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Matrix Metalloproteinase Mmp-1a Is Dispensable for Normal Growth and Fertility in Mice and Promotes Lung Cancer Progression by Modulating Inflammatory Responses*[S]

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Background: *MMP1* is overexpressed in malignant tumors and its levels are associated with poor prognosis.
Results: Mice deficient in Mmp-1a, the ortholog of human MMP-1, develop fewer lung carcinomas than controls and show a Th1 anti-inflammatory response.
Conclusion: Mmp-1a is a protumoral protease that alters Th1/Th2 cytokine balance.
Significance: *Mmp1a*-deficient mice are a new model for the functional analysis of this metalloproteinase in cancer.

Human MMP-1 is a matrix metalloproteinase repeatedly associated with many pathological conditions, including cancer. Thus, *MMP1* overexpression is a poor prognosis marker in a variety of advanced cancers, including colorectal, breast, and lung carcinomas. Moreover, MMP-1 plays a key role in the metastatic behavior of melanoma, breast, and prostate cancer cells. However, functional and mechanistic studies on the relevance of MMP-1 in cancer have been hampered by the absence of an *in vivo* model. In this work, we have generated mice deficient in *Mmp1a*, the murine ortholog of human *MMP1*. *Mmp1a*$^{-/-}$ mice are viable and fertile and do not exhibit obvious abnormalities, which has facilitated studies of cancer susceptibility. These studies have shown a decreased susceptibility to develop lung carcinomas induced by chemical carcinogens in *Mmp1a*$^{-/-}$ mice. Histopathological analysis indicated that tumors generated in *Mmp1a*$^{-/-}$ mice are smaller than those of wild-type mice, consistently with the idea that the absence of Mmp-1a hampers tumor progression. Proteomic analysis revealed decreased levels of chitinase-3-like 3 and accumulation of the receptor for advanced glycation end-products and its ligand S100A8 in lung samples from *Mmp1a*$^{-/-}$ mice compared with those from wild-type. These findings suggest that Mmp-1a could play a role in tumor progression by modulating the polarization of a Th1/Th2 inflammatory response to chemical carcinogens. On the basis of these results, we propose that *Mmp1a* knock-out mice provide an excellent *in vivo* model for the functional analysis of human MMP-1 in both physiological and pathological conditions.

The matrix metalloproteinases (MMPs)[2] are a family of structurally related enzymes that can collectively degrade the main protein components of the extracellular matrix and basement membranes (1). On the basis of these degrading activities, MMPs have been considered as essential enzymes in the invasive and metastatic properties of tumor cells (2). These findings, together with multiple clinical and experimental data associating MMPs with tumor progression, stimulated the search for MMP inhibitors with ability to block the activities of these enzymes in cancer. However, most clinical trials with synthetic MMP inhibitors failed to provide appreciable benefits to patients with advanced cancer (3). These negative results have forced a profound re-evaluation of the functional and clinical relevance of MMPs in cancer as well as a reformulation of the MMP inhibition strategies used in these clinical trials (3, 4). As a direct consequence of these post-trial studies, new paradigms have recently emerged in relation to the cancer relevance of the different members of this complex family of endoproteases. First, many experimental data have shown that MMPs are not exclusively implicated in the proteolytic breakdown of tissue barriers for metastatic spread. Thus, these enzymes may target a diversity of non-matrix substrates and influence other critical steps in tumor evolution such as cell proliferation, differentiation, angiogenesis, or apoptosis (5). Additionally, these new studies have revealed the occurrence of MMPs such as MMP-8, MMP-12, and MMP-26, which play a protective role during tumor progression (6). Furthermore, other MMPs such as MMP-3, MMP-9, and MMP-19, which were originally recognized as protumorigenic enzymes, may also function as tumor-suppressive proteases in some specific situations (7–10).

These recent experimental findings have provided some explanations for the lack of success of clinical trials based on the use of broad-range MMP inhibitors in patients with advanced stages of cancer, as they would also reduce the host-protective

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[2] The abbreviations used are: MMP, matrix metalloproteinase; RAGE, receptor for advanced glycation end products; CHI3L3, chitinase-3-like 3.

antitumor properties of certain MMPs (3, 4). Likewise, the administration of these MMP inhibitors to patients with advanced cancer would be of limited value for those cases in which MMPs play important roles during early stages of the disease (3, 4). Accordingly, it is necessary to perform a detailed analysis of the specific role of each individual MMP in the multiple stages of tumor evolution. This is the case for fibroblast or interstitial collagenase (MMP-1), the first member of the MMP family identified in human tissues and widely associated with cancer but whose functional relevance in the progression of the disease is still largely unknown (11, 12).

Human MMP-1 is produced by a variety of tumor cells as well as by adjacent stromal fibroblasts in response to factors derived from transformed cells. Clinical studies have reported that MMP-1 is overexpressed in a number of malignant tumors, and its presence is associated with poor prognosis in different cancers such as colorectal, breast, and lung carcinomas (13–15). Moreover, *MMP1* has been identified as one of the four key genes required for lung metastatic colonization in breast cancer (16). Additionally, MMP-1 together with ADAMTS-1 proteolytically engage EGF-like growth factors in an osteolytic signaling cascade, which facilitates bone metastasis (17). Furthermore, MMP-1 produced in the stromal tumor microenvironment activates the proinvasive functions of protease-activated receptor 1 and promotes invasion and tumorigenesis of breast cancer cells (18). Likewise, experimental manipulation of the expression levels of this protease alters the metastatic behavior of melanoma, breast, and prostate cancer cells (19–21). However, functional and mechanistic studies on the relevance of MMP-1 in cancer have been hampered by the absence of an *in vivo* model of *MMP1* deficiency. To address this question, we have undertaken studies to generate mutant mice deficient in *Mmp1a*, the mouse orthologous gene of human *MMP1* (22). In this work, we describe the generation of *Mmp1a*$^{-/-}$ mice and analyze their cancer susceptibility, with the finding that loss of *Mmp1a* partially protects mice against development of lung carcinomas induced by chemical carcinogens. We have also performed a series of histopathological and proteomic analysis that have provided information about the mechanisms underlying Mmp-1a implication in lung cancer development. Finally, we propose that *Mmp1a*$^{-/-}$ mice represent a novel *in vivo* model to elucidate the functional relevance of human MMP-1 in the context of the large complexity and diversity of proteolytic enzymes.

EXPERIMENTAL PROCEDURES

Animals—To generate *Mmp1a*$^{-/-}$ mice, we first isolated a genomic PAC clone encoding *Mmp1a* from a mouse 129/SvJ library (HGMP Resource Centre) by using a murine *Mmp1a* cDNA fragment as a probe. Then, we used the plasmid pKO scrambler V916 (Lexicon Genetics) to construct the *Mmp1a* targeting vector. A 1.4-kb HindIII fragment from the 5′-flanking containing part of exon 1, exons 2 and 3, and part of exon 4 was used as the 5′-homologous region, whereas a 6.8-kb fragment spanning from exon 6 to exon 7 was used as the 3′-region of homology (see Fig. 1*A*). The PGK-neo cassette was subcloned into an AscI site of the vector with the transcriptional orientation opposite to that of *Mmp1a* and replaced a 1.7-kb fragment containing exons 4, 5, and part of exon 6 of the gene.

The targeting vector was linearized by digestion with NotI and electroporated into HM-1 (129/Ola Hsd-Hprt⟨b-m3⟩) embryonic stem cells. Resistant clones were selected for homologous recombination with G418 and ganciclovir. Southern blot analysis of 198 neomycin-resistant colonies, using a 5′-external probe revealed homologous recombination in four independent clones. Correct recombination on the 3′-side was verified by PCR analysis. Two of these heterozygous stem cell clones were aggregated to CD1 morulas and transferred into uteri of pseudopregnant females to generate chimeras. Highly chimeric males were mated with C57BL/6J females, and the offspring was screened by Southern blot analysis of tail genomic DNA. Heterozygous mice from the two different clones were intercrossed to generate the colony of *Mmp1a*-deficient mice used in this study.

Northern Blot Analysis—Total RNA was isolated from frozen placenta samples obtained from wild-type and knock-out female mice at 13.5 days of embryonic development by using a commercial kit (RNeasy Mini kit; Qiagen). A total of 15 μg of denatured RNA was separated by electrophoresis on 1.2% agarose gels and transferred to Hybond N+ (Amersham Biosciences). Blots were prehybridized at 42 °C for 3 h in 50% formamide, 5× SSPE (1× = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10× Denhardt's solution, 2% SDS, and 100 μg/ml denatured herring sperm DNA, and then hybridized with a random primed ^{32}P-labeled cDNA probe for mouse *Mmp1a* (40090601, Geneservice) for 20 h under the same conditions. Blots were washed with 0.1× SSC, 0.1% SDS for 2 h at 50 °C and exposed to autoradiography. RNA integrity and equal loading was assessed by hybridization with a β-actin cDNA probe.

RT-PCR—Total RNA was reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen). A PCR reaction was then performed with the following *Mmp1a*-specific primers: *Mmp1a*-exon 4, 5′-GGACCTAACTATAAGCTTGCTCACA-3′; *Mmp1a*-exon 7, 5′-CTGGAAGATTTGGCCAGAGAATAC-3′. The PCR reaction was performed in a GeneAmp 9700 PCR system from Applied Biosystems for 35 cycles of denaturation (95 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 1 min). As a control, β-actin was PCR-amplified from all samples under the same conditions. Induction of cancer or other pathologies in mice was performed as indicated in the corresponding references: B16F10 and LLC lung cancer cell models (23), 3-methyl-cholanthrene-induced fibrosarcoma and 9,10-dimethyl-1,2-benzanthracene-induced skin cancer (24), *N*,*N*-diethylnitrosamine-induced hepatocarcinoma (25), 4-nitroquinoline 1-oxide-induced oral squamous carcinoma (26), CCl_4-induced hepatic fibrosis (27), for bleomycin-induced lung fibrosis (28), mouse mammary tumor virus-polyoma middle T antigen breast cancer (29), and keratin 14-human papillomavirus type 16 skin cancer (30).

Urethane Carcinogenesis Model—Mouse experimentation was done in accordance with the guidelines of the Universidad de Oviedo (Spain), regarding the care and use of laboratory animals. For urethane (ethylcarbamate; Sigma) chemical carcinogenesis, 12-week-old mice (15 animals per group) were

injected intraperitoneally with two doses (separated by 48 h) of a freshly prepared solution of 1 mg of urethane/g of body weight, dissolved in sterile 0.9% NaCl (saline). Mice were sacrificed 32 weeks after urethane exposure, and their lungs were either snap-frozen in liquid nitrogen for further RNA and protein analysis or fixed in 4% paraformaldehyde and processed for histological studies. Paraffin-embedded samples were cut in serial sections, stained with hematoxylin and eosin, and examined by two experienced pathologists without previous knowledge of mice genotypes. The total number of cells for each tumor was estimated from the number of cells present in the area of maximal section and tumors were then classified as small ($< 10^3$ cells) and large ($>10^3$ cells).

Difference Gel Electrophoresis—Lungs from WT and KO mice obtained 32 weeks after urethane injection were rinsed in TAM (10 mM Tris, pH 8.5, 5 mM magnesium acetate) and homogenized in TUCT (2 M thiourea, 7 M urea, 4% CHAPS, 30 mM Tris, pH 8.5). 50 μg of each sample were labeled with 400 pmol of a specific fluorophore (GE Healthcare): CyDye 3 (WT sample), CyDye 5 (KO sample), and CyDye 2 (pool of WT and KO sample, 1:1). Labeled samples were combined, and UCDA (8 M urea, 4% CHAPS, 130 mM DTT, 2% IEF buffer) was added in a 1:1 ratio. UCDA (8 M urea, 4% CHAPS, 13 mM DTT, 1% IEF buffer) was used to reach 450 μl of final volume. Samples were loaded in a strip holder, and 24-cm IPG strips, nonlinear pH gradient 3–11 (GE Healthcare), were placed over them. After strip rehydration, protein isoelectrofocusing was allowed to proceed for 26 h on an IPGphor Unit (GE Healthcare) in the dark at 18 °C. Then, strips were equilibrated for 15 min in SES-DTT (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris, pH 6.8, 0.5% DTT, and bromphenol blue) and 15 min in SES-IA (SES with 4.5% iodoacetamide), mounted on top of a 13% SDS-PAGE and electrophoresed at 80 V overnight in the dark at 18 °C. After SDS-PAGE, cyanine dye-labeled proteins were visualized directly by scanning using a Typhoon™ 9400 imager (GE Healthcare) and analyzed with Progenesis SameSpots software (Nonlinear Dynamics) and stained with SYPRO Ruby (Molecular Probes).

Tryptic Digestion and MALDI-TOF Analysis—Differential spots were manually excised over a transilluminator. Gel pieces were washed twice with 25 mM ammonium bicarbonate/acetonitrile (70:30), dried for 15 min at 90 °C, and incubated with 12 ng/μl trypsin (Promega) in 25 mM ammonium bicarbonate for 1 h at 60 °C. Peptides were purified with ZipTip C18 (Millipore) and eluted with 1 μl of α-cyano-4-hydroxycinnamic acid to be placed onto a MALDI-TOF plate. Once dried, they were analyzed by mass spectrometry on a time-of-flight mass spectrometer equipped with a nitrogen laser source (Voyager-DE STR, Applied Biosystems). Data from 200 laser shots were collected and analyzed with Data Explorer (version 4.0.0.0, Applied Biosystems).

Western Blotting—Samples were electrophoresed and transferred to PVDF (0.45-μm pore size) membranes (Millipore). Blots were blocked with 5% nonfat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with 3% BSA in TBS-T with either 0.2 μg/ml anti-CHI3L3, 0.2 μg/ml anti-S100A8 (R&D Systems), and 1:1000 anti-receptor for advanced glycation end-products (RAGE) (Cell Signaling). Finally, the blots were incubated for 1 h at room temperature in 2.5% nonfat dry milk in TBS-T buffer with 10 ng/ml of goat anti-rat horseradish peroxidase (GE Healthcare), rabbit anti-goat (Thermo Scientific), and donkey anti-rabbit (GE Healthcare), respectively. Then, blots were washed with TBS-T and developed with Immobilon Western chemiluminescent HRP substrate (Millipore). Chemiluminescent images were taken with a Fujifilm LAS3000 mini apparatus.

Enzymatic Assays—For *in vitro* proteolysis assays, we used recombinant S100A8 and S100A9 kindly provided by Dr. Philippe Tessier and recombinant CHI3L3 and MMP-1 from R&D Systems. Briefly, 100 ng of rat MMP-1 per reaction was activated with 4-aminophenylmercuric acetate at 37 °C for 2 h. Then, purified CHI3L3, S100A8, and S100A9 (1 μg) were incubated with activated MMP-1 at 37 °C for 24 h and analyzed by SDS-PAGE and Western blot.

Analysis of Cytokine Levels—To evaluate the levels of different Th1/Th2 cytokines, we used a mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit and a TGF-β1 kit (eBioscience), following the manufacturer's instructions. Briefly, snap-frozen lungs were homogenized at 4 °C in T-PER containing Complete Mini Protease Inhibitor Mixture tablets (1 tablet/50 ml of T-PER stock reagent) and centrifuged at 9,000 × *g* for 15 min. Total protein concentration in supernatant was determined using a BCA kit (Pierce). A total of 100 μg of each homogenate was incubated with antibody-coated bead complexes and biotinylated secondary antibody for 2 h. After washing, 100 μl streptavidin-phycoerythrin was added to each well and incubated for 1 h. Samples were then transferred to appropriate cytometry tubes and analyzed using the FC500 Cellular Cytomics analyzer (Beckman Coulter). A minimum of 300 events (beads) were collected for each cytokine/sample, and median fluorescence intensities were obtained. Cytokine concentrations were calculated based on standard curve data using FlowCytomix Pro software (version 3.0, eBioscience). The results were expressed as mean ± S.E. ($n = 5$).

RESULTS

Generation and Phenotype Analysis of Mmp1a-deficient Mice—To analyze the *in vivo* role in cancer of Mmp-1a, the murine counterpart of human MMP-1, we generated mutant mice deficient in *Mmp1a* by replacing exons 4, 5, and 6 encoding the catalytic domain, with a PGK-neomycin cassette (Fig. 1*A*). Following heterozygote intercrossing, *Mmp1a*-null, heterozygous, and wild-type mice were obtained in the expected Mendelian ratios. We verified homozygosity with respect to the mutated allele by Southern blot and the absence of functional transcripts by Northern blot analysis of placenta, a positive control for expression of this gene (Fig. 1, *B* and *C*). Despite the *Mmp1a* deficiency, these mutant mice developed normally and were fertile, and their long term survival rates were indistinguishable from those of their wild-type littermates. Furthermore, histopathological analysis of multiple tissues from $Mmp1a^{-/-}$ adult animals did not reveal any differences with wild-type tissues (data not shown). Taken together, these results demonstrate that Mmp-1a is absolutely dispensable for embryonic and adult mouse development as well as for normal growth and fertility.

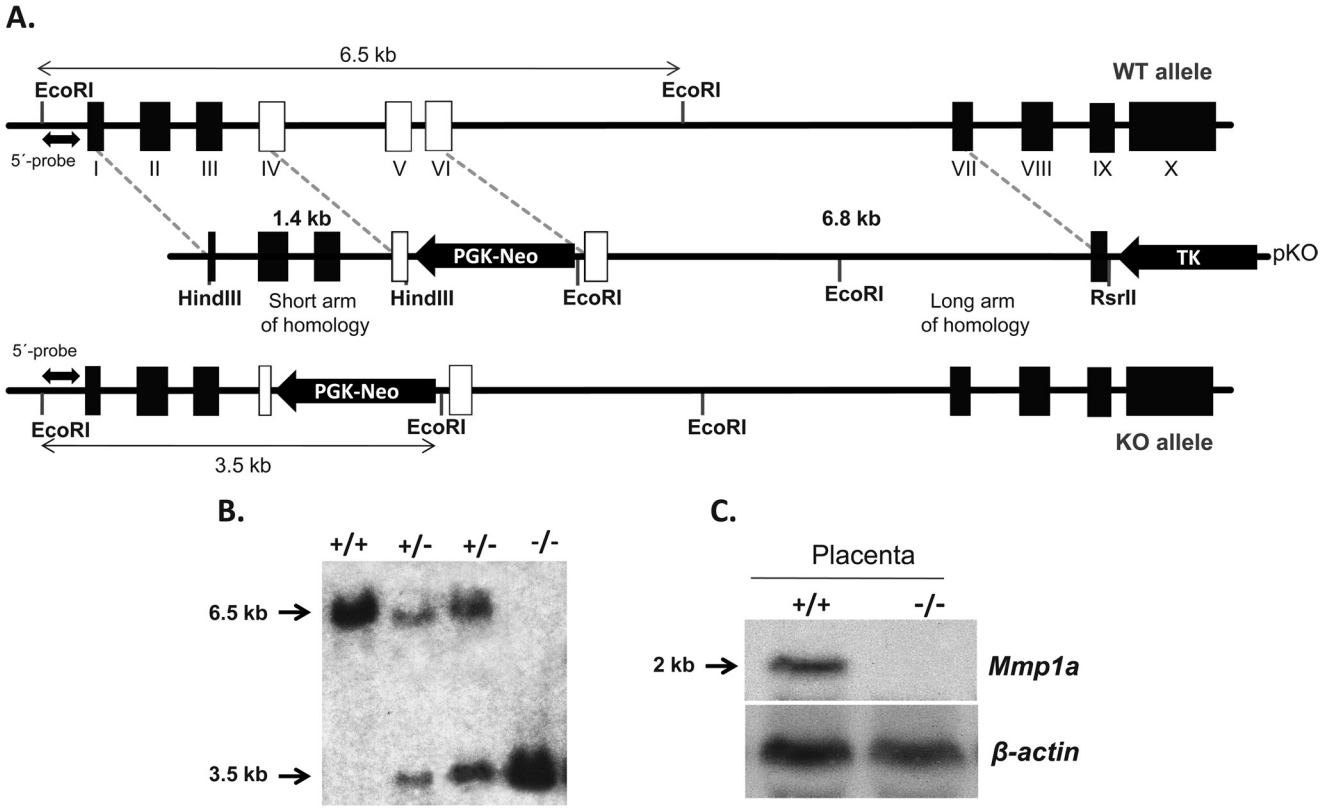

FIGURE 1. **Targeted disruption of mouse *Mmp1a* gene.** *A*, restriction maps of the *Mmp1a* gene region of interest (*top*), the targeting construct (*center*), and the mutant locus after homologous recombination (*bottom*). *B*, EcoRI Southern blot analysis of $Mmp1a^{+/+}$, $Mmp1a^{+/-}$, and $Mmp1a^{-/-}$ mice. *C*, detection of *Mmp1a* mRNA in placenta by Northern blot analysis.

Analysis of Cancer Susceptibility of Mmp1a-deficient Mice—The dispensability of Mmp-1a for mouse development, growth, and fertility opened the possibility to perform long term studies aimed at analyzing cancer susceptibility in mice deficient in this metalloproteinase. For this purpose, and because previous results had suggested that the expression of mouse *Mmp1a* in normal and pathological conditions could be more restricted than that of human *MMP1* (22), we first performed an RT-PCR expression analysis of *Mmp1a* in samples from wild-type mice in which cancer and other pathologies had been induced. As can be seen in Fig. 2*A*, *Mmp1a* expression was clearly detected in samples from lung carcinomas induced by chemical carcinogens or by Lewis cancer cells, in skin tumors induced by 9,10-dimethyl-1,2-benzanthracene/TPA or 3-methyl-cholanthrene, in oral carcinomas induced by 4-nitroquinoline 1-oxide, and in some acute pulmonary lesions induced by bleomycin treatment. In contrast, no significant expression of this mouse metalloproteinase gene was detected in mammary tumors induced by mouse mammary tumor virus-polyoma middle T antigen or in their metastasis (Fig. 2*A*). According to these results, we focused our study on the analysis of the susceptibility of *Mmp1a* mutant mice to lung cancer.

To this end, $Mmp1a^{-/-}$ mice and their wild-type littermates were subjected to a chemical carcinogenesis protocol with urethane and, after 8 months, mice were sacrificed, and the number and histopathological characteristics of their pulmonary lesions were examined. The penetrance of this carcinogenesis model was 100%, and all mice developed tumors. Nevertheless, as can be seen in Fig. 2, *B* and *C*, the number of lung tumors per mouse was higher in wild-type than in mutant mice, especially in the case of male mice, a gender difference that we had already observed in our previous studies with *Mmp8* mutant mice (24). Nevertheless, and beyond these sex-specific differences, we can conclude that the loss of Mmp-1a protects against lung cancer induced by chemical carcinogens such as urethane. To attempt to extend these observations, we next performed a histopathological analysis of tumors generated in both genetic backgrounds with the finding that the number of *Mmp1a* mutant mice that developed large tumors was notably lower than that of wild-type mice (Fig. 3*A*). Likewise, we could observe the presence of many inflammatory infiltrate foci in the lungs of knock-out mice (Fig. 3*B*). Further analysis revealed that the inflammatory infiltrate present in $Mmp1a^{-/-}$ mice was mainly constituted by mature lymphocytes. We also evaluated the possibility that this inflammatory response was secondary to bronchial damage caused by the lung carcinomas, which could result in obstructive pneumonia with intra-alveolar macrophages. However, we did not find any evidence in this regard. These characteristics are fully consistent with the idea that the absence of Mmp-1a hampers tumor progression.

Proteomic Analysis of Lung Tissues from Mmp1a-deficient Mice—As a first step to elucidate the molecular mechanisms underlying the above described tumor-promoting properties of Mmp-1a, we performed a proteomic analysis by difference gel electrophoresis of lung samples from urethane-treated mutant mice and the corresponding controls. After these difference gel

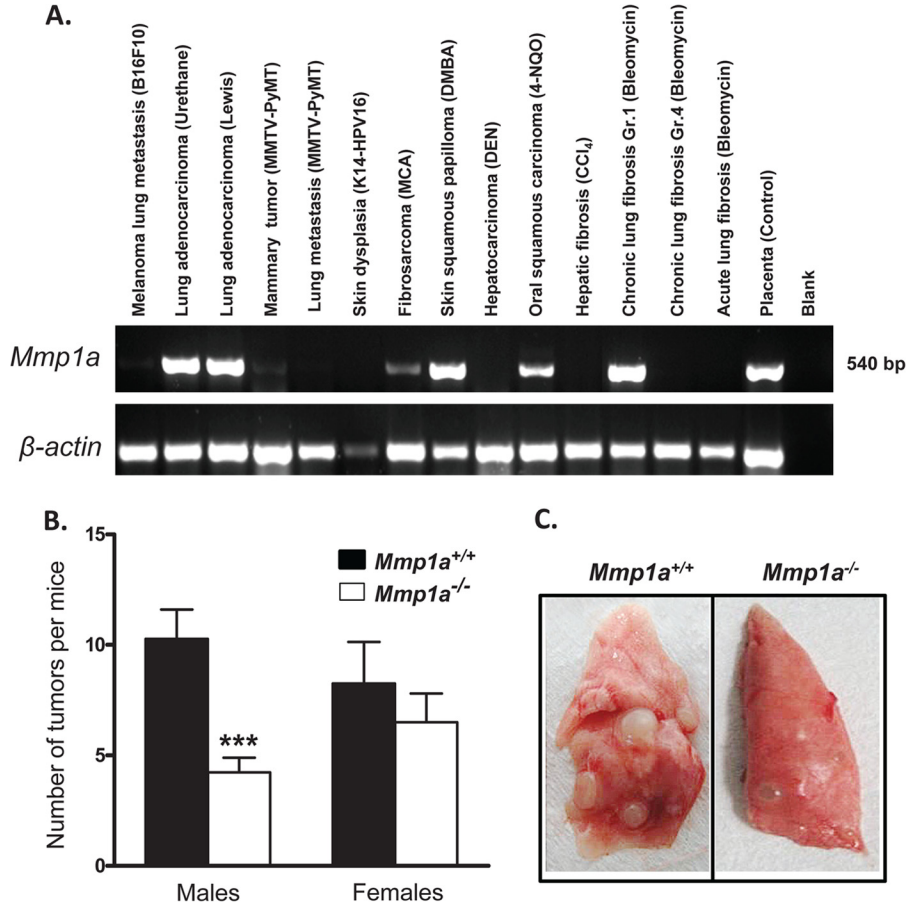

FIGURE 2. **Mmp1a relevance on cancer susceptibility in mice.** *A*, mRNA expression levels in tissues from wild-type mice subjected to different experimental protocols. *MMTV-PyMT*, mouse mammary tumor virus-polyoma middle T antigen; *K14-HPV16*, keratin 14-human papillomavirus type 16; *MCA*, 3-methylcholanthrene; *DMBA*, 9,10-dimethyl-1,2-benzanthracene. *DEN*, *N*,*N*-diethylnitrosamine; *4-NQO*, 4-nitroquinoline 1-oxide; CCl_4, carbon tetrachloride. *B*, number of total lung tumors per mouse after 8 months of urethane intraperitoneal treatment. *C*, representative images of lungs from mutant and control mice ($n = 15$ per group; ***, $p < 0.001$ by Student's *t* test.

electrophoresis experiments coupled to mass-spectrometry analysis, we could identify a series of differential proteins between tissues from normal and mutant mice (supplemental Table 1). Specifically, we focused on chitinase-3-like protein 3 (CHI3L3) and RAGE, which were up-regulated in wild-type and knock-out mice, respectively (Fig. 4*A* and supplemental Fig. 1*A*). To further validate these results, we performed two-dimensional Western blot analysis with antibodies specific against the differential proteins identified by difference gel electrophoresis in the urethane-treated mice lungs (supplemental Fig. 1*B*). These analyses, together with those performed with other urethane-treated littermates, corroborated that both CHI3L3 and RAGE protein levels were increased in lung samples from wild-type and *Mmp1a*$^{-/-}$ mice, respectively (Fig. 4*B*). Additionally, we observed that CHI3L3 was the target of a processing event in the urethane-treated lung samples from wild-type that was not present in the equivalent samples from *Mmp1a*$^{-/-}$ mice (Fig. 4*B*). By using mass spectrometry analysis, we identified these different forms of CHI3L3 as the complete protein or fragments thereof corresponding to the N-terminal and C-terminal region (supplemental Fig. 1*A*).We next evaluated the possibility that levels of the S100A8 and S100A9 ligands of the RAGE receptor could also be altered in the lung samples from knock-out mice. To this purpose, we performed a bidimensional Western blot analysis, which demonstrated the accumulation of high levels of S100A8, as well as the occurrence of a differential processing event of this chemotactic protein in samples from *Mmp1a*$^{-/-}$ mice (Fig. 4*C*). By contrast, S100A9 levels did not exhibit any significant difference in mice from both genotypes (data not shown). To investigate whether any of these proteins could be a substrate for MMP-1, we performed an *in vitro* digestion with the recombinant protease. As can be seen in Fig. 4*D*, incubation with recombinant MMP-1 caused a shift in the electrophoretic mobility of the large form of S100A8, similar to that produced by MMP-2, used as a control on the basis of its previously described cleavage of this protein (31). By contrast, S100A9 and CHI3L3 were not cleaved by this collagenolytic enzyme (Fig. 4*D*). Taken together, these results could indicate the occurrence of an imbalance in the inflammatory response induced in wild-type and *Mmp1a*$^{-/-}$ mice by chemical carcinogens.

Inflammatory Response Evaluation after Urethane-lung Carcinogenesis—Because the high levels of CHI3L3 in wild-type mice could be indicative of a Th2 protumoral inflammatory response (32, 33), and the increased levels of RAGE and its ligand S100A8 in mutant mice could point to the occurrence of a Th1 anti-tumoral response (33, 34), we next evaluated the levels of Th1 and Th2 cytokines in lungs from wild-type and *Mmp1a* knock-out mice. To this aim, a total of 14 different

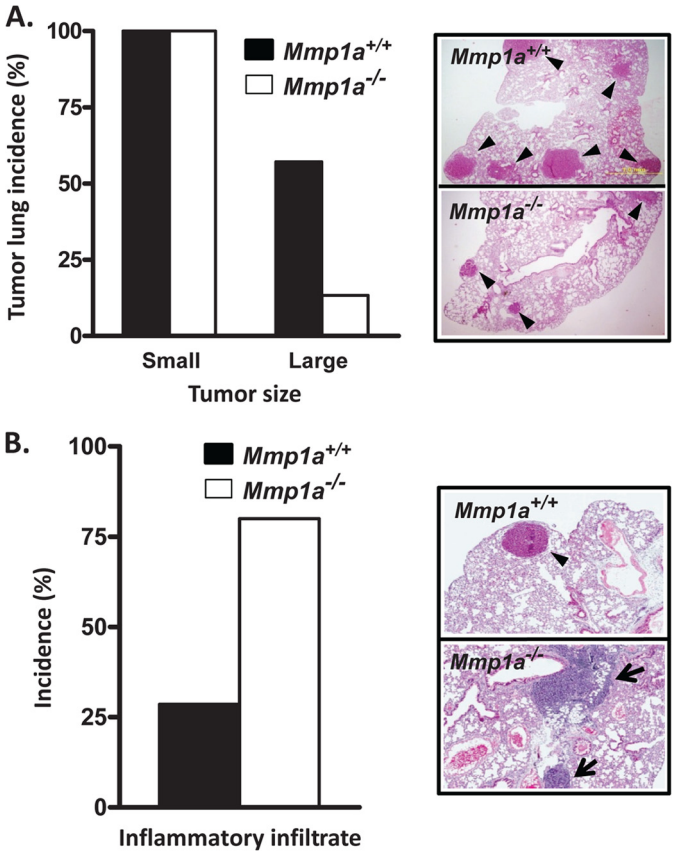

FIGURE 3. **Phenotypic features of urethane-induced tumors in *Mmp1a* wild-type and knock-out mice.** *A*, graphic representation of size tumor incidence in males. Knock-out mice show a lower incidence of large tumors ($>10^3$ cells). *B*, inflammatory infiltrate incidence in both sexes. We represent the number of mice which present inflammatory infiltrate foci in their lungs, showing an increased presence of inflammatory foci in lungs from knock-out mice. Inflammatory infiltrates are indicated with *arrows*, and tumors are shown with *arrowheads*.

mouse cytokines were simultaneously analyzed by flow cytometry. As can be seen in Fig. 5*A*, levels of well established Th1-associated cytokines such as IL-1α, IL-2, IL-27, and IFN-γ, are increased in lungs from *Mmp1a*$^{-/-}$ mice compared with their corresponding controls. Likewise, levels of IL-5, IL-10, IL-13, and TGF-β1, which are archetypal Th2-response cytokines, are consistently higher in wild-type mice than in *Mmp1a*-deficient mice, although the differences did not reach statistical significance due to high variability in the control samples. Notably, TNFα levels were not different in mice from both genotypes, thus indicating that this cytokine is not especially relevant in the generation of Th1/Th2 balance differences in this particular mouse model. Furthermore, and because IL-17 has been described to be highly dependent on IFN-γ (35), we evaluated the levels of both cytokines at different time points during the development of the urethane-induced lung cancer models, with the finding that IL-17 and IFN-γ showed a parallel increase in *Mmp1a*$^{-/-}$ mice in the course of the carcinogenesis experiment (Fig. 5*B*).

To further evaluate the putative Th1-polarized inflammatory response occurring in *Mmp1a*$^{-/-}$ mutant mice during urethane carcinogenesis, we analyzed levels of most significant cytokines found at increased levels in lung from *Mmp1a* knock-out mice. Thus, we measured IL-17, IL-27, and IFN-γ levels in the lungs from two different groups of mutant mice based on the lung inflammatory phenotype presented by them. To this end, we compared mutant mice showing a high number of inflammatory infiltrate foci in the lungs with those animals that did not present any of these foci. It is noteworthy that the group of mutant mice with lower number of inflammatory infiltrates presented the higher number of tumors (mean, 8 *versus* 3.8, respectively). Interestingly, the levels of these Th1-related cytokines displayed by the group with inflammatory infiltrate were higher than those found in the group in which that infiltrate was not observed (Fig. 5*C*). Taken together, all of these results, it seems that *Mmp1a*$^{-/-}$ mice develop a Th1 antitumoral response, which, in turn would contribute to explain the significantly low number of chemically induced lung carcinomas observed in mice deficient in this metalloproteinase.

DISCUSSION

In this work, we have generated mutant mice deficient in *Mmp1a* and demonstrated that they represent a new and valuable *in vivo* model for the functional analysis of MMP-1 in cancer. During the past few years, evidence has accumulated that MMP-1 is associated with tumor progression and metastasis (16, 17, 19). However, and somewhat surprisingly, very limited information is available about the putative functions mediated by MMP-1 during cancer development and progression. This is especially puzzling if we consider that MMP-1 was the first human MMP cloned and characterized at the biochemical level, and its correlative links with tumor invasion and metastasis were first reported more than 20 years ago (11, 36). One possibility to explain this lack of functional information about MMP-1 in cancer is the absence of an *in vivo* model of MMP-1 deficiency, an aspect that has been largely attributed to the wide assumption that no MMP-1 ortholog was present in rodents. However, our finding of two murine genes (*Mmp1a* and *Mmp1b*, also known as *McolA* and *McolB*) similar to human MMP-1 opened a series of further studies, which allowed us to conclude that *Mmp1a* is a *bona fide* counterpart of human MMP-1 (22). This finding was the starting point of a long term work that has now led us to the generation and analysis of mutant mice deficient in *Mmp1a*.

Similar to most cases of *Mmp* deficiency (1), *Mmp1a*$^{-/-}$ mice are viable and fertile. The absence of alterations in *Mmp1a*-null mice in processes such as uterine involution or mammary gland involution are absolutely consistent with previous findings demonstrating that functions originally ascribed to MMP-1 were in fact performed by other collagenases such as MMP-8 and MMP-13 (37–39). The current work describing the generation of the first *in vivo* model to differentially evaluate both collagenases may contribute to clarifying their relative importance in different biological processes. Notably, the lack of significant abnormalities in *Mmp1a*$^{-/-}$ mice has facilitated studies aimed at evaluating their cancer susceptibility. These studies first focused on lung carcinomas because our previous *Mmp1a* expression analysis in tissues from mice subjected to several carcinogenesis protocols revealed high expression levels of this murine metalloproteinase in different samples of lung cancer. After application of a urethane-based protocol of lung

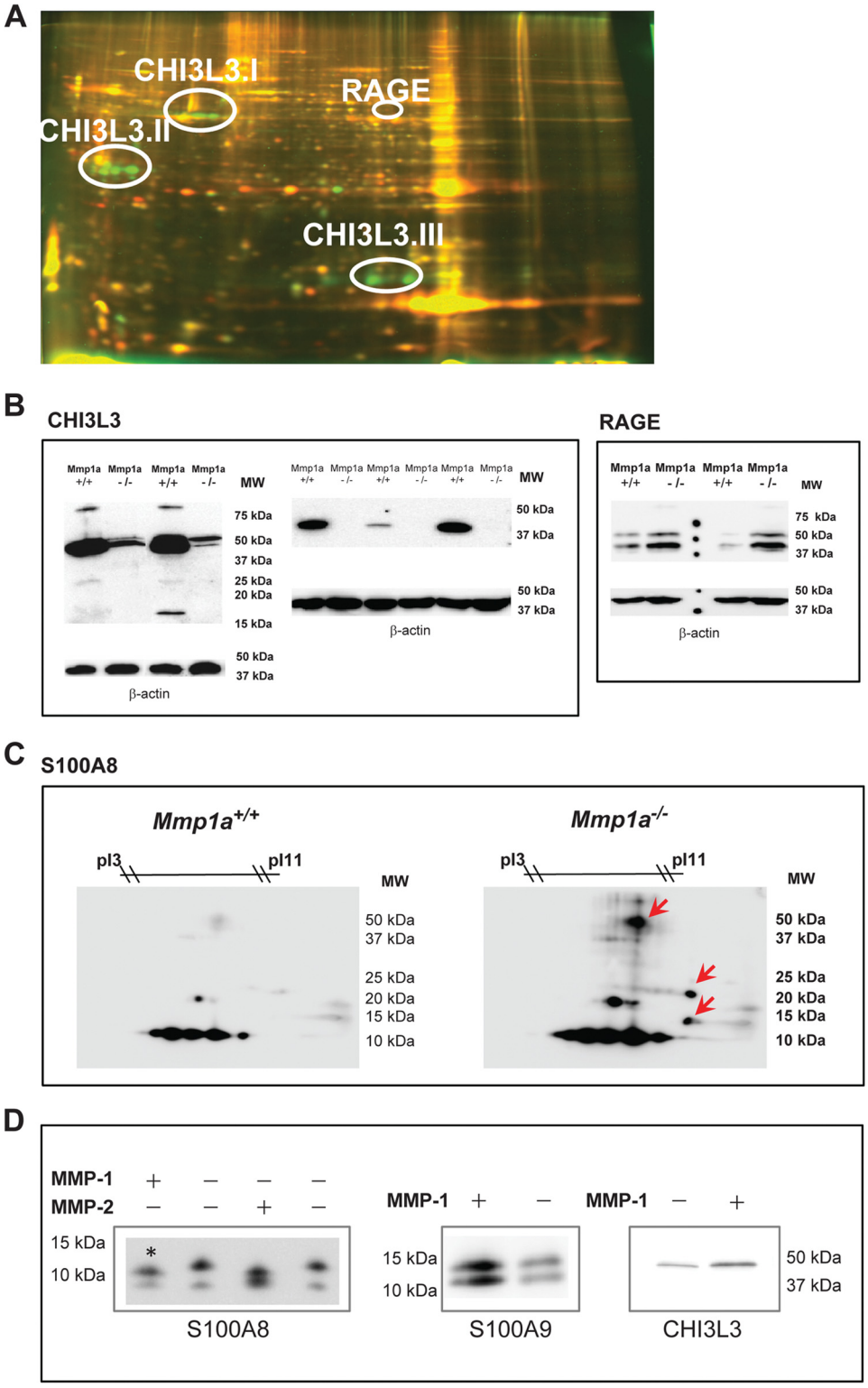

FIGURE 4. *A*, difference gel electrophoresis analysis of lung tissue from urethane-treated mice. Overlaying *green* and *red* image highlights differences between wild-type and *Mmp1a*$^{-/-}$ mice. *Yellow* indicates no change, *red spots* indicate more abundance in knock-out mice, and *green spots* more abundance in wild-type mice. Selected proteins are labeled with *white circles*, and bidimensional validation analysis is shown in supplemental Fig. 1. All differential analyzed spots are listed in supplemental Table 1. *B*, Western blot analysis extended to other urethane-treated littermates showing the increased CHI3L3 and RAGE levels in wild-type and knock-out mice, respectively, as well as the differential processing of CHI3L3 in wild-type lungs no present in the mutant lungs. Load control is shown at the *bottom* of each *panel*. *C*, Western blot analysis of the RAGE ligand S100A8 showing accumulation of different isoforms of this chemokine in lung from knock-out mice. *D*, *in vitro* cleavage assays with human MMP-1. Purified S100A8, S100A9, and CHI3L3 (1 μg) were incubated with 100 ng of activated MMP-1, which resulted in specific cleavage of S100A8 (*), but not S100A9 and CHI3L3. Recombinant MMP-2 was used as a positive control of S100A8 cleavage.

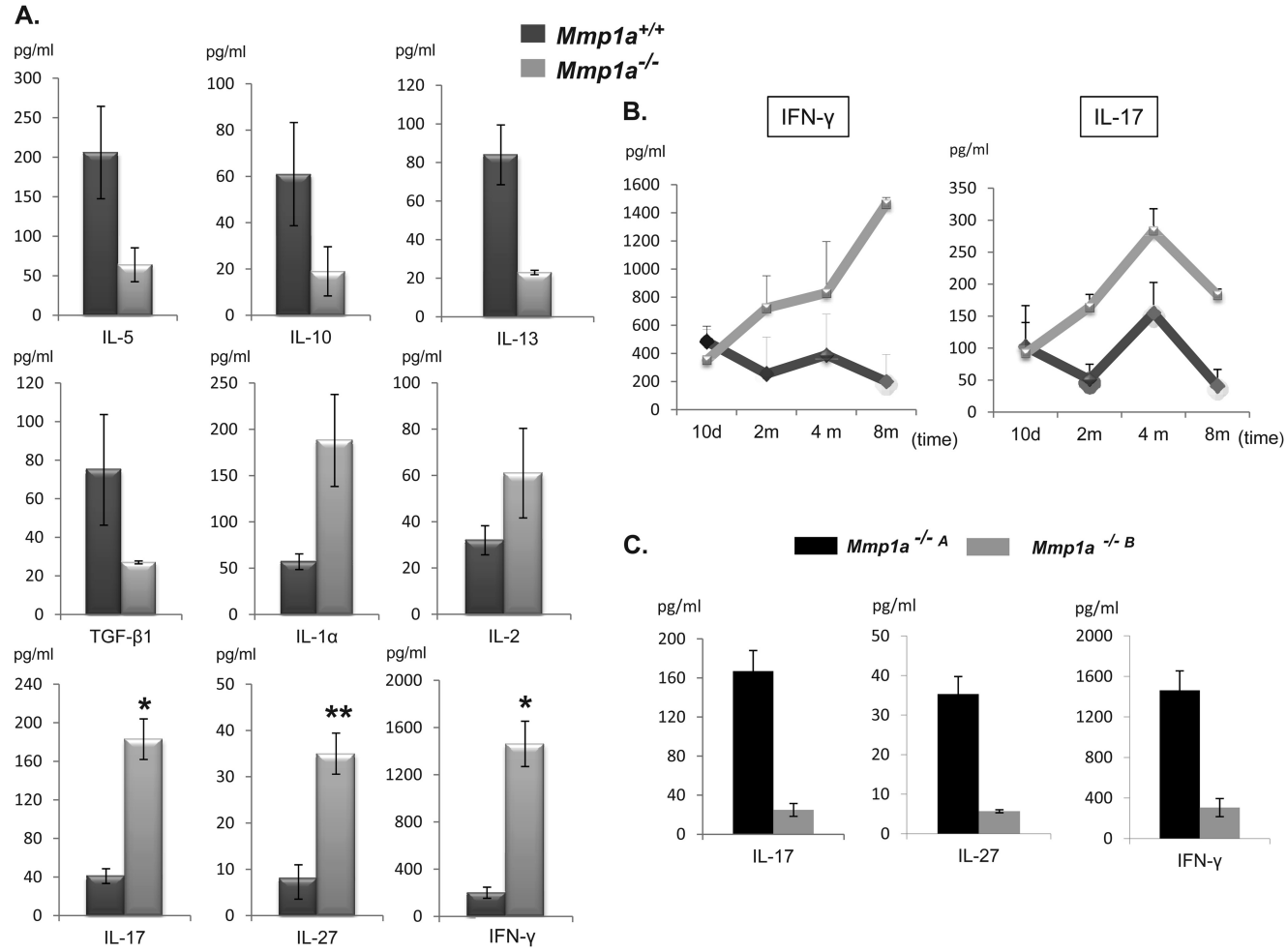

FIGURE 5. **Analysis of cytokine levels in lung from *Mmp1a* wild-type and knock-out mice treated with urethane.** *A*, cytokine levels measured in lung mice after 8 months of urethane intraperitoneal treatment. *B*, measurement of IFN-γ and IL-17 in the lungs of mutant and control mice at several time points along the urethane carcinogenesis model. *C*, levels of Th1 cytokines in lungs from two different groups of *Mmp1a*-deficient mice separated on the basis of their inflammatory phenotype. $Mmp1a^{-/-}$ group A ($Mmp1a^{-/-\,A}$) represents those mutant mice showing a notably high number of inflammatory infiltrate foci in the lungs, whereas $Mmp1a^{-/-}$ group B ($Mmp1a^{-/-\,B}$) represents those mutant mice with no overt infiltrate foci in their lungs. *, $p < 0.01$; **, $p < 0.001$ by Student's *t* test.

cancer induction, we observed that $Mmp1a^{-/-}$ mice showed a lower incidence of lung carcinomas than their corresponding wild-type littermates. Interestingly, this difference was more marked in male than in female mutant mice, indicating the occurrence of a gender difference that has been previously reported in other models of *Mmp* deficiency (24). The different susceptibility of male and female $Mmp1a^{-/-}$ mice to tumor development is likely due to hormonal factors as previously demonstrated in the *Mmp8*-null model, in which ovariectomy or estrogen receptor antagonist treatment increases the incidence of chemically induced tumors in $Mmp8^{-/-}$ female mice, and abrogates the gender differences in cancer susceptibility (22). Similar studies in *Mmp1a*-null mice will be necessary to confirm the hormonal basis of the gender differences in cancer susceptibility in these mutant animals. Interestingly, histopathological analysis showed that tumors generated in $Mmp1a^{-/-}$ mice are smaller than those of wild-type mice, a characteristic which is consistent with the idea that the absence of Mmp-1a hampers tumor progression. These *in vivo* findings agree perfectly with very recent *in vitro* data showing that silencing *Mmp1a* suppresses invasive growth of lung cancer cells in three-dimensional matrices, whereas ectopic expression of this murine metalloproteinase confers invasive properties to epithelial cells (40).

The protumorigenic role of MMPs was originally ascribed to their ability to break down tissue barriers for metastatic spread. Both human MMP-1 and mouse Mmp-1a are potent collagenases with the ability to degrade different types of fibrillar collagens consistent with a role for these metalloproteinases degrading the extracellular matrix and promoting tumor progression (16, 17, 19). Nevertheless, the growing evidence that virtually all MMPs, including MMP-1, target many other proteins distinct from extracellular matrix components (41–43), prompted us to perform comparative proteomic studies between samples of *Mmp1a* mutant and wild-type mice to try to identify putative *in vivo* substrates of Mmp-1a. Among the identified alterations, we found decreased CHI3L3 levels and increased RAGE levels in lung samples from urethane-treated mutant mice compared with controls. Further studies demonstrated that the S100A8 ligand for the RAGE receptor is also elevated in samples from *Mmp1a* mutant mice. Accordingly, this chemotactic protein could be a substrate directly targeted

by Mmp1a, a proposal that we further assessed by performing a series of enzymatic assays, which demonstrated that the recombinant collagenase cleaves S100A8, but no other related proteins such as S100A9. The impaired degradation of this immunoregulatory protein in *Mmp1a*-deficient mice could also contribute to the generation of marked differences in the inflammatory response induced by chemical carcinogens in *Mmp1a* wild-type and mutant mice. Thus, the increased levels of CHI3L3 together with high levels of several Th2-related cytokines in wild-type mice strongly suggests that urethane is inducing the archetypical Th2-polarized inflammatory response which operates under protumoral stimuli (44). In contrast, the absence of increased levels of CHI3L3 in $Mmp1a^{-/-}$ mice together with the presence of high levels of RAGE and its ligand S100A8, which are Th1 markers (33, 34), as well as high levels of IFN-γ and other antitumor cytokines such IL-1α and IL-2, is consistent with the possibility that *Mmp1a* deficiency hampers the Th2 response triggered by carcinogen injection in $Mmp1a^{-/-}$ mice. Moreover, further studies showed that mice that developed higher number of lung tumors did not display the Th1 cytokine profile observed in their mutant counterparts. Thus, it is tempting to speculate that inflammatory infiltrate in knock-out mice confers antitumor properties that finally result in the observed lower number of lesions in this group of animals. Likewise, our data are consistent with the hypothesis that IL-17 acts as a tumor-inhibiting cytokine in the lung carcinogenesis model used in this work.

MMPs have been associated with inflammatory responses in a wide variety of diseases (45). Likewise, several reports have described that cytokines released from different Th1/Th2 cell types can modulate MMP expression. Thus, it is well established that Th2 responses are associated with an increased expression of matrix degradative MMPs, including human MMP-1 (46). Moreover, MMPs may also act as direct inducers of this process, modulating chemokine gradients or processing specific cytokines, and finally switching the balance between both types of responses. This is the case of MMP-2, which is overexpressed in multiple cancers and induces a Th2 polarization through the degradation of type I IFN receptor in dendritic cells (47). In addition, MMP-9 proteolytically activates TFG-β, which in turn promotes differentiation toward the Th2 protumorigenic phenotype (48). Accordingly, lungs from *Mmp1a*-deficient mice exhibit lower levels of active TFG-β1 than controls suggesting that these mutant mice lack the capacity to proteolytically activate TFG-β1. Finally, the fact that human MMP-1 also cleaves the latent form of TGF-β and facilitates tumor invasion and angiogenesis (42) would agree very well with our proposal that its mouse ortholog Mmp-1a acts *in vivo* as a switching protease, which changes the Th1/Th2 balance toward a protumoral state. Nevertheless, further experimental work, now in progress, will be required to better understand the molecular mechanisms underlying this Mmp1a-mediated inflammatory response to chemical carcinogens.

In summary, the generation of the first mouse model of MMP-1 deficiency has contributed to the *in vivo* validation of this enzyme as a protumorigenic protease with potential impact in different stages of tumor progression, including cell proliferation and regulation of inflammatory responses. These studies have also validated the concept that human MMP-1 is a target protease, at least in some types of cancer, in which its expression is profoundly deregulated. Additional studies aimed at inducing other tumor types in $Mmp1a^{-/-}$ mice will be necessary to define the precise *in vivo* role of this protease in different malignancies because it could have dual roles in tumor development (49). Likewise, studies involving mice simultaneously deficient in *Mmp1a* and other protumorigenic MMPs will be required to evaluate the functional redundancy and relative relevance of MMP-1 in different stages of cancer progression. Hopefully, these studies will contribute to the appropriate targeting of this proteolytic enzyme as part of novel and combined targets for the treatment of malignant tumors (50).

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