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

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Abstract

Germline *BAP1* mutations predispose to several cancers, in particular malignant mesothelioma. Mesothelioma is an aggressive malignancy generally associated to 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 to 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.

Keywords

BAP1; BAP1 cancer syndrome; germline; mesothelioma; asbestos; dose; inflammation; macrophages; polarization; M1; M2

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

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Introduction

Malignant mesothelioma (MM) is a deadly cancer usually localized to the pleural and peritoneal linings[1]. In the US and in the UK, ~3 200 and ~2 500 individuals are diagnosed and die with MM each year, respectively[2, 3]. About 60–70% of mesotheliomas have been associated to 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, while alternatively activated (M2) MΦ are involved in immunosuppression and tissue repair[7]. Tumor-associated macrophages (TAM) 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 TAM 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 TAM, suggesting that they play 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.

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]. While homozygous BAP1 deficiency in mice results in embryonic lethality[19], BAP1$^{+/-}$ mice are viable and healthy. Compared to wild type littermates, BAP1$^{+/-}$ mice expressed about half the amount of BAP1 protein in relevant tissues (Suppl. Fig 1).

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In our experiments, we used 10–12 weeks 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 Hawai'i 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 five weeks to receive 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 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 macrophages polarization in BAP1$^{+/-}$ mice (Fig. 1a). In the macrophages from BAP1$^{+/-}$ mice exposed to asbestos fibers, the normalized mean fluorescence intensity (MFI) for CD206 (marker of M2 macrophages) was significantly higher compared to controls (197.1% [160.6–256.8] vs 163.1% [125.4–186.7], $P < 0.05$), whereas the normalized MFI for CD86 (marker of M1 macrophages) was significantly lower compared to controls (74.6% [57.6–90.3] vs 95.8% [77.4–109.1], $P < 0.05$) (Fig. 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 to 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 to wild type littermates (40.0% [30.7–47.0] vs 26.0% [13.3–37.6] of total macrophages, $P < 0.05$) (Fig. 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 to controls (0.54 [0.48–0.66] vs 0.36 [0.16–0.56], $P < 0.05$) (Fig. 1d).

Next, we compared the profiles of cytokines and chemokines present in peritoneal lavages of these same mice. 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], $P < 0.05$) (Fig. 2a). 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], $P < 0.05$) (Fig. 2b). Moreover, lavages from BAP1$^{+/-}$ mice exposed to asbestos contained significantly lower amounts of keratinocyte-derived

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chemokine (KC) compared to wild type littermates (253.4 pg/mL [19.5–557.1] vs 675.3 pg/mL [469.8–1741.5], $P < 0.05$) (Fig. 2c). 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], $P = 0.28$) (Fig. 2d). Levels of IL-6 also differed between genotypes upon asbestos exposure, though this difference did not reach nominal significance ($P = 0.08$) (Fig. 2e). Both IL-6 and LIF belong to the IL-6 family of cytokines, and in our samples their levels significantly correlated ($R^2 = 0.62$, $P < 0.0001$) (Fig. 2f). Finally, levels of G-CSF, IL-5, IP-10, and VEGF significantly increased after asbestos exposure, independently of the genotype (Suppl. Fig 2a-d). 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.

Therefore, we sought to experimentally study the relationship between asbestos dosage and MM carcinogenesis in the context of *BAP1* heterozygosis. Based on 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 ten 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 induced 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 to 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 to 14/50 (28.0%) BAP1$^{+/+}$ mice ($P = 0.011$) (Fig. 3a). Immunohistochemical staining of the tumors revealed expression of the mesothelial marker WT1 (Fig. 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 (Fig. 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, since 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, i.e. life-span, compared to BAP1$^{+/+}$ mice, both in the low-dose ($P < 0.01$) and the standard-dose group ($P < 0.001$) (Fig. 3d).

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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 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 KC, and two cytokines of the IL-6 family (IL-6 itself and LIF) 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, since lower levels of these and other inflammatory mediators in $BAP1^{+/-}$ mice are associated with higher M2/M1 macrophage ratio and higher MM incidence following asbestos exposure. Interestingly, BAP1 has been recently showed 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 to 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.* that were obtained in an independent murine model[29]. Both studies found a shorter lifespan of asbestos exposed BAP1 heterozygous mice compared to wild type littermates,

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suggesting that $BAP1^{+/-}$ mice might develop MM at an earlier age compared to wild type littermates. Similarly, individuals carrying germline *BAP1* mutations are diagnosed with MM at a much younger age compared to 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 to an average of 1 year in sporadic MM patients, the former die at an earlier age compared to the latter. Survival from diagnosis could not be evaluated in our model, as per IACUC requirements, mice were euthanized at the first clinical evidences of disease.

Mechanistically, Xu *et al.* suggest that the increased MM incidence in BAP1 heterozygous mice was partially related to BAP1-dependent transcriptional regulation of the tumor suppressor retinoblastoma protein[29]. 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.

Based on 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 eight-hour time-weighted average, as per US Occupational Safety & Health Administration standards[43]). Finally, while 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 arises in carriers of the BAP1 cancer syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAP1	BRCA1 associated protein-1
G-CSF	granulocyte-colony stimulating factor

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IL-5	interleukin 5
IL-6	interleukin 6
IP-10	interferon gamma-induced protein 10, CXCL10
LIF	leukemia inhibitory factor
MCP-1	monocyte chemoattractant protein-1, CCL2
MFI	mean fluorescence intensity
MM	malignant mesothelioma
MΦ	macrophages
KC	keratinocyte-derived chemokine, CXCL1
TAM	tumor-associated macrophages
VEGF	vascular endothelial growth factor

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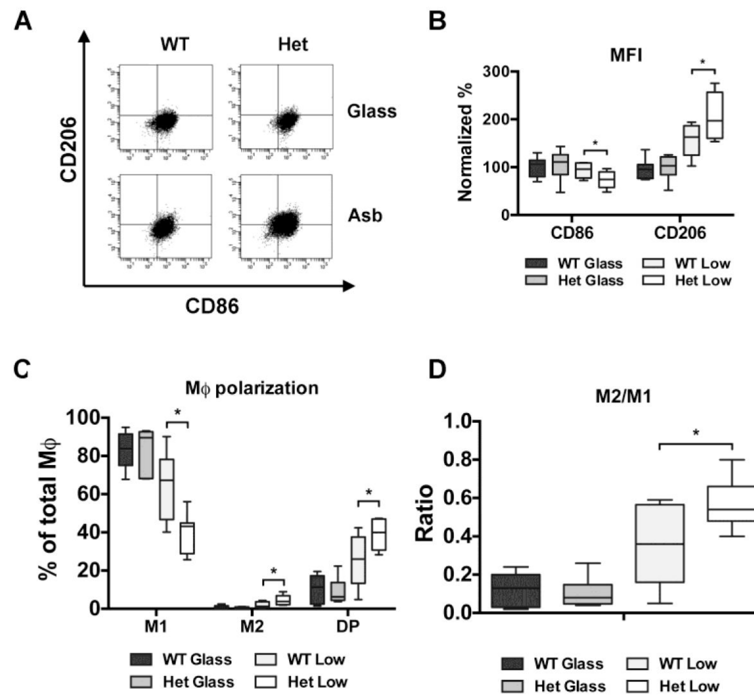

Figure 1. MΦ polarization is altered in BAP1$^{+/-}$ mice exposed to low doses of asbestos fibers

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), 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.

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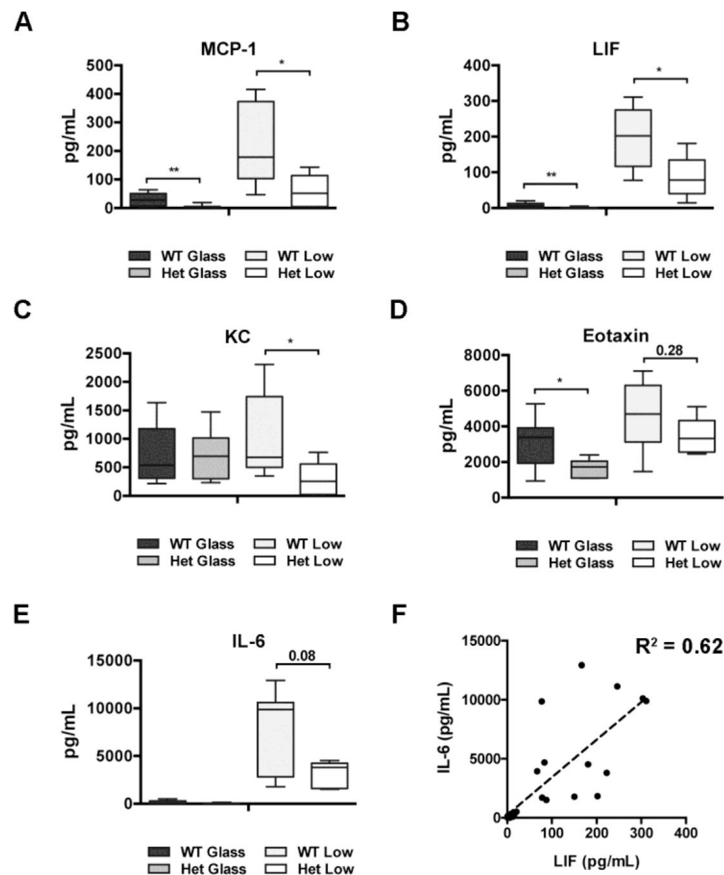

Figure 2. Several cytokines and chemokines are differentially expressed in lavage from BAP1$^{+/-}$ mice

The supernatants recovered from the peritoneal lavages were concentrated 45–60 times using Amicon Ultra Centrifuge Filters 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 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.

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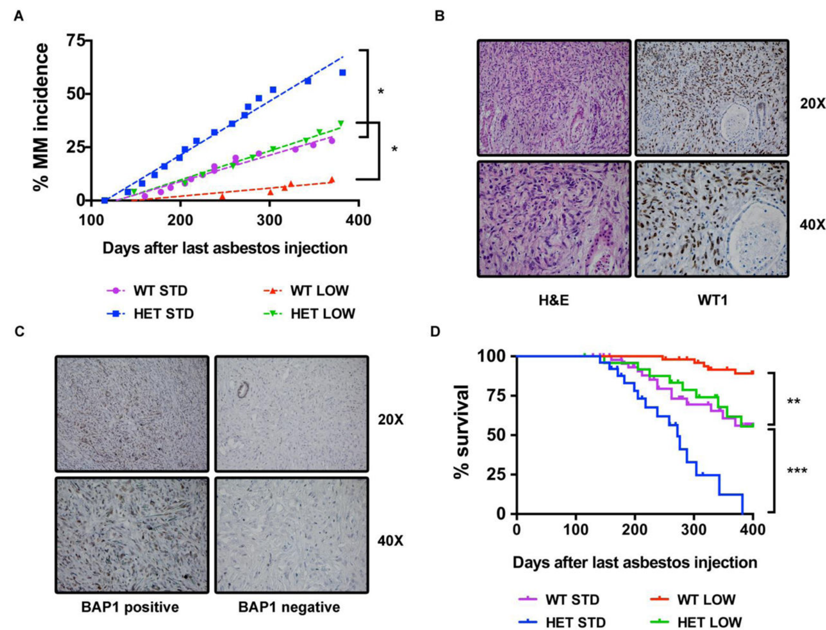

Figure 3. BAP1$^{+/-}$ mice develop more MMs and have shorter survival compared to wild type littermates

Briefly, BAP1$^{+/+}$ mice (n = 50 per group) and BAP1$^{+/-}$ mice (n = 25 per group) were injected intraperitoneally every week for ten 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 euthanized in presence of respiratory distress, gait instability, unresponsiveness to pain stimuli, or when tumor burden was obvious. Upon detection of illness, mice were sacrificed by CO2 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 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 M.C and A.P., both US board specialized pathologists with expertise in human and animal mesotheliomas[14, 45, 46] (**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.

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Table 1

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

$BAP1^{+/-}$ mice (n = 7 per group) and $BAP1^{+/+}$ (n = 9 per group) were injected intraperitoneally every week for five 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[44]. Next, mice were sacrificed by CO2 asphyxiation, and the abdominal cavity was washed with 5 ml of PBS. 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), F4/80 (MΦ; anti-F4/80-AlexaFluor®488, MCA497A488T, AbD Serotec), Ly-6G (neutrophils; anti-Ly6G-BV421, 562737, BD Biosciences), CD3 (T cells; anti-CD3-APC, 17-0032-80, eBioscience), 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].

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 (× 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
MF (%)	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