Mast cells mediate malignant pleural effusion formation

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Mast cells (MCs) have been identified in various tumors; however, the role of these cells in tumorigenesis remains controversial. Here, we quantified MCs in human and murine malignant pleural effusions (MPEs) and evaluated the fate and function of these cells in MPE development. Evaluation of murine MPE-competent lung and colon adenocarcinomas revealed that these tumors actively attract and subsequently degranulate MCs in the pleural space by elaborating CCL2 and osteopontin. MCs were required for effusion development, as MPEs did not form in mice lacking MCs, and pleural infusion of MCs with MPE-incompetent cells promoted MPE formation. Once homed to the pleural space, MCs released tryptase AB1 and IL-1β, which in turn induced pleural vasculature leakiness and triggered NF-κB activation in pleural tumor cells, thereby fostering pleural fluid accumulation and tumor growth. Evaluation of human effusions revealed that MCs are elevated in MPEs compared with benign effusions. Moreover, MC abundance correlated with MPE formation in a human cancer cell–induced effusion model. Treatment of mice with the c-KIT inhibitor imatinib mesylate limited effusion precipitation by mouse and human adenocarcinoma cells. Together, the results of this study indicate that MCs are required for MPE formation and suggest that MC-dependent effusion formation is therapeutically addressable.

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Mast cells (MCs) have been identified in various tumors; however, the role of these cells in tumorigenesis remains controversial. Here, we quantified MCs in human and murine malignant pleural effusions (MPEs) and evaluated the fate and function of these cells in MPE development. Evaluation of murine MPE–competent lung and colon adenocarcinomas revealed that these tumors actively attract and subsequently degranulate MCs in the pleural space by elaborating CCL2 and osteopontin. MCs were required for effusion development, as MPEs did not form in mice lacking MCs, and pleural infusion of MCs with MPE–incompetent cells promoted MPE formation. Once homed to the pleural space, MCs released tryptase AB1 and IL-1β, which in turn induced pleural vasculature leakiness and triggered NF-κB activation in pleural tumor cells, thereby fostering pleural fluid accumulation and tumor growth. Evaluation of human effusions revealed that MCs are elevated in MPEs compared with benign effusions. Moreover, MC abundance correlated with MPE formation in a human cancer cell–induced effusion model. Treatment of mice with the c-KIT inhibitor imatinib mesylate limited effusion precipitation by mouse MCs but MCs were found to be tumor-protective or indifferent in other settings (12–14). While the reasons for the divergent MC functions in cancer are not known, new models of MC deficiency lend promise to solve this riddle (15, 16).

Malignant pleural effusion (MPE) is extremely common in patients with lung, breast, or other adenocarcinomas (17, 18). No treatment exists, and palliative attempts may cause further morbidity and mortality (19, 20). MPE was recently reclassified as a separate stage of lung cancer, since it was acknowledged to represent a distinct form of metastatic disease with very short survival (18, 21, 22). Simultaneously, we and others used mouse models to hypothesize that MPE is primarily an immune- and vascular-

Introduction

Inflammation was recently recognized as an enabling hallmark of cancer that may mediate tumor growth and dissemination instead of tumor eradication (1). Inflammatory signaling networks in the tumor microenvironment can be initiated and orchestrated by malignant or immune cells; the networks conditionally facilitate tumor progression or regression depending on tumor type, immune effector cell type, and anatomic context (2–4). The identification of such inflammatory loops is of particular interest in the hunt for anticancer therapies that are anticipated to be more effective and less toxic than conventional chemotherapy (5). In addition to macrophages, neutrophils, and lymphocytes, mast cells (MCs) were recently found to be recruited to pancreatic and other tumors and to facilitate tumor growth (6, 7). Although they are relatively sparse, MCs are appealing candidates for tumor promotion, since they can release a battery of mediators to orchestrate the tumor milieu (8–11). However, MCs were found to be tumor-protective or indifferent in other settings (12–14). While the reasons for the divergent MC functions in cancer are not known, new models of MC deficiency lend promise to solve this riddle (15, 16).

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mediated manifestation of pleural-metastasized cancers (23, 24). However, the immune cells that drive MPE remain unidentified.

Here we describe the discovery of MCs in human and mouse MPEs and the elucidation of their fate and role. We show that MCs are driver cells required for MPEs and uncover the key messengers that instate tumor-MC circuitry during MPE development. Importantly, we provide proof-of-concept data supporting that MC-dependent MPEs are targetable using existing drugs; these data lend promise for translational applications of our findings.

Results

**MCs in MPEs.** MCs were encountered on May-Gruenwald-Giemsa–stained (MGG-stained) archival specimens of human MPEs from our pleural patient biobank. To rule out a local artifact, MCs were verified on metachromatic toluidine blue–stained (TB-stained) MPE samples from a different hospital. Systematic evaluation of samples and data from our pleural patient biobank revealed substantial MC numbers (millions/cavity) in MPEs that were significantly elevated compared with benign effusions from congestive heart failure (CHF; Figure 1A). Increased MC numbers were also identified in 2 different mouse models of MPEs that develop 14 days after intrapleural delivery of $1.5 \times 10^5$ syngeneic tumor cells ($n = 15$ mice per tumor cell type). Right: correlation between MPE and tumor-MC abundance and MPE volume, with linear regression line, sample size ($n$), probability value ($P$), and squared Pearson correlation coefficient ($r^2$). Hpf, high-power field. (C and D) Representative microphotographs of pleural fluid (C) and tumor (D) MCs from mice from B. Data presented as data points, mean ± SD. Numbers in boxes indicate sample size. Arrows indicate MCs. NS, $P > 0.05$; **$P < 0.01$; and ***$P < 0.001$, by 2-tailed Student’s $t$ test (A) or 1-way ANOVA with Bonferroni post hoc tests (B).

![Figure 1. MCs in human and murine MPEs.](image-url)
occasions (biological n = 2) by microarray analysis. Although 39 genes were overrepresented in MPE-competent adenocarcinoma cells on both occasions, only 2 RNAs possessed cytokine/chemokine activity required for systemic MC recruitment and were selected for further study: \( Spp1 \) and \( Ccl2 \) (encoding osteopontin, or secreted phosphoprotein 1 [SPP1], and CCL2, respectively; Figure 6A and Supplemental Tables 1 and 2). ELISA of tumor cell–conditioned media (CM) validated the microarray, and serum ELISA of pleural tumor–bearing C57BL/6 mice identified a significant difference in serum CCL2, but not SPP1, between adenocarcinoma- and melanoma-bearing mice (Figure 6, B and C). In modified mastotaxis assays (36), tracer BMMCs migrated toward LLC cells expressing random and anti-\( Spp1 \) shRNA (sh), but not toward B16F10 cells or LLC cells expressing \( shCcl2 \) (Figure 6D and Figure 7A), implicating CCL2 in MPE-directed mastotaxis. Indeed, forced expression of \( Ccl2 \) plasmid (p) in B16F10 cells restored, and forced expression of \( shCcl2 \) in LLC cells inhibited, MPE (25) and MC accumulation. (Figure 7, B and C). Moreover, treatment of mice harboring pleural LLC cells with i.p. CCL2- and/or CCL12-neutralizing Ab (37) blocked MPE (38) and MC accumulation, and direct pleural-delivered recombinant mouse (rm) CCL2 attracted MCs (Figure 7, D–F). Finally, CCL2 (but not SPP1) levels were correlated with MC abundance in human MPEs (Figure 7G). Collectively, these data indicated that CCL2 is a key tumor-secreted MC attractant to the pleural space.

MCs are required for MPEs. We next investigated MC effects on effusion formation. Pleural co-delivery of BMMCs facilitated induction of MPE by B16F10 cells, which are naturally MPE incompetent, without fully instilling the phenotype of adenocarcinoma cells (Figure 8A). Vice versa, both \( c-Kit^{\text{Wsh}} \) and \( Cpa3^{-/-} \) parietal and mediastinal, but not visceral, pleural tumors; most commonly resided in viable, but not necrotic, tumor tissue; and aggregated near or at the tumor front, forming chains or clusters (Figure 3). Hence, pleural MC accumulation is associated with MPE development in humans and mice. Moreover, MPE MCs appear to stream into the malignancy-affected pleural space via the parietal and mediastinal pleural surfaces.

Dynamic MC accumulation in the pleural space. To test MC kinetics during MPE development, we cultured murine BM-derived MCs (BMMCs) using c-KIT ligand (KITL) and interleukin-3 (IL-3), according to previously published protocols (31). BMMCs of C57BL/6 mice stained TB+ (>90%), CD45+c-KIT–Sca1–Lin(−) (>80%), and CD25+ (>50%) — and BMMCs of red-fluorescent mT/mG mice (32) — formed pseudopodia and moved, confirming the nature of these cells (Figure 4, A–C, and Supplemental Videos 1 and 2; supplemental material available online with this article; doi:10.1172/JCI79840DS1). BMMCs of luminescent CAG-luc-EGFP mice (33) emitted light proportional to cell number, and BMMCs of green fluorescent CAG-EGFP mice (34) were green fluorescent (Figure 4, D and E). When pulsed i.v. into irradiated C57BL/6 recipients adaptively reconstituted with \( c-Kit^{\text{Wsh}} \) BM (35), these tracer BMMCs distributed diffusely. However, when chimeras were challenged exclusively with pleural adenocarcinoma cells, BMMCs accumulated in the thorax concomitant with MPEs (Figure 5, A and B). Similar results were obtained with nonirradiated \( c-Kit^{\text{Wsh}} \) mice pulsed s.c. with tracer BMMCs (Figure 5C). Hence, pleural adenocarcinomas remotely mobilize/reruit MCs via circulating messengers.

**CCL2 as an adenocarcinoma-derived mastokine.** To identify these messengers, effusion-competent and effusion-incompetent tumor cells were transcriptionally profiled on 2 different occasions (biological n = 2) by microarray analysis. Although 39 genes were overrepresented in MPE-competent adenocarcinoma cells on both occasions, only 2 RNAs possessed cytokine/chemokine activity required for systemic MC recruitment and were selected for further study: \( Spp1 \) and \( Ccl2 \) (encoding osteopontin, or secreted phosphoprotein 1 [SPP1], and CCL2, respectively; Figure 6A and Supplemental Tables 1 and 2). ELISA of tumor cell–conditioned media (CM) validated the microarray, and serum ELISA of pleural tumor–bearing C57BL/6 mice identified a significant difference in serum CCL2, but not SPP1, between adenocarcinoma- and melanoma-bearing mice (Figure 6, B and C). In modified mastotaxis assays (36), tracer BMMCs migrated toward LLC cells expressing random and anti-\( Spp1 \) shRNA (sh), but not toward B16F10 cells or LLC cells expressing \( shCcl2 \) (Figure 6D and Figure 7A), implicating CCL2 in MPE-directed mastotaxis. Indeed, forced expression of \( Ccl2 \) plasmid (p) in B16F10 cells restored, and forced expression of \( shCcl2 \) in LLC cells inhibited, MPE (25) and MC accumulation. (Figure 7, B and C). Moreover, treatment of mice harboring pleural LLC cells with i.p. CCL2- and/or CCL12-neutralizing Ab (37) blocked MPE (38) and MC accumulation, and direct pleural-delivered recombinant mouse (rm) CCL2 attracted MCs (Figure 7, D–F). Finally, CCL2 (but not SPP1) levels were correlated with MC abundance in human MPEs (Figure 7G). Collectively, these data indicated that CCL2 is a key tumor-secreted MC attractant to the pleural space.

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determined the susceptibility of irradiated C57BL/6 or c-KitWsh recipients reconstituted with C57BL/6 or c-KitWsh BM (39) to LLC-triggered MPEs (Figure 8E). Consistent with prior reports, c-KitWsh mice with pleural tumors were MC-poor (40), whereas Cpa3C/+ mice were MC-eradicated (Figure 8, B and C). Indeed, donor genotype mice were protected against MPEs induced by both LLC and MC38 adenocarcinomas (Figure 8, B and C). In addition to MPE accumulation, MC deficiency resulted in retardation of pleural tumor growth, evident macroscopically (Figure 8, B and C), but also by decreased proliferating cell nuclear antigen (PCNA) immunoreactivity (Figure 8D). Since c-KIT is important for non-hematopoietic cells, too, we tested whether MCs are responsible for the phenotype of c-KitWsh mice. Indeed, donor genotype

Figure 3. MC topology in experimental MPEs. Whole thoracic sections from mice with pleural tumors and effusions induced by LLC and MC38 adenocarcinomas (Figure 8A–H). MCs (arrows) were found in parietal pleural tumors (ppt) and mediastinal tumors (mat), but not in visceral pleural tumors (vpt) (A–H). MCs appeared to stream in from intercostals vessels, sequentially invading intercostal tissues (fat and muscle) and ppt, forming chains invading into tumors or rings strategically positioned around tumors (I–Q). MCs were exclusively located in viable (vt), but not necrotic (nt), tumor tissues (R–T). All scale bars = 300 μm. B, D, F, H, J, L, N, and O, Q, and S and T: magnified inlays from A, C, E, G, I, K, M, P, and R, respectively. c, rib cartilage; cw, chest wall; ppm, parietal pleural mesothelium; pc, pleural cavity; bm, rib BM; scf, subcutaneous fat; icm, intercostal muscle; thy, thymus; sca, scalene muscle; tra, trachea; vpm, visceral pleural mesothelium; pv, pulmonary vein; icv, intercostal vein; d, dermis; r, rib; maf, mediastinal fat; mas, mediastinum.
MC-like innate immune cells described previously (29). We also sought to compare the relative contributions of MCs with those of macrophages during MPE development, since macrophages are the predominant cellular population in MPEs and are also chemoattracted to the pleural space by tumor-derived CCL2 (23, 25). For this, mice expressing Cre recombinase under the drive of the Lyz2 promoter were crossed with mice expressing Diphtheria toxin selectively in somatic cells that undergo Cre-mediated recombination to generate a macrophage ablation model (Lyz2-Cre and R26-DTA, respectively; refs. 41, 42). Pleural macrophages of naive C57BL/6 mice were predominantly F4/80+ and CD11b+, but not CD11c−; F4/80−CD11b+ pleural cells were markedly diminished in Lyz2-Cre R26-DTA mice compared with single transgenic controls (Figure 9A). Interestingly, Lyz2-Cre R26-DTA mice were protected against MPEs induced by both LLC and MC38 adenocarcinomas to a degree comparable to both mouse models of MC deficiency (Figure 9B). Hence, MCs are required for effusion formation, and they are equally important with much more prevalent cell types, such as macrophages. In addition, c-Kit+ and Cpa3Cre+ mice can serve as tumor models of MC depletion and eradication, respectively.

Tumor-secreted osteopontin causes MC degranulation. We next examined the role of tumor-originated osteopontin (encoded by the SPP1 gene in humans and the Spp1 gene in mice) — the other candidate detected by tumor cell microarray — in MC-dependent effusions. We had previously identified that SPP1 is a marked mediator of vascular permeability that leads to MPE accumulation (43). In addition to its vasoactive effects, we determined here in multiple ways that tumor-secreted SPPI promoted MC activation and degranulation. Adenocarcinoma cell-CM caused SPPI-dependent BMMC degranulation, and rmSPPI directly degranulated BMMCs (Figure 10 and Supplemental Video 3). SPPI effects were only partial, indicating the presence of additional tumor-elicited players in MC activation. Collectively with our past work, these data established dual functions of tumor-derived SPP1 during effusion development: in addition to inducing vascular leakage, osteopontin degranulates MCs.

Adenocarcinoma-primed MCs secrete tryptase AB1 and IL-1β to foster MPEs. To identify how pleural adenocarcinoma–recruited and pleural adenocarcinoma–primed MCs mediate effusion development, 2 different BMMC cultures were exposed to tumor-CM and were profiled transcriptionally (biological n = 2). Four BMMC transcripts were induced specifically and consistently by adenocarcinoma-CM, including secretory genes Tpsab1 and Il1b and membrane/granule-associated Cd68 (Figure 11A and Supplemental Tables 3–5). Indeed, adenocarcinoma-CM caused IL-1β release by BMMCs in a SPP1-dependent fashion, and adenocarcinoma-induced MPEs featured substantial IL-1β levels, which were reduced in c-KitWsh mice (Figure 11, B and C). Staining of C57BL6 BMMC and MPE cells for IL-1β, c-KIT, CD68, and the granule tag avidin localized IL-1β both in granules and the cytoplasm, and identified that c-KIT+CD68+ MCs are a subset of IL-1β–expressing cells in MPEs (Figure 11, D-F). Importantly, Ilb+/− mice (44) were protected from MPEs induced by LLC cells, similar to c-KitWsh and Cpa3Cre+ mice. In multiple MC reconstitution experiments, C57BL6 — but not...
c-KitWsh or Il1b−/−—BMMCs could restore effusion formation (Figure 11, G and H). Both rmIL-1β and BMMC-CM selectively enhanced LLC and MC38 adenocarcinomas, but not B16F10 melanoma proliferation, in vitro (Figure 11I). rmIL-1β did not induce skin vessel leakage in C57BL/6 mice; on the contrary, rm tryptase AB1 (TPSAB1) did not affect cell proliferation (data not shown), but induced marked vascular hyperpermeability comparable to rmVEGF (Figure 11J). These results indicated that c-KIT–competent MCs facilitate MPE development by secret- ing TPSAB1 and IL-1β to foster vascular permeability and tumor growth, respectively.

MC-derived IL-1β activates NF-κB in pleural adenocarcinoma cells. NF-κB responds to IL-1α in cancer cells (45) and augments tumor growth and MPEs (46, 47). To test whether MC-derived products activate NF-κB and other important transcription pathways of tumor cells, such as STAT3 and NOTCH, we assessed the expression of 12 target genes of the above pathways by qPCR before and after 4 hours of treatment with BMMC-CM. Interestingly, none of the 12 genes examined was inducible by BMMC-CM in MPE-defective B16F10 cells; however, both NF-κB-target gene Ccl2 and STAT3-target gene Myc were strongly upregulated by BMMC-CM selectively in MPE-competent adenocarcinoma cells (Figure 12A). By imaging a NF-κB reporter (pNF-κB-Luc; refs. 47, 48) and by immunoblotting, IL-1β (and IL-1α) and BMMC coculture induced and/or sustained NF-κB

Figure 5. Dynamic MC trafficking to the pleural space. (A) Bioluminescence of C57BL/6 chimeras engrafted with c-KitWsh BM that received 1.5 × 10^5 pleural B16F10 (n = 9), LLC (n = 9), or MC38 (n = 11) tumor cells and same-day i.v. 5 × 10^5 CAG-luc-EGFP BMMCs at day 30 after transplant. Note the increased chest signal in mice with adenocarcinoma-induced MPEs (arrows). (B) Pleural tumor sections (with magnified inlays) from chimeras as in (A) treated with pleural tumor cells followed by i.v. CAG-luc-EGFP BMMCs (n = 5/group). Note GFP+ BMMCs in adenocarcinomas 12 days later (arrows). (C) Bioluminescence of c-KitWsh mice that received 8 × 10^5 s.c. CAG-luc-EGFP BMMCs followed by next-day pleural injections of PBS (n = 6) or B16F10 (n = 6), LLC (n = 7), or MC38 (n = 8) tumor cells. Note the increased chest signal in mice with adenocarcinomas 13 days later (arrows). Data presented as data points, mean ± SD. Numbers in boxes indicate sample size. NS, P > 0.05; **P < 0.01; and ***P < 0.001, by 1-way ANOVA with Bonferroni post hoc tests.
available mouse model of MPEs caused by human cancer cells (38); A549 lung adenocarcinoma, but not SKMEL2 melanoma cells, elaborated SPP1/CCL2 and selectively caused MPE and MC accumulation upon pleural inoculation into NOD/SCID mice (Figure 15B). In addition, A549-induced MPEs were responsive to IM regression treatment (1 mg/kg daily) initiated on day 15 after establishment of pleural tumors (Figure 15C). Moreover, human MPEs from our biobank contained significantly elevated IL-1β levels compared with matched sera, a phenomenon not observed when CHF or IL-1α levels were examined (Figure 15D).

In summary, our present work shows that MCs are required for MPEs, attracted to and activated in the pleural space by CCL2 and SPP1 of tumor cell origin. Once in the pleura, MCs secrete TPSAB1 and IL-1β to foster a hyperpermeable microenvironment and tumor NF-κB activation, respectively (Figure 15E).

Discussion
Here we report a connection between inflammation and cancer: MCs feed the inflammatory pleural environment fostering MPEs. We consistently found MCs in MPEs of patients and mice, and identified a plausible reason for their previous neglect. We also investigated MC trafficking and determined that these BM cells (49, 50) stream into the pleural cavities primarily attracted by tumor-originated CCL2. MCs were required for MPEs, since c-KitWsh and Cpa3Cre/+ mice were protected from adenocarcinoma-induced effusions and adoptive transfer of WT BM, as well as MC reconstitution, reinstalled MPEs in protected c-KitWsh and Il1b−/− mice. c-KIT signaling was necessary for mastopoiesis and/or MC maintenance, since c-KitWsh mice had reduced BMMC yield and decreased MC-like cells (29) in MPEs. Hence, it was shown here that c-KIT–intact MCs are essential for MPE formation.
Indeed, MCs secreted multiple inflammatory and vasoactive mediators upon adenocarcinoma encounter: histamine, a known permeability factor (9); TPSAB1 shown here to induce strong vasoactive effects; and IL-1β, a well-known NF-κB stimulus (45). Interestingly, the intrapleural levels of MC-originated TPSAB1 identified here may be responsible for the clinically well-known failure of normal, benign, and malignant pleural fluid to spontaneously coagulate, since TPSAB1 was recently found to cleave fibrinogen (53). Hence, tumor site likely determines MC function in cancer, and the pleural space probably constitutes a preferential theater for deployment of protumor MC effects.

Regardless of the tumor milieu, only certain tumor cells were able to initiate bidirectional signaling with MCs in our hands. First, only tumor cells competent of CCL2 secretion were able to attract substantial numbers of MCs to the pleural cavities. The finding of CCL2 as a cardinal MPE mastokine corroborates development. Moreover, it was shown that this less prevalent cellular population is as important for effusion formation as predominant immune cells such as macrophages.

Our data support that substantial populations of MCs exist in MPEs of humans and mice, and that they play a functional tumor-promoting role in the pleural space when the latter is taken over by metastatic tumors. This protumor role of pleural MCs appears to be conditional on the pleural space, since MCs were tumor-indifferent or even exerted antitumor functions in other tumor models (6, 12). The marked effusion-promoting effects of pleural-accrued MCs may be explained by the vast abundance of MCs in serosal cavities, a preferential site for their isolation (15). However, it is also conceivable that MCs, potent inflammatory regulators and vasoactors (51, 52), impact effusion formation more heavily than other tumor models, since MPE is mainly caused by inflammation and vasoactive signaling, and since the pleural cavities feature an extensive vascular bed (23, 24).

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Our data support that substantial populations of MCs exist in MPEs of humans and mice, and that they play a functional tumor-promoting role in the pleural space when the latter is taken over by metastatic tumors. This protumor role of pleural MCs appears to be conditional on the pleural space, since MCs were tumor-indifferent or even exerted antitumor functions in other tumor models (6, 12). The marked effusion-promoting effects of pleural-accrued MCs may be explained by the vast abundance of MCs in serosal cavities, a preferential site for their isolation (15). However, it is also conceivable that MCs, potent inflammatory regulators and vasoactors (51, 52), impact effusion formation more heavily than other tumor models, since MPE is mainly caused by inflammation and vasoactive signaling, and since the pleural cavities feature an extensive vascular bed (23, 24). Indeed, MCs secreted multiple inflammatory and vasoactive mediators upon adenocarcinoma encounter: histamine, a known permeability factor (9); TPSAB1 shown here to induce strong vasoactive effects; and IL-1β, a well-known NF-κB stimulus (45). Interestingly, the intrapleural levels of MC-originated TPSAB1 identified here may be responsible for the clinically well-known failure of normal, benign, and malignant pleural fluid to spontaneously coagulate, since TPSAB1 was recently found to cleave fibrinogen (53). Hence, tumor site likely determines MC function in cancer, and the pleural space probably constitutes a preferential theater for deployment of protumor MC effects.
together, our data suggest that MPE-prone adenocarcinomas may initiate circuitry with MCs due to coordinated expression of MC chemoattractants (i.e., CCL2) and effectors (i.e., SPP1), as well as the cognate receptors of MC-originated ligands such as IL-1β. The reason behind the propensity of the adenocarcinoma cells studied here to initiate crosstalk with MCs remains to be determined but can explain, together with the site-specificity discussed above, the differential impact of MCs on different tumors. In addition, these results can serve as a paradigm of how tumor-associated inflammatory cells can conditionally orates and expands work that identified CCL2 as the culprit for MC accrual to pancreatic tumors (6, 7). However, CCL2-mediated MC recruitment was not enough for full-blown tumor-MC interactions during MPE development; murine adenocarcinoma cells also secreted SPP1 that facilitated MC degranulation. On the contrary, B16F10 cells were unable to activate codelivered BMMCs, thus failing to mount full effusion-inducing properties, since they were found not to express SPP1. Finally, adenocarcinomas were selectively responsive to MC-originated IL-1β and expressed Il1r1, while melanoma cells did not. Taken together, our data suggest that MPE-prone adenocarcinomas may initiate circuitry with MCs due to coordinated expression of MC chemoattractants (i.e., CCL2) and effectors (i.e., SPP1), as well as the cognate receptors of MC-originated ligands such as IL-1β. The reason behind the propensity of the adenocarcinoma cells studied here to initiate crosstalk with MCs remains to be determined but can explain, together with the site-specificity discussed above, the differential impact of MCs on different tumors. In addition, these results can serve as a paradigm of how tumor-associated inflammatory cells can conditionally

**Figure 8. MCs are required for MPEs.** (A) MPEs and pleural tumors of C57BL/6 mice at day 14 after pleural BMMCs (n = 5), B16F10 cells (n = 11), or both (n = 12). (B and C) MPEs, pleural tumors, and TB-stained pleural fluid cells including MCs of C57BL/6 (n = 45), c-Kit<sup>inn</sup> (n = 37), and Cpa3<sup>Cre</sup> (n = 14) mice at day 14 after pleural LLC (B) and MC38 (C) cells. (D) PCNA immunoreactivity of pleural tumors from B and C (n = 7/group). (E) MPEs (graph) and TB-stained pleural tumor MCs (images) of irradiated C57BL/6 and c-Kit<sup>inn</sup> recipients of BM transplants from C57BL/6 and c-Kit<sup>inn</sup> donors at day 14 after pleural LLC cells (n = 6–7/group). (F) BMMC yield of C57BL/6 and c-Kit<sup>inn</sup> mice (n = 6 each) at 1 month; data summary, TB-staining, and flow cytometry of c-Kit<sup>inn</sup> BMMCs. Arrows indicate shift toward c-KIT<sup>Lin</sup> phenotype, as compared with Figure 4B. Shown throughout are MPEs (dashed lines), lungs (l), pleural tumors (t), and MCs (arrows). Numbers below columns: percentile MPE inhibition and pleural MCs (thousands) of c-Kit<sup>inn</sup> (gray font) and Cpa3<sup>Cre</sup> (black font) mice. Data presented as data points, mean ± SD. Numbers in boxes indicate sample size. NS, P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001, by 2-tailed Student’s t-test (D) or 1-way ANOVA with Bonferroni post hoc tests (all other graphs).
modulate metastatic disease.

NF-κB integrates inflammatory stimuli from the tumor microenvironment to pivotally influence tumor cell survival and paracrine inflammatory signaling (54). In turn, immune cells stimulate tumor NF-κB, reinforcing a “vicious cycle” between tumor progression and inflammation (5, 55). NF-κB affects MPE progression, stimulated by tumor- and host-originated ligands of an ever-expanding cellular origin (26, 47, 48). Here we show that MC-secreted IL-1β upregulates NF-κB and cellular proliferation of pleural adenocarcinomas. Using in vivo imaging, we show in real time how MCs fuel tumor cell NF-κB and identify that IKKβ relies on MC-derived IL-1β to sustain the transcription factor. These findings are consistent with the recently reported Nlrp3-mediated IL-1β release by skin MCs (56), and they also strengthen the link between inflammation and cancer by positioning MCs as “feeder cells” of oncogenic factors, such as STAT3 (57).

Importantly, the requirement for MCs during MPE development was actionable. Encouraging benefits were obtained from imatinib treatment of mice with syngeneic effusions, and concordant findings were recapitulated in human cancer cell–induced MPEs. These data strengthen the proposed connection and show how MC-targeted therapies can impact nongastrointestinal stromal tumors. The finding of significantly increased MCs and IL-1β in human MPEs compared with both matched serum samples and corresponding samples from patients with CHF suggests that our findings may be applicable to humans with established or impending MPEs, a possibility worth exploring.

In conclusion, we identified the conditional initiation and execution of a circuitry of tumor-initiated, MC-perpetuated inflammatory signaling events that occur during MPE formation. We show how tumor cells co-opt MCs to drive effusion development. In addition to the surprising discovery of MCs as culprits of MPEs, we identify CCL2, SPP1, TPSAB1, IL-1β, and IKKβ as key players in tumor cell–MC interactions in the pleural space. MCs per se, as well as each of the above targets, may present candidates for ameliorating the requirement for MCs during MPE formation.

Methods

Further information can be found in Supplemental Methods.

Reagents. CCL2 and CCL12 neutralizing and IgG2a control Abs (37) were a gift from Oncology Discovery Research, Janssen Research & Development LLC. rmCCL2 was from Peprotech; rmIL-1β, rm IL-3, and rmKIF1 were from Immunotools; rmSPP1 and rmTPSAB1 were from R&D Systems; C48/80 and Evans’ blue were from Sigma-Aldrich; ELISA kits were from Peprotech and R&D Systems; IM was from Selleckchem; and Boyden chambers were from Millipore.

Cells. LLC, B16F10, A549, and SKMEL2 (NCI) and MC38 cells (a gift from Barbara Fingleton, Vanderbilt University, Nashville, Tennessee, USA) (38) were cultured and tested as described in the Supplemental Methods. In vivo injections are described elsewhere (25, 26, 38, 47, 48). BM cells were flushed from femurs and tibias and cultured in full DMEM with rmIL-3 ± rmKITL (100 ng/ml each). Nonadherent cells were passaged for 4–6 weeks (31).

Animals. C57BL/6, c-Kit+/+; NOD/SCID, CAG-luc-EGFP, CAG-EGFP, mt+/mG, Lyz2-Cre (39), and R26-DTA (40) mice (The Jackson Laboratory); Cpa3cre/+; mice (a gift from Hans-Reimer Rodewald, Heidelberg, Germany) (15); and Il1β−/− mice (a gift from Yoichiro Ikawara, Tokyo University of Science, Tokyo, Japan) (44) were bred at the Center for Animal Models of Disease of the University of Patras. Experiments were approved a priori by the local Veterinary Administration and were conducted according to 2010/63/EU. Experimental mice and littermate controls were sex-matched, weight-matched (20–25 g), and age-matched (6–12 weeks). For MPE generation, mice received 150,000 murine or 1,000,000 human cancer cells intrapleurally and were sacrificed after 14 or 30 days, respectively (38). IM (1 mg/kg) was given daily i.p. Anti–mouse CCL2 and CCL12 (a murine CCL2 ortholog) and IgG2a control Abs were delivered i.p. at 50 mg/kg every 3 days (37, 38). Harvest and pleural lavage are described elsewhere (25, 26, 38, 47, 48).

Humans. Pleural fluid was obtained during diagnostic thoracenteses in patients with MPEs (due to lung cancer [n = 14], breast cancer [n = 6], and malignant pleural mesothelioma [n = 4]) or CHF treated at General Hospital Evangelismos between 2006 and 2008. Samples from Hospital G. Papanikolaou were diagnostic MPE cyto-
Figure 10. Tumor-secreted osteopontin causes MC degranulation. (A) Histamine content of C57BL/6 BMMCs after 2-hour incubation with indicated CM or C48/80 compound (potent MC degranulating compound used as positive control, n = 3/group). Note the partial effect of osteopontin silencing (shSpp1), indicating that additional tumor-elaborated factors degranulate MCs. (B and C) C57BL/6 (B) and mT/mG (C) BMMC morphology after 2-hour incubation with indicated CM or C48/80 compound indicates that osteopontin is, at least in part, responsible for MC activation. (D) Time-lapse phase-contrast images of C57BL/6 BMMCs after direct exposure to rm SPP1 showing 2 consecutive waves of granule ejection (arrows). See also Supplemental Video 3. (E) C57BL/6 BMMC histamine content after 4-hour incubation with indicated rm mediators or C48/80 compound (n = 3/group) shows direct MC activation by osteopontin, which is only partial compared with C48/80. Data shown represent 1 representative of 3 experiments and are presented as data points, mean ± SD. Numbers in boxes indicate sample size. nd, not detected; NS, P > 0.05; *P < 0.05; and ***P < 0.001, by 1-way ANOVA with Bonferroni post hoc tests.
Figure 11. Adenocarcinoma-primed MCs secrete TPSAB1 and IL-1β. (A) Microarray (Venn and PCA diagrams, n = 2) and qPCR (n = 3) of BMMC differential gene expression (ΔGE) after tumor-CM exposure. Comparisons with naive BMMCs. (B) C57BL/6 BMMC-CM IL-1β ELISA after 4 hours of sham or tumor-CM exposure (n = 3/treatment). (C) MPE IL-1β ELISA of mice from Figure 8, B and C (n = 5/group). (D and E) IL-1β and CD68 immunolocalization in C57BL/6 BMMCs counterstained with Hoechst 33258 (nuclei) and avidin (granules). (F) IL-1β colocalization with c-KIT and CD68 in MPE cells. (G and H) MPEs of C57BL/6 (n = 45), c-Kit−/− (G, n = 33), and IL1b−/− (H, n = 51) mice 14 days after pleural LLC cells with or without s.c. C57BL/6, c-Kit−/−, or IL1b−/− BMMCs. (I) Proliferation of B16F10, LLC, and MC38 cells (n = 3/cell line) at 100 ng/ml IL-1β (top; comparisons of adenocarcinomas with melanoma) and at increasing BMMC-CM concentrations (bottom; comparisons of LLC [stars] and MC38 [number sign] cells with 0% BMMC-CM [control]). (J) C57BL/6 skin Evans’ blue leak (color-coded areas) induced by BSA or rm cytokines (comparisons with BSA, n = 6/group). (A, B, and I) Shown are 1 representative of 3 experiments. Data are presented as data points, mean ± SD. Numbers in boxes indicate sample size. NS, P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001, by 2-way (I) or 1-way (all other graphs) ANOVA with Bonferroni post hoc tests.
logic specimens from 20 patients with lung adenocarcinoma aspirated in 2013. Diagnosis and sample handling are described elsewhere (25, 26, 48). All protocols abided by the Helsinki Declaration were approved a priori by the relevant IRB and by all patients via written informed consent.

**Cytology and histology.** Cell and tissue specimens, prepared as described in the Supplemental Methods, were stained with MGG, TB (0.05%; 5–15 minutes), or the indicated Abs (Supplemental Table 6) and counterstained with hematoxylin, Hoechst 33258 (Sigma-Aldrich; dilution 1:5000), Envision (Dako), and/or avidin (Vector Laboratories). MCs were counted as a percentage of 10,000 cells on cytology or of all cells on histology. Microscopy was done on an AxioObserver D1 (Zeiss) or an SP5 (Leica Microsystems) microscopes.

**Flow cytometry.** After NH$_4$Cl red blood cell lysis, cells were suspended in PBS 2% FBS, stained with the indicated Abs (Supplemental Table 6) for 20 minutes, fixed in 1% paraformaldehyde (10 minutes), and analyzed on a FACScalibur (BD Biosciences). Data were examined using FlowJo.

**Figures.**

**Figure 12.** MC-derived IL-1β activates NF-κB in adenocarcinoma cells. (A) Tumor cell mRNA expression levels by qPCR of 12 target genes of the NF-κB, STAT3, and NOTCH pathways before and 4 hours after exposure to BMCC-CM. n = 3/data point. (B and C) Cancer cell NF-κB reporter (pNF-κB-Luc) activity induced by rmIL-1β (B) and BMCC-CM (C). n = 3/data point. (D) Tumor cell pNF-κB-Luc activity induced by C57BL/6, c-KitWsh, or Il1b–/– BMCC coculture. n = 3/data point. (E) Il1r1 qPCR of tumor cell RNA. n = 3/data point. Significance indicators stand for comparison of color-matched data at indicated time point compared with baseline (A), B16F10 cells (B and C) or with C57BL/6 BMCC (D). Shown is 1 representative of 3 experiments. Data presented as mean ± SD (A–D) or data points, mean ± SD (E). Numbers in boxes indicate sample size. NS, P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001, by 1-way (E) or 2-way (all other graphs) ANOVA with Bonferroni post hoc tests.

*Constructs.* Random (shC), anti-Ccl2 (shCcl2), or anti-Spp1 (shSpp1) shRNAs, as well as Ccl2 and β-gal expression vectors, have been described (25, 41). pEGFP and pEGFP.Ikbb (Addgene IDs 58249 and 58251) were cloned from MC38 total RNA using specific primers (Supplemental Table 7). Cells were transfected with 5 μg DNA by X-fect reagent (BD Biosciences — Clontech) and selected by puromycin.

**BM cell transfer.** For adoptive BM replacement (Figure 5, A and B, and Figure 8E), mice received 10 million BM cells i.v. 12 hours after total-body irradiation (1100 Rad; ref. 35). For MC pulse and chase (Figure 5), irradiated C57BL/6 chimeras engrafted with c-KitWsh BM (39) received pleural tumor cells at day 30 after transplant, followed by same-day 5 × 10$^5$ i.v. CAG-luc-EGFP or CAG-EGFP BMCCs; nonirradiated c-KitWsh mice received 8 × 10$^5$ s.c. CAG-luc-EGFP BMCCs, followed by next-day pleural tumor cells. For intrapleural BMCC delivery (Figure 8A), C57BL/6 mice received 10$^5$ intrapleural BMCCs, with or without B16F10 cells. For BMCC give-back (Figure 11, G and H), 2.5 × 10$^5$ BMCC were administered s.c.
electroblotted to PVDF membranes (Millipore). Membranes were probed with specific Ab (Supplemental Table 6), and were visualized by enhanced chemiluminescence.

**Cellular assays.** Tumor cell proliferation in response to IL-1β or BMMC-CM was determined using MTT reduction (Promega). MC migration was studied in Boyden chambers with 8.0 μm pores (36): cancer cells were cultured in the lower and bioluminescent BMMCs in the upper chambers. After 48 hours, the upper chambers were removed and the bioluminescent signal of transmigrated BMMCs was measured by imaging. MC histamine was measured by the o-phthalaldehyde method (58). All cellular experiments were done at least 3 times, while 1 representative experiment is shown.

**Statistics.** Sample size was calculated using G*power (http://www.gpower.hhu.de/; ref. 59) assuming α = 0.05, β = 0.8, and ρ = 0.3. No data were excluded. Animals were allocated to treatments by alternation, and transgenic animals were enrolled case-control-wise. Data acquisition was blinded on samples previously coded by a nonblinded investigator. All data were examined for normality by Kolmogorov-Smirnov test. Values are given as mean ± SD and median ± interquartile range, as indicated. Sample size (n) refers to biological replicates. Differences in means were examined by

**Bioluminescence imaging.** Cells and mice were imaged after the addition of 300 μg/ml D-luciferin to culture media or i.v. delivery of 1 mg D-luciferin on a Xenogen Lumina II. Data were analyzed on Living Image v.4.2 (PerkinElmer) (47, 48).

**Vascular permeability assays.** Mice with MPEs received 0.8 mg Evans’ blue i.v. and were killed after 1 hour for determination of MPE levels of the albumin tracer (48). Intradermal injections of test molecules (1.5 ng/50 μl PBS), cell-free MPEs (50 μl), or cancer cell–conditioned media (50 μl) were followed by Evans’ blue injection as above, and euthanasia, skin inversion and imaging after 1 hour (25). Dye leak was determined using Fiji (http://fiji.sc/Fiji).

**qPCR and microarray.** RNA was isolated using Trizol (Invitrogen) followed by RNAeasy (QIAGEN), RNA was reverse transcribed using Superscript III (Invitrogen), and reverse transcriptase or qPCR was performed using specific primers (Supplemental Table 7). For microarray, 5 μg RNA pooled from triplicate samples was tested for quality, labeled, and hybridized to GeneChip Mouse Gene 1.0 or 2.0 ST arrays (Affymetrix). Data (http://www.ncbi.nlm.nih.gov/geo/; Accession ID: GSE58190) were analyzed as detailed in the Supplemental Methods.

**Immunoblotting.** Nuclear and cytoplasmic extracts were prepared using NE-PER (Thermo), separated by 10% SDS-PAGE, and

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**Figure 13. Tumor cell NF-κB subunit profiling after exposure to MC-conditioned media and IL-1β.** Immunoblots of cytoplasmic (c) and nuclear (n) tumor cell extracts for NF-κB pathway components after C57BL/6 or Il1b−/− BMMC-CM exposure (A) or treatment with 100 ng/ml rmIL-1β (B).
Study approval. All animal experiments were approved a priori by the Veterinary Administration of Western Greece according to a full and detailed protocol (approval 276134/14873/2). Human studies were approved a priori by the Ethics Committee of the General Hospital of Athens Evangelismos (Athens, Greece; approval 379-7/12/2006 and extension 323-4/12/2012).

2-tailed Student’s t test, or 1-way or 2-way ANOVA with Bonferroni post-tests, as appropriate, and in medians by Mann-Whitney U test or Kruskal-Wallis test with Dunn’s post hoc tests. Correlations were done using Pearson’s r or Spearman’s p. P values are 2-tailed, and P < 0.05 was considered significant. Analyses and plots were done on Prism v5.0 (GraphPad Software).

Figure 14. MC-derived IL-1β promotes effusion development via IkB kinase (IKK) β. (A) Bioluminescence of C57BL/6 (n = 14) and c-Kit+ (n = 12) mice 14 days after pleural LLC cells expressing constitutive (pCAG-Luc) or NF-κB-dependent (pNF-κB-Luc) reporters. (B) Validation of adenocarcinoma cells overexpressing pEGFP and pEGFP.IkBkb. (C) Bioluminescence of pNF-κB-Luc LLC cells expressing pEGFP or pEGFP.IkBkb in response to 100 ng/ml IL-1β, with comparisons of pEGFP.IkBkb with pEGFP cells (n = 3/data-point). Shown is 1 representative of 3 experiments. (D) MPEs of C57BL/6 (n = 36), c-Kit+ (n = 21), and Cpa3Cre+ (n = 15) mice 14 days after pleural pEGFP- or pEGFP.IkBkb-expressing LLC cells. Shown are MPEs (dashed lines), lungs (l), and pleural tumors (t). Numbers below columns indicate percentile MPE inhibition of c-Kit+ (gray font) and Cpa3Cre+ (black font) mice. Data presented as data points, mean ± SD. Numbers in boxes indicate sample size. NS, P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001, by Student’s t test (A), 1-way (D), or 2-way (C) ANOVA with Bonferroni post hoc tests.
Figure 15. MC-mediated MPEs are actionable in mice and humans. (A) MPEs, MPE MCs, and Evans' blue content after i.v. delivery of 0.8 mg of the dye of C57BL/6 mice at day 14 after pleural delivery of $1.5 \times 10^5$ LLC cells and daily treatment with intraperitoneal PBS or IM, as indicated ($n = 14$/group). Percentage indicates IM-mediated MPE inhibition. (B) MCs in pleural tumors (arrows) and MPE volume of NOD/SCID mice 30 days after pleural human cancer cells, and SPP1/CCL2 ELISA of human tumor cell–CM ($n = 5$/group). (C) A549-induced MPEs of NOD/SCID mice at day 30 after establishment of A549 cells, after treatment with PBS ($n = 7$) or IM ($n = 6$) starting at day 15 after establishment of A549 cells. Shown are MPEs (dashed lines), lungs (l), and pleural tumors (t). Percentage indicates IM-mediated MPE inhibition. (D) Pleural and serum IL-1α/β of patients with CHF ($n = 26$) and MPEs ($n = 24$) from Figure 1A. (E) Graphical summary of present work: pleural adenocarcinomas secrete CCL2 and SPP1, which facilitate, respectively, pleural MC accumulation and activation. Upon tumor cell encounter, MCs release TPSAB1 and IL-1β, which increase vascular leakage and tumor NF-κB activation, respectively. Data presented as data points and median ± interquartile range (D) or mean ± SD (all other graphs). Cytokine measurements in (B) were repeated 3 times; shown are data from 1 experiment. Numbers in boxes indicate sample size. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$, by Student’s t test (A-C) or Kruskal-Wallis ANOVA with Dunn’s post hoc tests (D).
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