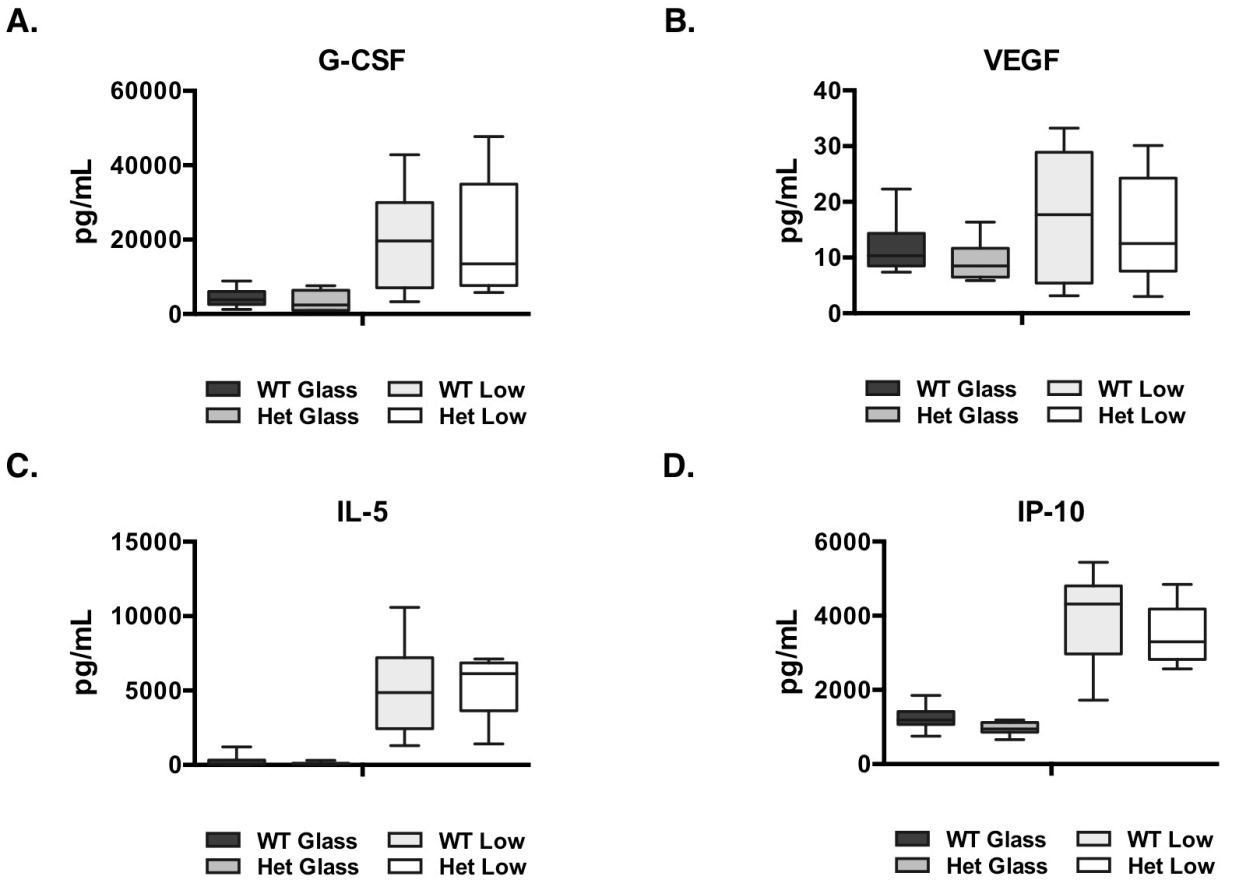

Figure 7. Levels of other cytokines and chemokines are not differentially expressed.
(A), G-CSF (B), VEGF (C), IL-5 (D) IP-10 in lavages from BAP1 wild type and heterozygous mice after short-term exposure to glass beads or asbestos fibers. Comparisons between heterozygous and wild type groups were calculated using Mann-Whitney U test for rank comparisons. No statistically significant differences were observed. The experiment was replicated two times

In summary, we discovered that BAP1+/- mice exposed to low doses of asbestos developed MMs at a similar rate as BAP1+/+ mice exposed to 10 times higher doses. Therefore, although it is not possible to directly compare the low-dose exposure in mice to indoor and/or outdoor environmental exposure in humans, our findings support our hypothesis that germline BAP1 heterozygosity increases susceptibility to the carcinogenic effects of low doses of asbestos. Moreover, our results suggest a novel, complex model of asbestos-induced MM pathogenesis, in which the chronic inflammatory response can have preferentially anti-tumoral or pro-tumoral roles, depending on the cellular and soluble mediators involved

Key Research Accomplishments:

We have accomplished the proposed experiments and achieved the following findings.

(I) BAP1 silencing induces more foci formation in HM cells exposed to asbestos.

(II) Both HM and macrophages cells release more HMGB1 into the extra cellular space following BAP1 silencing.

(III) HM cells with silenced BAP1 are more resistant to asbestos induced cytotoxicity, therefore less HM die: consequently, the amount HMGB1 passively released by dying cells following asbestos exposure, and measured in the tissue culture medium, is decreased in cells carrying BAP1 mutations compared to cells with wild type BAP1.

(IV) BAP1+/- mice develop more MMs and have shorter survival compared to wild type littermates. Moreover, BAP1 germline mutations increase the susceptibility to low dose of asbestos and mesothelioma.

(V) Several cytokines and chemokines are differentially expressed in lavage from BAP1+/- mice following asbestos exposure, in particular MCP-1, LIF, KC, eotaxin and IL-6, suggesting that they play an important role in the mechanisms responsible for the increased susceptibility of BAP1+/- mice to asbestos and mesothelioma.

Reportable Outcomes:

Our findings suggest that minimal exposure to carcinogenic fibers may significantly increase the risk of malignant mesothelioma in genetically predisposed individuals carrying germline *BAP1* mutations. Thus we proposed that carriers of germline BAP1 mutations should avoid jobs in trades were even minimal exposure to asbestos may occur –such as mechanics, electricians, and certain military branches, such as military working on ships and submarines.

Our suggestion that BAP1 mutant carriers should avoid trades in which a minimal exposure to asbestos – i.e., an amount of exposure that would not be considered to increase mesothelioma risk among the population at large- may occur, was reviewed and agreed upon by a board of experts and has now been reported as suggestive guideline for carriers of BAP1 mutations (see below “Other achievements”).

Our results elucidated some of the mechanisms of how BAP1 loss influences the cellular responses to asbestos and provide a rationale for the increased the susceptibility of carriers of BAP1+/- mutations to asbestos and MM.

Conclusions:

We found that BAP1 silenced HM cells (and macrophages) release more HMGB1 into the extra cellular space. These in vitro findings suggested that germline BAP1 mutations by increasing the release of HMGB1 create an environment favorable to malignant transformation. Moreover, since we found that BAP1 silenced HM cells are much less sensitive to asbestos induced cytotoxicity compared to cells with wild type BAP1, a larger pool of cells survives asbestos exposure increasing the probability of malignant transformation. Accordingly we found that

BAP1 silenced HM cells exposed to asbestos form significantly more foci in tissue culture compared to cells containing wild type BAP1. Together these in vitro studies suggested that germline BAP1 mutations would increase susceptibility to asbestos carcinogenesis, an hypothesis that we tested in Aim 3 in mice and that was proven correct.

Briefly, we found that $BAP1^{+/-}$ mice develop more MMs and had shorter survival (probably related to earlier tumor development) compared to wild type littermates. Moreover, BAP1 loss increased the susceptibility to low doses of asbestos that rarely cause MM in animals carrying wild type BAP1. Mechanistically, we linked the increased susceptibility of BAP1+/- mice to asbestos carcinogenicity to differences in the chronic inflammatory response, and to the release of specific cytokines and chemokines that follows asbestos exposure in BAP1 +/- mice.

References:

Publications, Abstracts, and Presentations:

(I) Peer-Reviewed Scientific Journals:

1. Napolitano A, Pelegrini L, Anwesha D, Larson D, Tanji M, Flores EG, Kendrick B, Lapid D, Powers A, Kanodia S, Pastorino S, Pass HI, Dixit V, Yang H and Carbone M. Minimal asbestos exposure in germline BAP1 heterozygous mice is associated with deregulated inflammatory response and increased risk of mesothelioma. Oncogene. 2015 Jun 29. (Epub ahead of print) PMID: 26119930.
2. Bononi A, Napolitano A, Pass HI, Yang H, Carbone M. Latest developments in our understanding of the pathogenesis of mesothelioma and the design of targeted therapies. Expert Rev Respir Med. 2015 Oct;9(5):633-54. PMID: 26308799
3. Carbone M, Chao A, Kanodia S, Miller A, Wali A, Weissman D, Adjei A, Baumann F, Boffetta P, Buck B, Perrot M, Dogan AU, Gavett S, Gualtieri A, Hassan R, Hesdorffer M, Hirsch FR, Larson D, Mao W, Masten S, Pass HI, Peto J, Pira E, Steele I, Tsao A, Woodard GA, Yang H and Malik S. Consensus Report of the 2015 Weinman International Conference on Mesothelioma. J Thorac Oncol. 2016 (accepted for publication pending minor revisions)

(II) Abstracts

Napolitano A, Pelegrini L, Anwesha D, Larson D, Tanji M, Flores EG, Kendrick B, Lapid D, Powers A, Kanodia S, Pastorino S, Pass HI, Dixit V, Yang H and Carbone M. Germline BAP1 heterozygous mice are sensitive to low dose of asbestos and have increased risk of mesothelioma. AACR 2015

(III) Meeting Presentations:

I was invited to give talks at several National and International meetings, where I presented (or will present) these data:

1. University of Ferrara. 2015, April, Ferrara, Italy.
2. New Frontiers in Oncology. 2015, April, Rome, Italy.
3. Mt Sinai Hospital. 2015, June. New York, NY.
4. Magna Graecia University and Tommaso Campanella Cancer Center. 2015, September. Catanzaro, Italy.
5. 7th International Symposium DAMPS and HMGB. 2015, September. Bonn, Germany.

6. Weinman International Conference on Mesothelioma, Honolulu, Hawaii, Nov 2015.
7. University of Torino. 2015, November, Turin, Italy.
8. International Mesothelioma Interest Group. Birmingham, UK May 1-4, 2016

Appendices:

Inventions, patents and licenses:

Methods and Kits for Analysis of HMGB1 Isoforms
Inventors: Carbone, M., Yang, H.
Filing #: US Provisional Patent application no. 62/106,092
Year Filed: 2015
Abstract: Methods of determining signatures of HMGB1 isoforms in a subject, and the use of HMGB1 and its isoforms as biomarkers for asbestos exposure and mesothelioma detection

Biomarker of Asbestos Exposure and Mesothelioma
Inventors: Carbone, M., Yang, H., Pass, H. I.
Filing #: US Application no. 14/123,722
Year Filed: 2013 (Notice of allowance issued in 2015)
Abstract: Methods of diagnosing asbestos exposure or mesothelioma, and methods of differentiating whether a tumor of the lung is lung cancer or mesothelioma:.

Treatment and Prevention of Cancer with HMBG1 Antagonists
Inventors: Carbone, M., Yang, H., Bianchi, M.E.
Filing #: US Application no. 14/123,607
Year Filed: 2013
Abstract: Methods and Compositions for treating and preventing cancer; more particularly to treating or preventing malignant mesothelioma with antagonists of HMGB1

Oncogene (2015), 1–7

www.nature.com/onc

SHORT COMMUNICATION

Minimal asbestos exposure in germline *BAP1* heterozygous mice is associated with deregulated inflammatory response and increased risk of mesothelioma

A Napolitano[1,2], L Pellegrini[1], A Dey[3], D Larson[1], M Tanji[1], EG Flores[1], B Kendrick[1], D Lapid[1], A Powers[1], S Kanodia[4], S Pastorino[1], HI Pass[5], V Dixit[3], H Yang[1] and M Carbone[1]

Germline *BAP1* mutations predispose to several cancers, in particular malignant mesothelioma. Mesothelioma is an aggressive malignancy generally associated with professional exposure to asbestos. However, to date, we found that none of the mesothelioma patients carrying germline *BAP1* mutations were professionally exposed to asbestos. We hypothesized that germline *BAP1* mutations might influence the asbestos-induced inflammatory response that is linked to asbestos carcinogenesis, thereby increasing the risk of developing mesothelioma after minimal exposure. Using a $BAP1^{+/-}$ mouse model, we found that, compared with their wild-type littermates, $BAP1^{+/-}$ mice exposed to low-dose asbestos fibers showed significant alterations of the peritoneal inflammatory response, including significantly higher levels of pro-tumorigenic alternatively polarized M2 macrophages, and lower levels of several chemokines and cytokines. Consistent with these data, $BAP1^{+/-}$ mice had a significantly higher incidence of mesothelioma after exposure to very low doses of asbestos, doses that rarely induced mesothelioma in wild-type mice. Our findings suggest that minimal exposure to carcinogenic fibers may significantly increase the risk of malignant mesothelioma in genetically predisposed individuals carrying germline *BAP1* mutations, possibly via alterations of the inflammatory response.

Oncogene advance online publication, 29 June 2015; doi:10.1038/onc.2015.243

INTRODUCTION

Malignant mesothelioma (MM) is a deadly cancer usually localized to the pleural and peritoneal linings.[1] In the US and in the UK, ~3200 and ~2500 individuals are diagnosed with and die because of MM each year, respectively.[2,3] About 60–70% of mesotheliomas have been associated with exposure to carcinogenic mineral fibers, mainly asbestos.[1] Nevertheless, the risk of developing MM in high-risk cohorts professionally exposed to asbestos is ~5%, suggesting that other factors contribute to MM pathogenesis.[1] Mineral fibers promote mesothelioma inducing a chronic inflammatory reaction: on one hand, this results in the production of mutagenic oxygen and nitrogen radicals, and on the other hand, it provides damaged mesothelial cells with important survival signals.[4] Although chronic inflammation has been associated with the pathogenesis of several cancers, competent inflammatory cells also provide immunosurveillance, the host's protection process against nascent transformed cells expressing altered antigens.[5] In fact, different functional and phenotypical cell subtypes are associated to anti-tumoral or pro-tumoral immunity.[6] Macrophages (MΦ) can undergo different types of polarization based on the kind and levels of cytokines present in the local tissue environment. Classically activated (M1) MΦ have a pro-inflammatory anti-tumoral phenotype, whereas alternatively activated (M2) MΦ are involved in immunosuppression and tissue repair.[7] Tumor-associated macrophages represent one of the major populations of immune cells infiltrating tumors, and usually acquire functional characteristics similar to M2 MΦ.[8] The ratio between M2-like and M1-like tumor-associated macrophages has prognostic value in MM and other cancers, with the former usually associated with a worse prognosis.[9–11] However, the contribution of different MΦ subpopulations to the initiation of inflammation-induced cancers is still unclear. MM has a large number of tumor-associated macrophages, suggesting that they have an important role in this malignancy.[12]

Recently, we identified germline mutations in the tumor suppressor gene *BRCA1 associated protein-1* (*BAP1*) as causative of a novel hereditary cancer syndrome characterized by a very high risk of MM, uveal and cutaneous melanoma, several other malignancies and characteristic benign melanocytic tumors we named MBAITs.[13–15] The penetrance of the BAP1 cancer syndrome is ~100%, and several patients carrying germline *BAP1* mutations develop multiple cancers.[16] Notably, none of the germline *BAP1* heterozygous patients who developed MM reported professional exposure to asbestos fibers,[13,16] suggesting that either these MMs were not caused by asbestos or that minimal amounts of asbestos—as in the case of some indoor exposure[17] or naturally occurring outdoor environmental exposure[18]—may be sufficient to cause MM in germline *BAP1* mutation carriers. Here, we experimentally tested in a $BAP1^{+/-}$ murine model whether germline *BAP1* heterozygosity would result in alterations of the asbestos-induced inflammatory response, and whether low doses of asbestos might be sufficient to cause MM.

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E-mail: mcarbone@cc.hawaii.edu
Received 14 January 2015; revised 16 March 2015; accepted 20 April 2015

We used constitutive BAP1$^{+/-}$ mice (C57BL/6 background) generated by breeding mice with *loxP* sites flanking *BAP1* exons 4 and 5 with mice expressing a constitutive general Cre deleter.[19] Although homozygous BAP1 deficiency in mice results in embryonic lethality,[19] BAP1$^{+/-}$ mice are viable and healthy. Compared with wild-type littermates, BAP1$^{+/-}$ mice expressed about half the amount of BAP1 protein in relevant tissues (Supplementary Figure 1).

In our experiments, we used 10–12-week-old mice of either sex equally distributed in the experimental groups using a computational random number generator. All the experiments were approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC). Unless otherwise specified, results are presented as median (interquartile range).

RESULTS

First, we exposed BAP1$^{+/-}$ mice and BAP1$^{+/+}$ for 5 weeks to injections with glass beads or a low amount of crocidolite asbestos fibers (0.05 mg/week). After performing a peritoneal lavage, we counted the total number of peritoneal cells and determined via flow cytometry the percentage of total and subset-specific leukocytes. CD45+ leukocytes represented 95–99% of the total cells recovered in each group. In the glass control groups, macrophages and B cells represented the most abundant population, regardless of genotype (Table 1). Upon exposure to low-dose crocidolite fibers, the cellular inflammatory response was largely overlapping in mice with either genotype. We observed a significant increase in the total number of leukocytes and in the relative percentage of neutrophils, and, at the same time, a significant decrease in the percentage of B cells and macrophages (Table 1). Further characterization of the cell types revealed that exposure to crocidolite fibers induced significant alterations in macrophage polarization in BAP1$^{+/-}$ mice (Figure 1a). In the macrophages from BAP1$^{+/-}$ mice exposed to asbestos fibers, the normalized mean fluorescence intensity for CD206 (marker of M2 macrophages) was significantly higher compared with controls (197.1% (160.6–256.8) vs 163.1% (125.4–186.7), $P < 0.05$), whereas the normalized mean fluorescence intensity for CD86 (marker of M1 macrophages) was significantly lower compared with controls (74.6% (57.6–90.3) vs 95.8% (77.4–109.1), $P < 0.05$) (Figure 1b). Accordingly, the percentage of M1 macrophages (CD206 − CD86+ cells) was significantly lower in BAP1$^{+/-}$ mice (43.2% (28.9–44.9) vs 67.3% (46.7–78.2) of total macrophages, $P < 0.05$). On the other hand, the percentage of M2 macrophages (defined as CD206+ CD86 − cells) was significantly higher in BAP1$^{+/-}$ mice compared with wild-type littermates (3.8% (2.1–6.8) vs 1.2% (0.5–3.6) of total macrophages, $P < 0.05$). Double positive (CD206+ CD86+) macrophages, which represent a transition state from M1 to M2, were also more represented in BAP1$^{+/-}$ mice compared with wild-type littermates (40.0% (30.7–47.0) vs 26.0% (13.3–37.6) of total macrophages, $P < 0.05$) (Figure 1c). Moreover, the M2/M1 ratio (overall percentage of CD206+ cells divided by overall percentage of CD86+ cells) was significantly higher in asbestos-exposed BAP1$^{+/-}$ mice compared with controls (0.54 (0.48–0.66) vs 0.36 (0.16–0.56), $P < 0.05$) (Figure 1d).

Next, we compared the profiles of cytokines and chemokines present in peritoneal lavages of these same mice. Compared with wild-type littermates, the levels of monocyte chemoattractant protein-1 (MCP-1) were significantly lower in BAP1$^{+/-}$ mice exposed to glass (2.5 pg/ml (2.3–5.2) vs 33.6 pg/ml (6.5–51.7), $P < 0.01$) and in BAP1$^{+/-}$ mice exposed to asbestos (52.4 pg/ml (4.7–113.4) vs 178.5 pg/ml (102.9–373.2), $P < 0.05$) (Figure 2a). Analogously, compared with wild-type littermates, the levels of leukemia inhibitory factor were significantly lower in the BAP1$^{+/-}$ mice exposed to glass (0.9 pg/ml (0.9–1.0) vs 6.9 pg/ml (1.1–13.5), $P < 0.01$), and in the BAP1$^{+/-}$ mice exposed to asbestos (78.2 pg/ml (41.0–134.4) vs 201.9 pg/ml (116.9–274.8), $P < 0.05$) (Figure 2b). Moreover, lavages from BAP1$^{+/-}$ mice exposed to asbestos contained significantly lower amounts of keratinocyte-derived chemokine compared with wild-type littermates (253.4 pg/ml (19.5–557.1) vs 675.3 pg/ml (469.8–1741.5), $P < 0.05$) (Figure 2c). We also observed that eotaxin levels were significantly lower in BAP1$^{+/-}$ mice compared with wild-type littermates in the glass-exposed control group (1.73 ng/ml (1.11–2.06) vs 3.27 ng/ml (1.94–3.92), $P < 0.05$); the same trend, although non-significant, was retained following asbestos exposure (3.33 ng/ml (2.56–4.33) vs 4.70 ng/ml (3.13–6.30), $P = 0.28$) (Figure 2d). Levels of interleukin (IL)-6 also differed between genotypes upon asbestos exposure, though this difference did not reach nominal significance ($P = 0.08$) (Figure 2e). Both IL-6 and leukemia inhibitory factor belong to the IL-6 family of cytokines, and in our samples, their levels significantly correlated ($R^2 = 0.62$, $P < 0.0001$) (Figure 2f). Finally, levels of granulocyte colony-stimulating factor, IL-5, IP-10 and vascular endothelial growth factor significantly increased after asbestos exposure, independently of the genotype (Supplementary Figures 2a-d). Levels of several other cytokines were below the lower limit of detection of our assay. Together, these results

Table 1. Major subpopulations of peritoneal leukocytes are not influenced by germline *BAP1* heterozygosity

Cells	*WT Glass*	*WT Asb*	*Het Glass*	*Het Asb*	P *value*			
					WT (G vs A)	*Het (G vs A)*	*Glass (WT vs Het)*	*Asb (WT vs Het)*
Total leukocytes ($\times 10^6$)	2.7 (1.3–3.6)	6.1 (3.5–14.2)	2.7 (1.3–4.9)	8.5 (4.9–12.7)	< 0.01	< 0.05	ns	ns
Neut (%)	1.8 (1.6–2.4)	13.0 (11.3–16.4)	1.1 (0.8–2.2)	10.4 (9.9–16.6)	< 0.0001	< 0.001	ns	ns
B cells (%)	20.4 (17.5–26.3)	12.7 (9.9–14.2)	19.4 (17.8–21.3)	10.3 (8.6–12.6)	< 0.01	< 0.01	ns	ns
T cells (%)	7.0 (5.1–10.4)	5.0 (3.8–6.4)	6.4 (4.1–10.8)	7.7 (4.3–8.4)	ns	ns	ns	ns
MΦ (%)	33.4 (27.0–38.5)	21.3 (18.6–27.5)	24.2 (20.1–45.2)	19.2 (14.6–22.8)	< 0.01	< 0.05	ns	ns

Abbreviation: WT, wild type. BAP1$^{+/-}$ mice ($n = 7$ per group) and BAP1$^{+/+}$ ($n = 9$ per group) were injected intraperitoneally every week for 5 weeks with 0.05 mg of inert glass beads or crocidolite asbestos fibers, for a total dose of 0.25 mg per mouse. Sample size was estimated hypothesizing a 60% difference in the levels of at least one cytokine. Full mineralogical characterization of crocidolite fibers used in these experiments was reported previously.[46] Next, mice were killed by CO_2 asphyxiation, and the abdominal cavity was washed with 5 ml of phosphate-buffered saline. The peritoneal cells obtained were pelleted and supernatant was removed for later cytokine analysis. Cells were blindly characterized with the following antibodies: CD45 (leukocytes; anti-CD45-BV711, 563709, BD Biosciences, San Jose, CA, USA), F4/80 (MΦ; anti-F4/80-AlexaFluor488, MCA497A488T, AbD Serotec, Raleigh, NC, USA), Ly-6G (neutrophils; anti-Ly6G-BV421, 562737, BD Biosciences), CD3 (T cells; anti-CD3-APC, 17-0032-80, eBioscience, San Diego, CA, USA) and B220 (B cells; anti-B220-PE, 561878, BD Biosciences). Comparisons between groups were calculated using Mann–Whitney U test for rank comparisons. Results are presented as median (interquartile range).

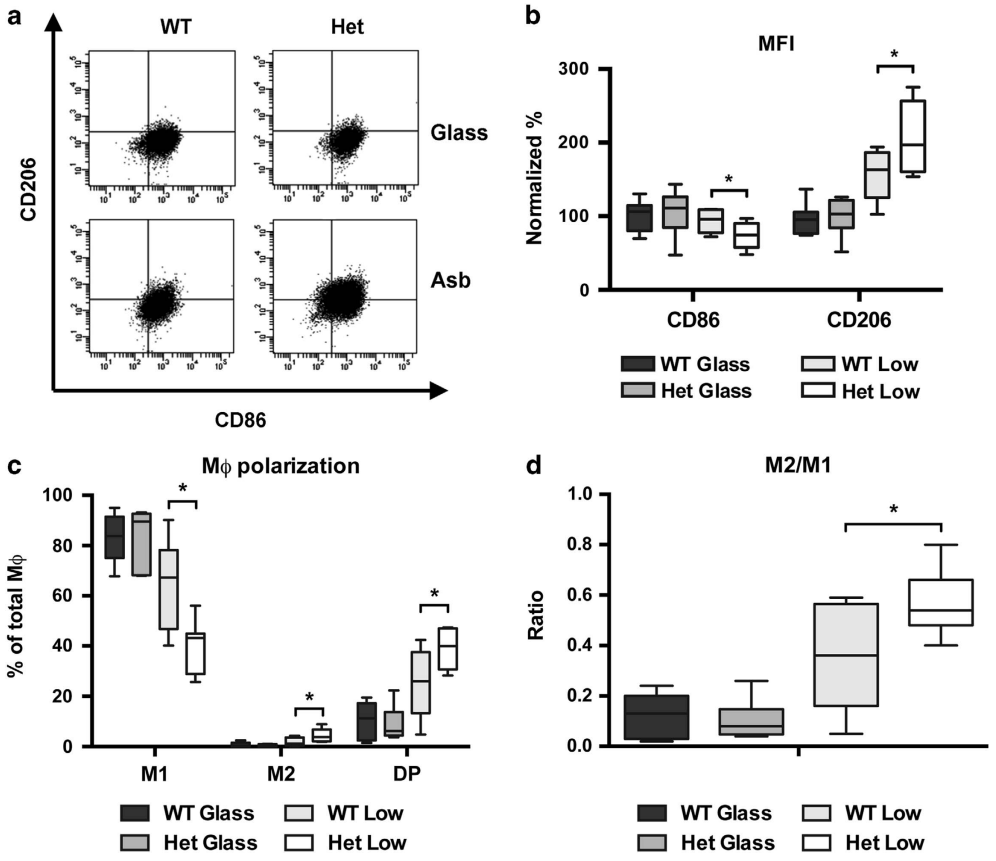

Figure 1. MΦ polarization is altered in BAP1$^{+/-}$ mice exposed to low doses of asbestos fibers. Macrophages and macrophage subtypes were identified using a separate tube of peritoneal cells stained for general MΦ markers CD11b (anti-CD11b-Bv711, 563168, BD Biosciences) and F4/80, CD206 (M2 marker; anti-CD206-APC, 141707, BioLegend, San Diego, CA, USA) and CD86 (M1 marker; anti-CD86-PE, 561963, BD Biosciences). (**a**) Representative flow cytometry dot plot of peritoneal MΦ in BAP1$^{+/-}$ mice and wild-type littermates after short-term treatment with glass beads or crocidolite asbestos. (**b**) Mean fluorescence intensities of CD86 and CD206. (**c**) Percentage of MΦ subpopulations: M1 (CD86+ CD206-), M2 (CD86- CD206+), double positive (DP) (CD86+ CD206+). (**d**) M2/M1 ratio (overall percentage of CD206+ cells divided by overall percentage of CD86+ cells). Comparisons between heterozygous and wild-type groups were calculated using Mann–Whitney U test for rank comparisons. *$P < 0.05$. The experiment was replicated two times.

indicated that germline *BAP1* heterozygosity significantly influenced the peritoneal inflammatory response upon asbestos exposure.

Therefore, we sought to experimentally study the relationship between asbestos dosage and MM carcinogenesis in the context of *BAP1* heterozygosis. On the basis of previous publications on murine models,[20,21] and on our own experience (Carbone, unpublished observations), doses of asbestos ranging from 3 to 5 mg induce MM in ~ 20–40% of exposed animals, while 0.5 mg of asbestos induce MM in 0–10% of exposed animals. BAP1$^{+/+}$ mice and BAP1$^{+/-}$ mice received 10 weekly injections of 0.5 mg of crocidolite asbestos fibers (total of 5 mg, referred to as 'standard-dose' as it is the dose most commonly used to induce MM in rodents), 0.05 mg of crocidolite fibers (total of 0.5 mg, referred to as 'low-dose') or 0.5 mg of inert glass beads (total of 5 mg, negative control). During the 13 months of follow-up after the last injection, we did not observe MM or any other spontaneous tumor in the glass control groups. In mice exposed to asbestos fibers, MM was the only malignancy observed. In the low-dose group, crocidolite fibers caused pathologically confirmed MM in 9/25 (36.0%) BAP1$^{+/-}$ mice compared with 5/50 (10.0%) BAP1$^{+/+}$ mice ($P = 0.010$). Similarly, in the standard-dose group, MM was diagnosed in 15/25 (60.0%) BAP1$^{+/-}$ mice compared with 14/50 (28.0%) BAP1$^{+/+}$ mice ($P = 0.011$) (Figure 3a). Immunohistochemical staining of the tumors revealed expression of the mesothelial marker WT1 (Figure 3b), supporting the histologic diagnosis of MM. In sporadic human MM, somatic *BAP1* inactivation is one of the most frequent events, and it has been reported in about 40–60% of the cases.[13,22–27] Consistent with these human data, BAP1 nuclear staining was absent in all MM analyzed arising from BAP1$^{+/-}$ mice and in 66.7% from BAP1$^{+/+}$ mice (Figure 3c). With regard to histology, all the MMs we observed in human germline *BAP1* mutation carriers were epithelioid.[13] In sporadic human MMs, several groups have reported that mutations of BAP1 occur primarily in epithelioid MM,[24,25] although this is not unequivocal.[28] All the MMs we observed in our BAP1$^{+/-}$ and BAP1$^{+/+}$ mice displayed, totally or partially, sarcomatoid features. This is likely due to interspecies differences, because sarcomatoid features, contrary to what happens in human MMs, were also prevalent in MMs arising from other independent murine models of asbestos-induced MM.[29,30] BAP1$^{+/-}$ mice had also a significantly shorter survival, that is, life span, compared with BAP1$^{+/+}$ mice, both in the low-dose ($P < 0.01$) and the standard-dose group ($P < 0.001$) (Figure 3d).

DISCUSSION

Taken together, our results showed that germline *BAP1* heterozygosity is associated with a significantly altered peritoneal inflammatory response upon exposure to asbestos fibers and to

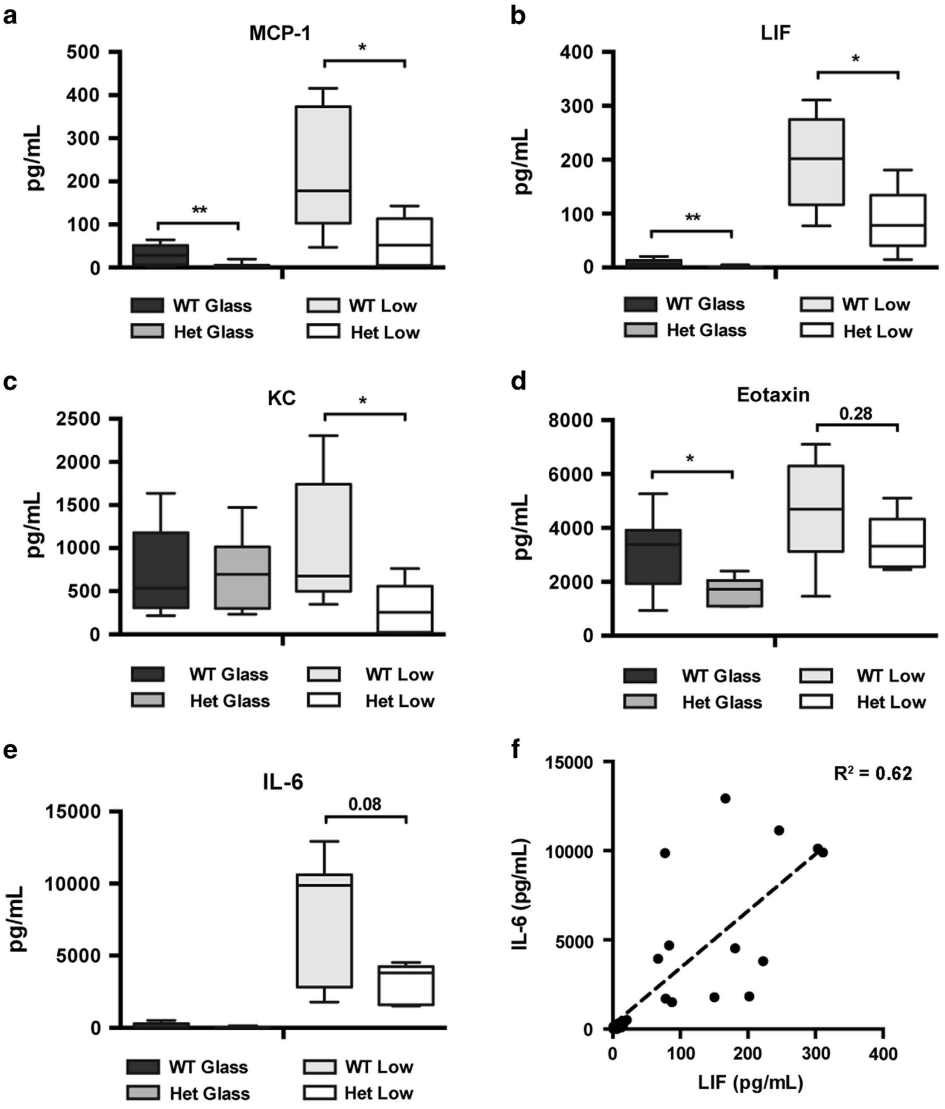

Figure 2. Several cytokines and chemokines are differentially expressed in lavage from BAP1$^{+/-}$ mice. The supernatants recovered from the peritoneal lavages were concentrated 45–60 times using Amicon Ultra Centrifuge Filters (EMD Millipore Corporation, Billerica, MA, USA) with a 3000 Dalton cutoff. Levels of 32 cytokines and chemokines were detected in concentrated lavages using human cytokine multiplex kits (EMD Millipore Corporation). Levels of MCP-1 (**a**), leukemia inhibitory factor (LIF) (**b**), keratinocyte-derived chemokine (KC) (**c**), eotaxin (**d**) and IL-6 (**e**) in lavages from *BAP1* wild-type and heterozygous mice after short-term exposure to glass beads or crocidolite fibers. Comparisons between heterozygous and wild-type groups were calculated using Mann–Whitney U test for rank comparisons. *$P < 0.05$, **$P < 0.01$. (**f**) Correlation of IL-6 and LIF levels (both belonging to the IL-6 family of cytokines) calculated using linear regression. The experiment was replicated two times.

an increased risk of MM following exposure to minimal amounts of asbestos that rarely cause MM in wild-type animals. BAP1 is a nuclear deubiquitinating enzyme and an important epigenetic regulator via deubiquitination of histone H2A.[31] Originally discovered in 1998,[32] it has several cell-intrinsic tumor-suppressive functions, such as regulation of gene transcription,[33] cell cycle and replication,[34–36] and DNA damage response.[37,38] BAP1 knockdown in MM cell lines has been paradoxically associated to a decreased proliferation, with an accumulation of cells in the S phase of the cell cycle,[22] suggesting that BAP1 loss might promote tumorigenesis inducing a delayed, but more permissive, G1/S checkpoint.[22] Heterozygous germline mutations of other important tumor-suppressor genes, such as *BRCA1*, *CDKN2A* and *RB1*, increase risk of cancer specifically to one or very few anatomical sites.[39] One of the few tumor-suppressor genes whose germline heterozygosity, similar to BAP1, is associated to increased risk of cancer to several sites is *TP53*, which encodes p53.[39] Besides its well-known intrinsic functions, recently a novel non-cell-autonomous tumor-suppressor effect of p53 has been described, via regulation of macrophage polarization and cytokine release.[40] Our results suggest that germline *BAP1* heterozygosity, similarly to *TP53*, influences *in vivo* macrophage polarization and cytokine release. Indeed, BAP1$^{+/-}$ mice exposed to asbestos had significantly more M2-like pro-tumoral macrophages. Also, the chemokines MCP-1 and keratinocyte-derived chemokine, and two cytokines of the IL-6 family (IL-6 itself and leukemia inhibitory factor) are soluble mediators significantly reduced in BAP1$^{+/-}$ mice exposed to asbestos. MCP-1 and IL-6 have been reported to increase following asbestos exposure and have been linked to asbestos pathogenesis.[41,42] Our results support these findings and also suggest that this inflammatory response might be associated with increased immunosurveillance, because lower levels of these and other inflammatory mediators in BAP1$^{+/-}$ mice are associated with higher M2/M1 macrophage

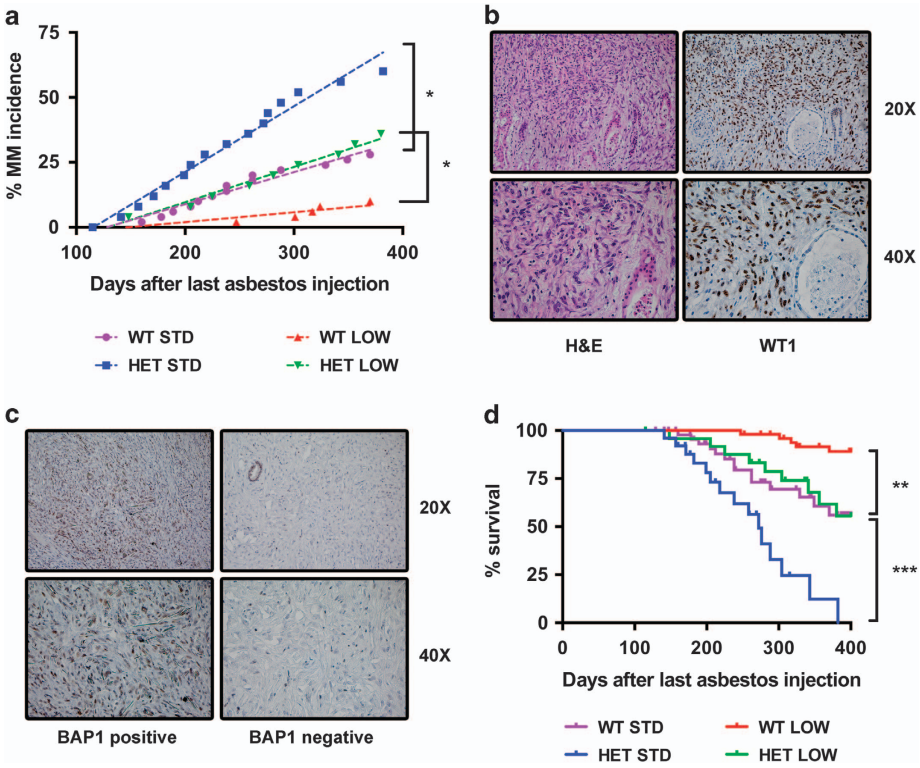

Figure 3. BAP1$^{+/-}$ mice develop more MMs and have shorter survival compared with wild-type littermates. Briefly, BAP1$^{+/+}$ mice ($n=50$ per group) and BAP1$^{+/-}$ mice ($n=25$ per group) were injected intraperitoneally every week for 10 weeks with 0.05 mg (low dose) or 0.5 mg (standard dose) of UICC crocidolite; 0.5 mg of glass beads were injected at the same schedule as control. Sample size was estimated to detect a difference in MM incidence between the low-exposed groups ⩾ 25%. Mice were monitored daily for clinical evidence of abdominal swelling, and killed in presence of respiratory distress, gait instability, unresponsiveness to pain stimuli or when tumor burden was obvious. Upon detection of illness, mice were killed by CO_2 asphyxiation, and all the major organs were evaluated histologically. (**a**) MM incidence in BAP1$^{+/-}$ mice and wild-type littermates after long-term exposure to glass beads or asbestos fibers (standard and low dose) was compared using Fisher's exact test. *$P<0.05$. (**b**) Formalin-fixed/paraffin-embedded samples were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) according to the standard procedure. The pathological diagnosis of mesothelioma was based on H&E staining and supported by WT1 nuclear staining in tumor cells. H&E and immunostainings were blindly interpreted by MC and AP, both US board specialized pathologists with expertise in human and animal mesotheliomas.[14,44,45] (**c**) Tumors were also stained with a rabbit polyclonal anti-BAP1 antibody to evaluate presence and localization of BAP1. (**d**) Survival curves of BAP1$^{+/-}$ mice and wild-type littermates after long-term exposure to asbestos fibers (standard and low dose) were compared using log-rank (Mantel-Cox) test. **$P<0.01$, ***$P<0.001$. The experiment was performed one time.

ratio and higher MM incidence following asbestos exposure. Interestingly, BAP1 has been recently shown to regulate the myeloid stem cell compartment via complex alterations of the transcriptional profile, possibly via its interaction with transcriptional co-regulators such as Host Cell Factor-1 (HCF-1) and Additional Sex Combs Like-1 (ASXL1).[19]

Altogether, our results suggest a novel, complex model of asbestos-induced MM pathogenesis, in which the chronic inflammatory response can have preferentially anti-tumoral or pro-tumoral roles, depending on the cellular and soluble mediators involved. To explain the observed intra- and inter-familial variability of cancer types in germline *BAP1*-mutated carriers, we hypothesized that MM might be more prevalent in individuals/families exposed to low levels of asbestos,[15] levels that are not, or only marginally, carcinogenic for the population at large. Our results support our hypothesis, as we found that 36% of BAP1$^{+/-}$ mice exposed to low doses of asbestos developed MM, compared with 10% of wild-type mice. Moreover, we found that MM is significantly more frequent in BAP1$^{+/-}$ mice exposed to standard doses of asbestos. This finding corroborates the recent results of Xu *et al.*[29] that were obtained in an independent murine model. Both studies found a shorter life span of asbestos exposed BAP1 heterozygous mice compared with wild-type littermates, suggesting that BAP1$^{+/-}$ mice might develop MM at an earlier age compared with wild-type littermates. Similarly, individuals carrying germline *BAP1* mutations are diagnosed with MM at a much younger age compared with sporadic MM cases (mean age 55 years vs 72 years, respectively).[16] Accordingly, although MMs in carriers of germline *BAP1* mutations are less aggressive and are associated with survivals from diagnosis of 5–10 years,[16] compared with an average of 1 year in sporadic MM patients, the former die at an earlier age compared with the latter. Survival from diagnosis could not be evaluated in our model, as per IACUC requirements, mice were killed at the first clinical evidences of disease.

Mechanistically, Xu *et al.*[29] suggest that the increased MM incidence in BAP1 heterozygous mice was partially related to BAP1-dependent transcriptional regulation of the tumor suppressor retinoblastoma protein. Our findings expand what was previously reported by implicating novel tumor-suppressor effects of BAP1 mediated via the microenvironment.

Moreover, we discovered that BAP1$^{+/-}$ mice exposed to low doses of asbestos developed MMs at a similar rate as BAP1$^{+/+}$ mice exposed to 10 times higher doses. Therefore, although it is not possible to directly compare the low-dose exposure in mice to indoor and/or outdoor environmental exposure in humans, our

findings support our hypothesis that germline *BAP1* heterozygosity increases susceptibility to the carcinogenic effects of low doses of asbestos.

On the basis of our results, we suggest that prevention programs of MM in individuals carrying germline *BAP1* mutations should focus on reducing exposure to even minimal sources of carcinogenic fibers, levels that are within the acceptable 'safe' limits for the population at large (0.1 fibers/cc of air as an 8-hour time-weighted average, as per US Occupational Safety & Health Administration standards[43]). Finally, although our model focuses on asbestos as a trigger, this novel non-cell-autonomous tumor-suppressive function of BAP1 may not be restricted to the peritoneal compartment or to the asbestos stimulation, and may contribute to the large numbers and diverse types of tumors that arise in carriers of the BAP1 cancer syndrome.

CONFLICT OF INTEREST

M Carbone has pending patent applications on BAP1 and provides consultation for mesothelioma expertise and diagnosis. The remaining authors declare no conflicts of interests.

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