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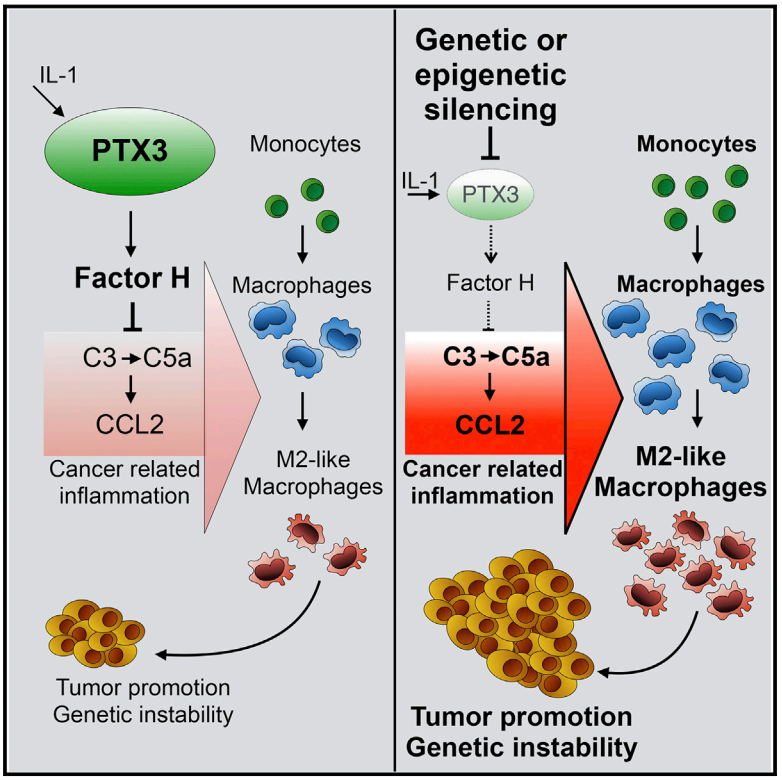
PTX3 Is an Extrinsic Oncosuppressor Regulating Complement-Dependent Inflammation in Cancer

Graphical Abstract



Highlights

- PTX3 deficiency unleashes Complement-dependent tumor-promoting inflammation
- Tumors developed in a PTX3-deficient context have higher frequency of mutated *Trp53*
- PTX3 expression is epigenetically repressed in selected human tumors
- Complement is an essential component of tumor-promoting inflammation

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In Brief

PTX3 deficiency triggers Complement-dependent tumor-promoting inflammation, with enhanced tumor burden, macrophage infiltration, cytokine production, angiogenesis, and genetic instability, revealing the role of this innate immunity mediator as an extrinsic oncosuppressor.

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PTX3 Is an Extrinsic Oncosuppressor Regulating Complement-Dependent Inflammation in Cancer

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SUMMARY

PTX3 is an essential component of the humoral arm of innate immunity, playing a nonredundant role in resistance against selected microbes and in the regulation of inflammation. PTX3 activates and regulates the Complement cascade by interacting with C1q and with Factor H. PTX3 deficiency was associated with increased susceptibility to mesenchymal and epithelial carcinogenesis. Increased susceptibility of *Ptx3*$^{-/-}$ mice was associated with enhanced macrophage infiltration, cytokine production, angiogenesis, and *Trp53* mutations. Correlative evidence, gene-targeted mice, and pharmacological blocking experiments indicated that PTX3 deficiency resulted in amplification of Complement activation, CCL2 production, and tumor-promoting macrophage recruitment. PTX3 expression was epigenetically regulated in selected human tumors (e.g., leiomyosarcomas and colorectal cancer) by methylation of the promoter region and of a putative enhancer. Thus, PTX3, an effector molecule belonging to the humoral arm of innate immunity, acts as an extrinsic oncosuppressor gene in mouse and man by regulating Complement-dependent, macrophage-sustained, tumor-promoting inflammation.

INTRODUCTION

Inflammatory cells and molecules are an essential component of the tumor microenvironment (Coussens et al., 2013; Grivennikov et al., 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008). The connection between inflammation and cancer can be viewed as consisting of two pathways. Selected inflammatory conditions, the prototype of which is ulcerative colitis, increase the risk of developing cancer (extrinsic pathway). On the other hand genetic events that cause cancer orchestrate the construction of an inflammatory microenvironment even in tumors that are epidemiologically unrelated to inflammation (Mantovani et al., 2008). Genetic approaches have unequivocally proven the role of cells and mediators (e.g., cytokines) of the innate and adaptive immune system in sustaining tumor-promoting inflammation. However, no dominant or suppressor oncogene encodes for effector molecules of the humoral innate immune system.

The innate immune system includes a cellular and a humoral arm (Garlanda et al., 2005). The humoral arm of innate immunity is constituted of diverse molecules including Complement components, collectins, ficolins, and pentraxins (Bottazzi et al., 2010). The long pentraxin PTX3 is an essential component of the humoral arm of innate immunity (Bottazzi et al., 2010; Garlanda et al., 2005). PTX3 acts as a functional ancestor of antibodies: it interacts with selected microbial molecules (Jeannin et al., 2005); it has opsonic activity via Fcγ receptors (Moalli et al., 2010); it activates and regulates the Complement cascade by interacting with C1q and Factor H (Bottazzi et al., 1997; Deban et al., 2008); and it regulates inflammation by interacting with P-selectin via its glycosidic moiety (Deban et al., 2010). PTX3 plays a nonredundant role in resistance against selected microbial pathogens in mouse and man (Chiarini et al., 2010; Cunha et al., 2014; Garlanda et al., 2002) and is a candidate therapeutic agent against *Aspergillus fumigatus* and *Pseudomonas aeruginosa*.

In the context of an effort to translate this molecule to the clinic as a novel candidate antimicrobial agent in cancer patients at risk of infection (Cunha et al., 2014), it was important to assess its role in carcinogenesis. We found that the humoral pattern recognition molecule PTX3 acts as an oncosuppressor in mice and humans by regulating Complement-dependent tumor-promoting inflammation. This observation represents a missing link in the connection between inflammation and cancer by providing genetic evidence that an effector molecule of humoral innate immunity can act as a cancer gene.

RESULTS

PTX3 Deficiency Increases Susceptibility to Cancer Development and Growth

In order to address the role of PTX3 in cancer-related inflammation (CRI) and tumor development, we analyzed the susceptibility

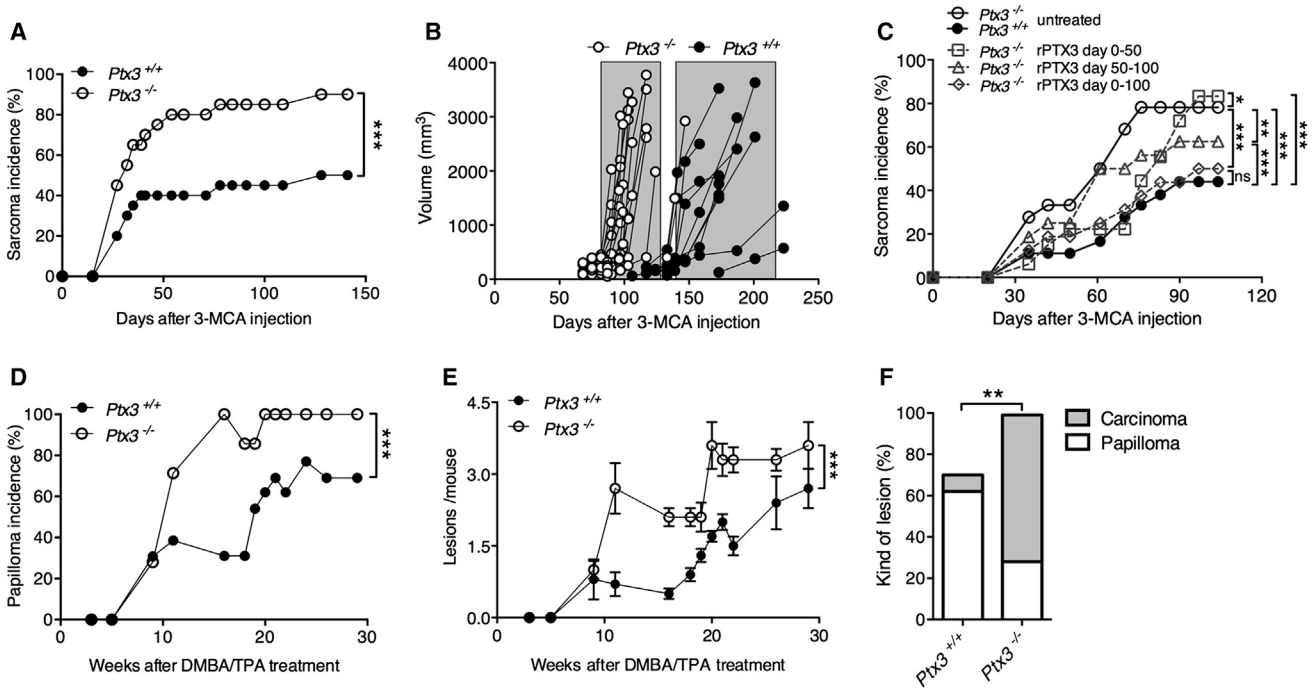

Figure 1. PTX3 Deficiency Increases Susceptibility to Carcinogenesis

(A and B) Incidence (A) and tumor volume (B) of 3-MCA-induced sarcoma in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice. In (B), the volume of each tumor is shown. One experiment with 20 mice/group out of ten performed with similar results is shown.

(C) Effect of recombinant PTX3 (9 μg/day, s.c. by osmotic pump) administered from day 0 to 50,–100, or from 0–100 on tumor incidence.

(D and E) Incidence (D) and number of lesions (mean ± SEM) (E) of DMBA/TPA-induced skin papillomas in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice. One experiment with ten mice/group out of five performed with similar results is shown.

(F) Histological classification of lesions developed in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice. **p < 0.01, Chi-square test. Two pooled experiments.

(A–E) *p < 0.05, **p < 0.01, ***p < 0.001 paired Student's t test. See also Figure S1.

of *Ptx3*$^{-/-}$ mice in different models of mesenchymal or epithelial carcinogenesis. As shown in Figure 1A, in the model of 3-Methylcholanthrene (3-MCA)-induced carcinogenesis, 80% *Ptx3*$^{-/-}$ mice developed a sarcoma in comparison to 40% *Ptx3*$^{+/+}$ mice (p = 0.0002). *Ptx3*$^{-/-}$ sarcomas grew faster than *Ptx3*$^{+/+}$ tumors and reached the endpoint (3 cm^3) in 100–120 days, whereas *Ptx3*$^{+/+}$ tumors reached it in 170–220 days (Figure 1B). Treatment with recombinant PTX3 subcutaneously (s.c.) throughout the carcinogenesis process (days 0–100) completely rescued the phenotype of *Ptx3*$^{-/-}$ mice (Figure 1C). Early treatment (days 0–50) inhibited the early difference, with an increase in incidence following interruption of treatment. A minor decrease in tumor incidence was also observed when treatment was begun on day 50 through day 100 (Figure 1C). The impact of PTX3 administration after day 50 may well reflect a role of Complement (see below) in promotion of established transplanted tumors (Markiewski et al., 2008). Treatment of *Ptx3*$^{+/+}$ mice caused some delay in tumor appearance but did not affect incidence (Figure S1A available online).

We next addressed the relevance of PTX3 deficiency in a model of 7,12-dimethylbenz [α] anthracene/terephthalic acid (DMBA/TPA)-induced skin carcinogenesis. As shown in Figures 1D and 1E, both the incidence and the multiplicity of papillomas were significantly increased in *Ptx3*$^{-/-}$ mice in comparison to *Ptx3*$^{+/+}$ mice (p = 0.0004 and p = 0.0005, respectively). In addition, the number of lesions evolving to skin carcinomas was significantly higher in *Ptx3*$^{-/-}$ mice (p = 0.009), suggesting a more aggressive behavior of DMBA/TPA-induced skin lesion in PTX3-deficient hosts (Figure 1F).

These data indicate that PTX3 is involved in controlling the incidence and growth of tumors of both mesenchymal and epithelial origin.

Production of PTX3 during Carcinogenesis

The following experiments, aimed at defining the underlying cellular and molecular mechanisms, were focused on the 3-MCA-induced carcinogenesis model. We first analyzed the production of PTX3 during carcinogenesis. 3-MCA is a carcinogenic compound acting as initiator and promoter, metabolized by the liver. PTX3 levels were increased in serum (Figure 2A) and liver (not shown) a few hours (8 and 24) after 3-MCA treatment, whereas after 7 days they had returned to basal levels. At the injection site, the levels of PTX3 were higher 24 hr after 3-MCA treatment in comparison to vehicle and further increased at day 7. PTX3 was originally cloned as an IL-1 inducible gene (Garlanda et al., 2005). It was therefore important to assess whether IL-1 was upstream of PTX3 in 3-MCA carcinogenesis. Both systemic and local PTX3 levels were significantly lower in *Il1r1*$^{-/-}$ mice compared to wild-type mice, suggesting that in this model, IL-1 is involved in PTX3 induction (Figure 2A). In established

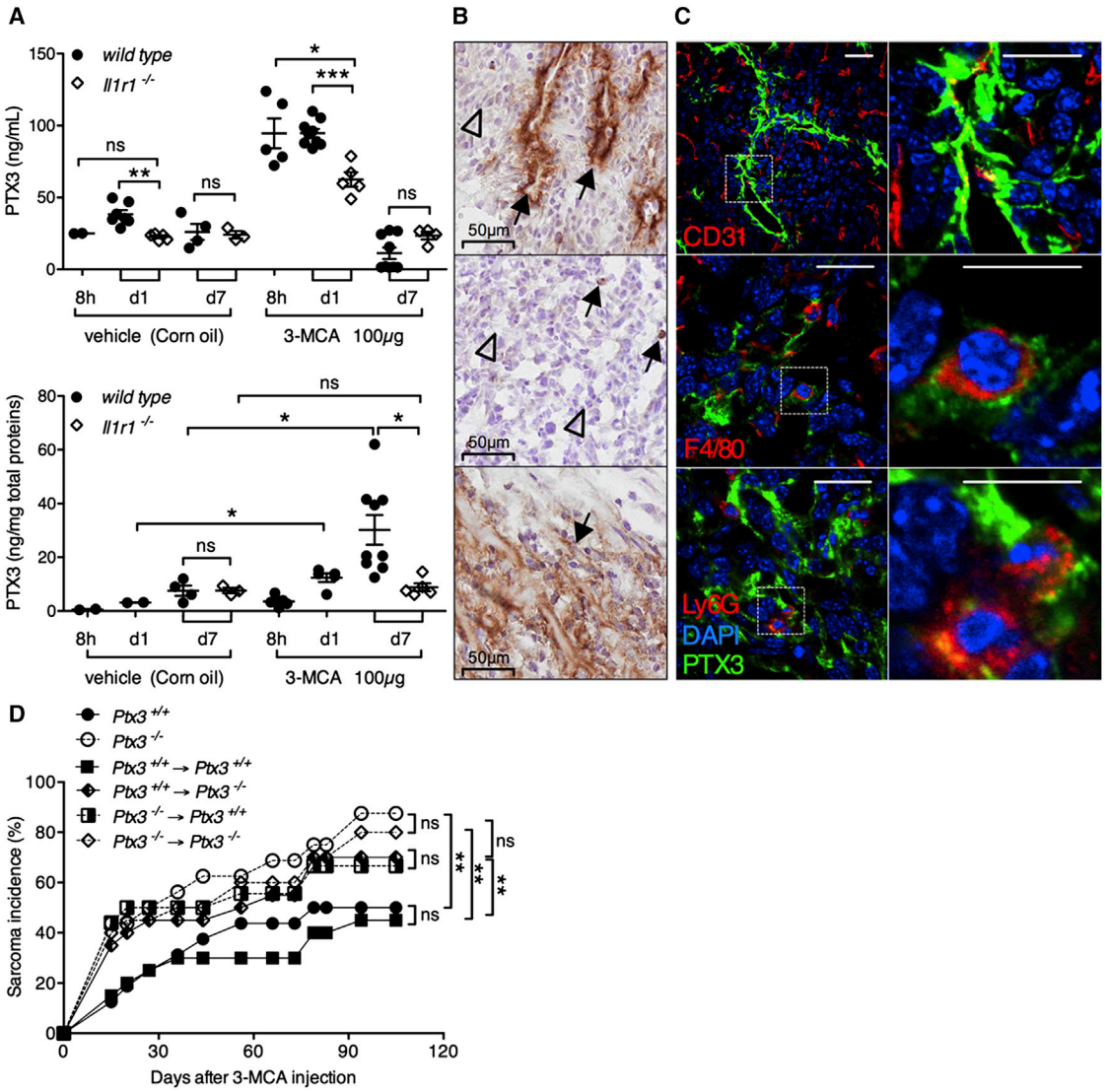

Figure 2. Analysis of the Cellular Source of PTX3 and of Its Role in 3-MCA-Induced Carcinogenesis

(A) Systemic (upper panel) and local (lower panel) PTX3 production at different time points upon vehicle or 3-MCA injection in wild-type and *Il-1r1*$^{-/-}$ mice (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student's t test.

(B) Immunohistochemical analysis of PTX3 expression in 3-MCA-induced sarcoma. Arrows show vessels (upper panel), infiltrating leukocytes (central panel), and interstitial stroma (lower panel). Arrowheads show tumor cells (upper and central panels).

(C) Immunofluorescence analysis of PTX3 expression in blood vessels (CD31^{+} cells), macrophages (F4/80^{+} cells), and neutrophils (Ly6G^{+} cells). Scale bar represents 150 µm.

(D) 3-MCA-induced sarcoma incidence in PTX3-chimeric mice (Donor → Recipient). **p < 0.01, paired Student's t test.

See also Figure S2.

tumors (day 100), PTX3 immunostaining was associated with vessels, macrophages, neutrophils, and interstitial stroma, but not to tumor cells (Figures 2B and 2C). Accordingly, most sarcoma cell lines isolated from *Ptx3*$^{+/+}$ tumor-bearing mice did not produce PTX3 in basal or inflammatory conditions (Figure S2C). PTX3 expression in cells of the monocyte-macrophage lineage was confirmed by RT-PCR analysis, with highest levels in MCHII^{low} macrophages (see below) (Figure S2D). At early time points (e.g., day 15), PTX3 expression was strongly induced in F4/80^{+} cells (macrophages) in the skin and to a lesser extent in liver, as well as in stromal cells (Figure S2A). In the latter cell type, substantial coexpression of a marker of DNA damage response (DDR), γ-H2Ax, was observed only at early time points to wane thereafter (Figure S2B). Collectively these results suggest that infiltrating leukocytes and endothelial cells are a major source of PTX3 and that silencing of PTX3 expression in tumor cells occurs generally during progression.

Bone marrow chimeras were used to assess the relative importance of hematopoietic and nonhematopoietic (including tumor) cells as a source of PTX3 and in the protection against

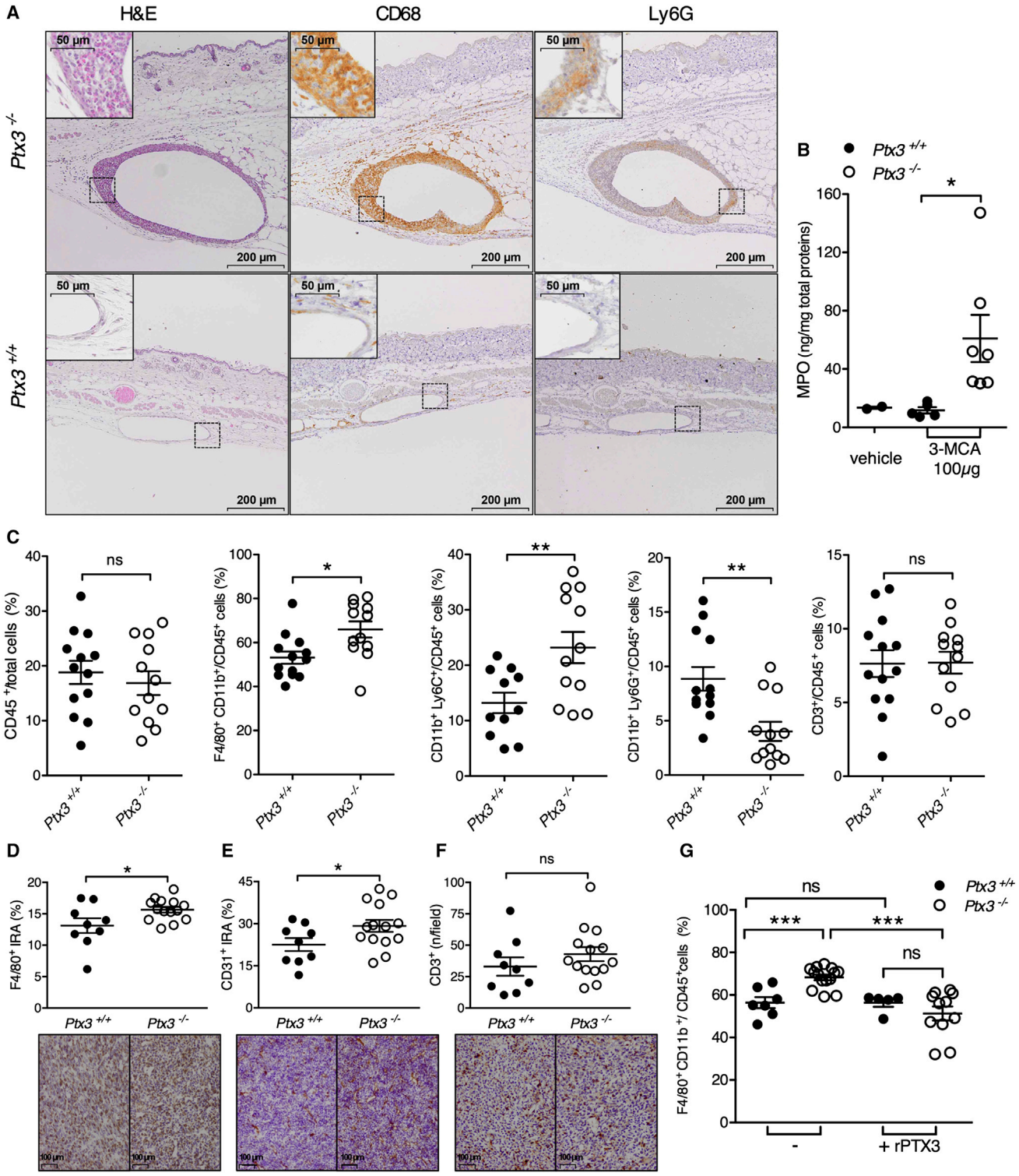

Figure 3. PTX3 Deficiency Is Associated to Increased CRI

(A) Histological and immunohistochemical analysis of macrophage ($CD68^+$) and neutrophil ($Ly6G^+$) infiltration in $Ptx3^{-/-}$ and $Ptx3^{+/+}$ 3-MCA-injection site at 7 days.

(B) MPO levels in 3-MCA-injection site at 7 days (mean ± SEM). See also Table S1.

(C) Analysis of the leukocyte infiltrate in 3-MCA-sarcoma by FACS. Total leukocytes ($CD45^+$), macrophages ($F4/80^+/CD11b^+$), monocytes ($CD11b^+/Ly6C^+$), neutrophils ($CD11b^+/Ly6G^+$), and lymphocytes ($CD3^+$) were analyzed (mean ± SEM).

(legend continued on next page)

carcinogenesis. PTX3 tumor concentration in the two chimeric groups (*Ptx3*$^{+/+}$ mice receiving *Ptx3*$^{-/-}$ bone marrow and *Ptx3*$^{-/-}$ mice receiving *Ptx3*$^{+/+}$ bone marrow) were comparable (2.15 ng/mg ± 0.8 ng/mg and 1.89 ng/mg ± 0.1 ng/mg) and significantly lower than in *Ptx3*$^{+/+}$ tumors (6.61 ng/mg ± 0.2 ng/mg, $p < 0.003$), suggesting that both nonhematopoietic cells and hematopoietic cells produce PTX3 in tumors. PTX3 competence in hematopoietic cells or in stromal cells was insufficient to reconstitute the full "protected" phenotype observed in *Ptx3*$^{+/+}$ mice or in reconstituted fully competent mice (Figure 2D). Thus, PTX3 derived from both hematopoietic and nonhematopoietic cells contributes to protection against carcinogenesis.

PTX3 Deficiency Is Associated with Increased Cancer-Related Inflammation

PTX3 does not affect the proliferation of normal and transformed cells (Figure S1B). It interacts with Fcγ receptors (Lu et al., 2008; Moalli et al., 2010) and has antibody-like properties (Bottazzi et al., 2010) including the capacity to regulate inflammatory reactions. Therefore, we focused on CRI in *Ptx3*$^{-/-}$ mice. We first evaluated leukocyte recruitment into the 3-MCA-injection site at early time points in *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ mice. As shown in Figure 3A, on day 7 after injection macrophages (CD68^{+} cells) and neutrophils (Ly6G^{+} cells) recruited around 3-MCA-containing corn oil droplets were more abundant in *Ptx3*$^{-/-}$ compared to *Ptx3*$^{+/+}$ mice. In agreement, the MPO content (Figure 3B), as well as the levels of CCL2 and CXCL2 (Table S1), were significantly higher in homogenates of tissues collected from *Ptx3*$^{-/-}$ mice, compared to *Ptx3*$^{+/+}$ mice.

We next analyzed the leukocyte infiltrate of tumors collected at their maximum volume (3 cm^3) by flow cytometry. As shown in Figure 3C, the percentage of macrophages (F4/80^{+}/CD11b^{+} cells) and monocytes (Ly6C^{high}/CD11b^{+} cells) in CD45^{+} cells was higher in *Ptx3*$^{-/-}$ compared to *Ptx3*$^{+/+}$ mice, whereas the percentage of neutrophils (Ly6G^{high}/CD11b^{+} cells) was significantly lower. The percentage of T lymphocytes (CD3^{+} cells) was comparable in the two groups. Similar results were obtained when the analysis was performed at day 100 after 3-MCA-treatment in tumors of different size in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice (Figure S3A). Histological analysis confirmed increased macrophage infiltration in *Ptx3*$^{-/-}$ sarcomas and showed higher density of CD31^{+} vessels (Figures 3D and 3E). Similar lymphocyte infiltration in the two groups was confirmed by histological analysis (Figure 3F).

In addition, the concentrations of proinflammatory cytokines TNFα, IL-1β and IL-6, CCL2, and the proangiogenic vascular endothelial growth factor (VEGF) were significantly higher in *Ptx3*$^{-/-}$ tumor homogenates than in *Ptx3*$^{+/+}$. IL-6 and VEGF levels were significantly higher also at the systemic level in *Ptx3*$^{-/-}$ mice (Table S1). Treatment with recombinant PTX3 reduced macrophage infiltration and angiogenesis abolishing the difference between the two genotypes (Figures 3G and S3B).

PTX3 Deficiency Is Associated to Increased Complement Activation

PTX3 has been shown to tune inflammatory responses by modulating P-selectin-dependent leukocyte recruitment and Complement activation (Deban et al., 2010; Inforzato et al., 2013). We first addressed the role of PTX3-P-selectin interaction in leukocyte recruitment and in tumor growth by evaluating sarcoma incidence in P-selectin-deficient and in PTX3/P-selectin-double-deficient mice. As shown in Figure S3C, we found that PTX3 deficiency was associated to higher tumor incidence regardless of P-selectin competence. Moreover, P-selectin deficiency did not affect susceptibility to 3-MCA carcinogenesis.

Next, we analyzed Complement activation. Confocal microscopy of the injection site revealed significantly higher C3 immunoreactivity in areas surrounding 3-MCA-containing oil droplets in *Ptx3*$^{-/-}$ mice, in comparison to *Ptx3*$^{+/+}$ mice, where C3 deposition was negligible (Figure 4A). In tumors, C3 deposition occurred also in *Ptx3*$^{+/+}$ lesions, but it was significantly lower than in *Ptx3*$^{-/-}$ sarcomas (Figure 4B, two pooled experiments). An analysis of C3 deposition and PTX3 expression in each *Ptx3*$^{+/+}$ lesion was performed on samples from one experiment and showed that C3 and PTX3 were significantly inversely correlated (Pearson $r = -0.65$, $p = 0.03$). Treatment with recombinant PTX3 reduced C3 immunoreactivity in *Ptx3*$^{-/-}$ tumors to levels observed in *Ptx3*$^{+/+}$ tumors (Figure 4C).

In addition, a higher C5a concentration was measured in *Ptx3*$^{-/-}$ tumor homogenates compared to *Ptx3*$^{+/+}$ ones (Figure 4D). In contrast, the immunoreactivity for C5b-9, which was associated with necrotic tumor areas, did not differ in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ tumors (Figure 4E). We next analyzed the immunoreactivity for Factor H and C4BP, because PTX3 interacts with these two Complement-regulatory proteins without interfering with their functional activity and facilitates their deposition on damaged cells, amplifying their regulatory potential (Braunschweig and Józsi, 2011; Deban et al., 2008). As shown in Figure 4F, the immunostaining for Factor H in tumors from *Ptx3*$^{-/-}$ mice was significantly lower than in *Ptx3*$^{+/+}$ tumors. A correlation analysis showed that PTX3 expression and Factor H deposition were significantly positively correlated (Pearson $r = 0.71$, $p = 0.01$). We also observed lower C4BP deposition in *Ptx3*$^{-/-}$ than in *Ptx3*$^{+/+}$ tumors, but the difference did not reach statistical significance ($p = 0.07$) (Figure S4A).

Role of Complement

In the effort to assess the pathogenic role of increased Complement activation associated to PTX3 deficiency, we evaluated the susceptibility to 3-MCA of C3-deficient and PTX3/C3-double-deficient mice. Carcinogenesis by 3-MCA and macrophage recruitment in *C3*$^{-/-}$ mice were significantly lower than in wild-type animals (Figures 4G and 4H). In addition, C3 deficiency reduced the susceptibility to 3-MCA of *Ptx3*$^{-/-}$ mice to levels comparable to that of *Ptx3*$^{+/+}$ mice and double deficiency was

(D–F) Analysis of macrophages (F4/80^{+} immunoreactive area, IRA), angiogenesis (CD31^{+} IRA), and lymphocytes (CD3^{+} cells) in 3-MCA-sarcoma by immunohistochemistry (each dot represents the average of ten fields, mean ± SEM).

(G) Analysis by FACS of the macrophage infiltrate (mean ± SEM) in 3-MCA-sarcoma of *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice, after treatment with recombinant PTX3 (from day 0 to 100). *$p < 0.05$, **$p < 0.01$, unpaired Student's t test.

See also Figure S3.

associated to macrophage recruitment comparable to that of *C3*$^{-/-}$ mice. Finally, treatment with the C5a receptor antagonist PMX53 reduced the susceptibility of *Ptx3*$^{-/-}$ mice, reverting the phenotype, without affecting the tumor growth in wild-type mice (Figure 4I).

Factor H-deficient mice are depleted in circulating C3 and suffer from spontaneous nephropathy (Pickering et al., 2002). Therefore, a genetic double deficiency approach would not be informative as to the actual role of Factor H. Thus, we set up an in vitro model of C3 deposition on *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ cell lines, in the presence of recombinant PTX3 and a recombinant Factor H inhibitor (SRC19-20) (Banda et al., 2013). For these experiments, we selected one wild-type sarcoma line (Wt-2), generated on day 100, which, unlike most wild-type cell lines (Figure S2C), produces detectable levels of PTX3 (8 ng/ml in 24 hr). In line with results obtained in vivo (Figure 4B), C3 deposition was significantly higher on *Ptx3*$^{-/-}$ than on *Ptx3*$^{+/+}$ producing cells (Figures 4J and 4K). Recombinant PTX3 reduced C3 deposition on *Ptx3*$^{-/-}$ cells abrogating the difference between *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ cells, and SRC19-20 that interferes with the interaction between PTX3 and Factor H (Deban et al., 2008), caused higher C3 deposition on *Ptx3*$^{+/+}$ cells and did not further increase C3 deposition on *Ptx3*$^{-/-}$ cells. Notably, the rescue of C3 deposition on *Ptx3*$^{-/-}$ cells obtained with recombinant PTX3 was abolished in the presence of SRC19-20.

To further address whether PTX3 regulates immune complexes (IC)-dependent Complement and leukocyte activation, we measured circulating IC and analyzed their composition and proinflammatory activity. PTX3 deficiency was associated with normal levels of total IC and C3-containing IC (Figure S4C). Qualitative analysis revealed the presence of PTX3 in IC, but the amount of C3, C1q, and total Ig did not differ between *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ IC (Figure S4D). Finally, *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ IC equally activated macrophages (expression of polarization markers and cytokines) indicating that PTX3 does not regulate CRI through IC in this cancer model (Figure S4E).

All together, these data suggest that Complement is an essential component of tumor-promoting inflammation and that the increased Complement activation associated to PTX3 deficiency due to lack of recruitment of Factor H has a major role in sustaining an exacerbated inflammatory response and enhanced carcinogenesis.

Other Components of Cancer-Related Inflammation

As shown in Figure 3C and Table S1, PTX3 deficiency was associated with increased macrophage infiltration and increased levels of the monocyte attracting chemokine CCL2. Complement activation and C5a in particular induce chemokine production. It was therefore important to assess the role of CCL2 in the increased susceptibility to carcinogenesis exhibited by PTX3-deficient mice. To this aim, we treated *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice with an anti-CCL2 Ab. As shown in Figure 5A, the anti-CCL2 treatment did not modify the tumor incidence of *Ptx3*$^{+/+}$ mice, whereas it reduced the susceptibility of *Ptx3*$^{-/-}$ mice, completely reverting the phenotype.

CCL2 has been shown to skew macrophage polarization in an M2-like direction (Roca et al., 2009; Sierra-Filardi et al., 2014), we therefore characterized the phenotype of tumor-associated macrophages (TAM) in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice. Upon purification of CD11b^{+}/Ly6G^{-} cells, MHCII^{high} (CD11b^{+}/Ly6C^{-}/MHCII^{high}) and MHCII^{low} (CD11b^{+}/Ly6C^{-}/MHCII^{low}) macrophages and monocytes (CD11b^{+}/Ly6C^{+}/MHCII^{-}) were sorted to >98% purity to analyze the expression of M1- and M2-like markers as described (Laoui et al., 2014). As shown in Figures 5B–5D and Table S2, *Ptx3*$^{-/-}$ MHCII^{high} and MHCII^{low} macrophages and monocytes expressed higher levels of M2-like markers (in particular *Arg1*, *Ym1*, *Fizz1*, *Il10*, and *Mcr1* in macrophages and *Ym1* and *Fizz1* in monocytes) and lower levels of M1 markers (in particular *Il12a* and *Ifng*) in comparison to *Ptx3*$^{+/+}$ macrophages and monocytes. Moreover *Ptx3*$^{-/-}$ macrophages (MHCII^{high} or MHCII^{low}) expressed higher levels of Nos2, which is overexpressed by TAMs (Murray et al., 2014).

Increased *Trp53* Mutations and DNA Damage in *Ptx3*$^{-/-}$ Mice

Because CRI is potentially a cause of gene instability (Colotta et al., 2009), we next addressed whether the increased tumor incidence observed in *Ptx3*$^{-/-}$ mice was associated to increased gene mutation. To this aim, we evaluated the frequency of mutations in *Trp53* and *Kras*, which are target genes mutated by 3-MCA, in sarcoma cell lines isolated from *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ tumor-bearing mice. The genomic DNA of 10 *Ptx3*$^{+/+}$ and 12 *Ptx3*$^{-/-}$ 3-MCA-induced sarcomas was examined by direct sequencing of exons 5 to 8 of the *Trp53* gene and of exons 1 and 2 of *Kras* gene. In line with previous studies (Watanabe et al., 1999), eight out of ten wild-type sarcomas (80%) carried mutations of *Trp53* gene in 12 different codons, whereas *Kras* mutations were less frequent (20%) and were restricted to two codons (Table S3). As shown in Figure 6A, the number of *Trp53* mutations was significantly higher in *Ptx3*$^{-/-}$ than in *Ptx3*$^{+/+}$ sarcoma cell lines, whereas no differences were observed in *Kras* gene mutations (Figure 6B). Interestingly, similar numbers of mutations were observed in *Ptx3*$^{-/-}$ cell lines collected 100 days upon treatment or at the end point (tumor volume 3 cm^3) suggesting that the increased *Trp53* gene instability is not due to accumulation of mutations during tumor progression (Table S3). We finally addressed the functional consequences of *Trp53* mutations. As shown in Figure 6C, the expression of *Mdm2*, *Bax*, and *Cdkn1a* (p21) downstream p53 activation, was significantly reduced in *Ptx3*$^{-/-}$ tumor cell lines in comparison to *Ptx3*$^{+/+}$, indicating that the higher incidence of *Trp53* mutations observed in *Ptx3*$^{-/-}$ is likely associated with increased loss of onco-suppressor function.

To further investigate whether and to what extent the increased inflammation in *Ptx3*$^{-/-}$ mice translated into DNA damage we studied DNA damage by performing immunohistochemical analysis of 8-OH-deoxiguanosine (8-OH-dG), a modified DNA base generated during oxidative damage. As shown in Figure 6D, the number of 8-OH-dG-positive nuclei was significantly higher in *Ptx3*$^{-/-}$ than in *Ptx3*$^{+/+}$ tissues 15 days after 3-MCA injection. In addition, the expression of the DDR markers, γ-H2Ax and 53BP1 was significantly higher in *Ptx3*$^{-/-}$ tissues (Figures 6E and 6F). The relevance of damage caused by reactive species was addressed by treating 3-MCA-injected mice with amminiguanidine hemisulphate (AG), an irreversible inhibitor of iNOS. AG significantly reduced the tumor incidence in *Ptx3*$^{-/-}$ mice (Figure 6G). In addition, treatment with AG

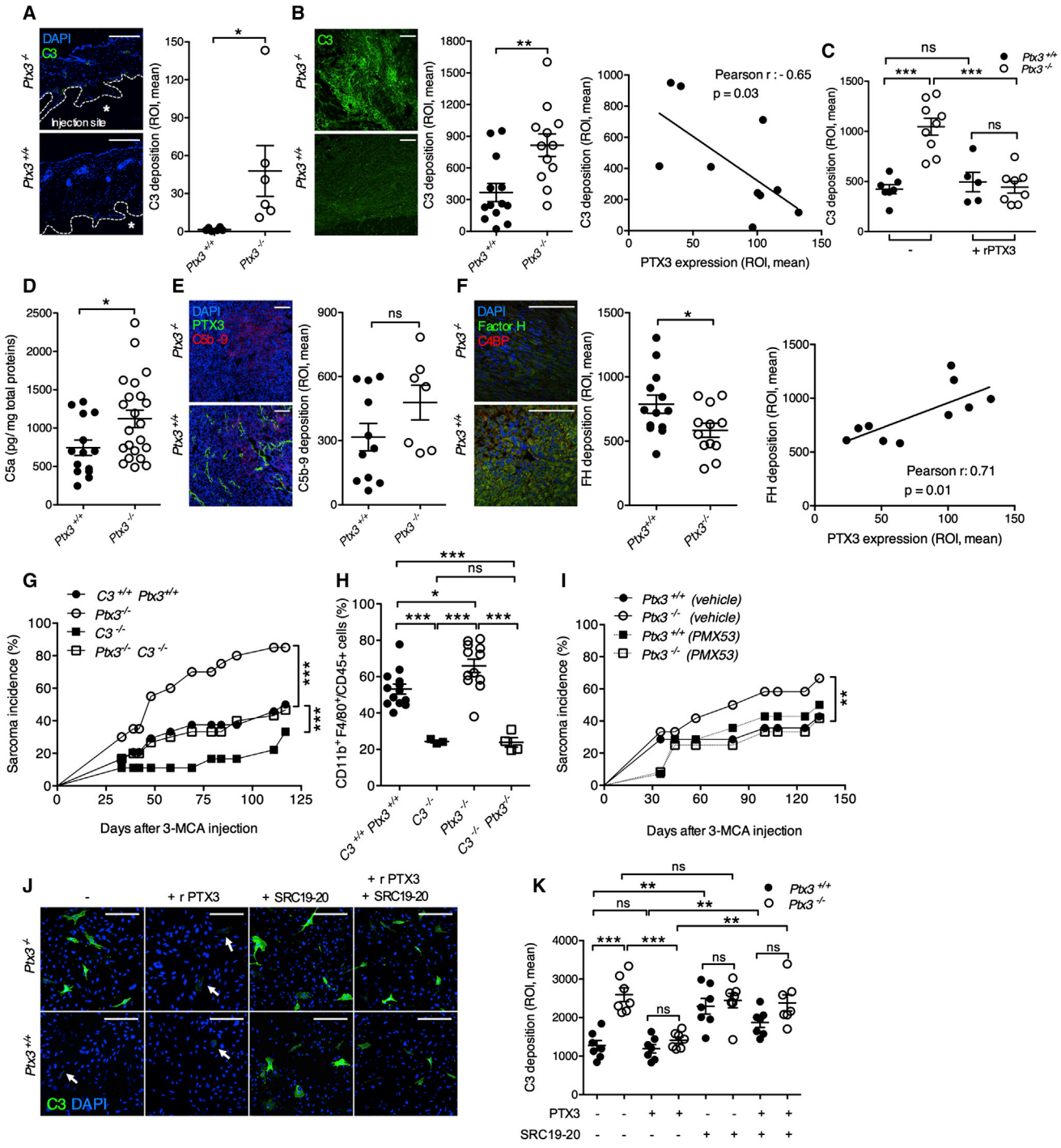

Figure 4. PTX3 Deficiency Is Associated to Increased Complement Deposition in 3-MCA-Sarcoma

(A–C) Analysis by confocal microscopy of C3 deposition in the injection site (*) at 7 days (A) or in tumors (B and C) of *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice, after treatment with recombinant PTX3 (from day 0 to 100) (C). Correlation analysis between C3 and PTX3 expression was performed in wild-type tumors (B).

(D) Analysis by ELISA of C5a concentrations, (mean ± SEM, two pooled experiments), in 3-MCA-tumors.

(E and F) Analysis by confocal microscopy of C5b-9 (E) and Factor H (F) in 3-MCA-tumors. Correlation analysis between Factor H and PTX3 expression was performed in wild-type tumors.

(G) Incidence of 3-MCA-sarcoma in *C3*$^{-/-}$ and/or *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice (n = 10–15).

(H) Analysis of the macrophage (F4/80^{+}/CD11b^{+}) infiltrate in 3-MCA-sarcoma by FACS in *C3*$^{-/-}$ and/or *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice.

(I) 3-MCA-sarcoma incidence in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice treated with the C5aR antagonist PMX53 (n = 7).

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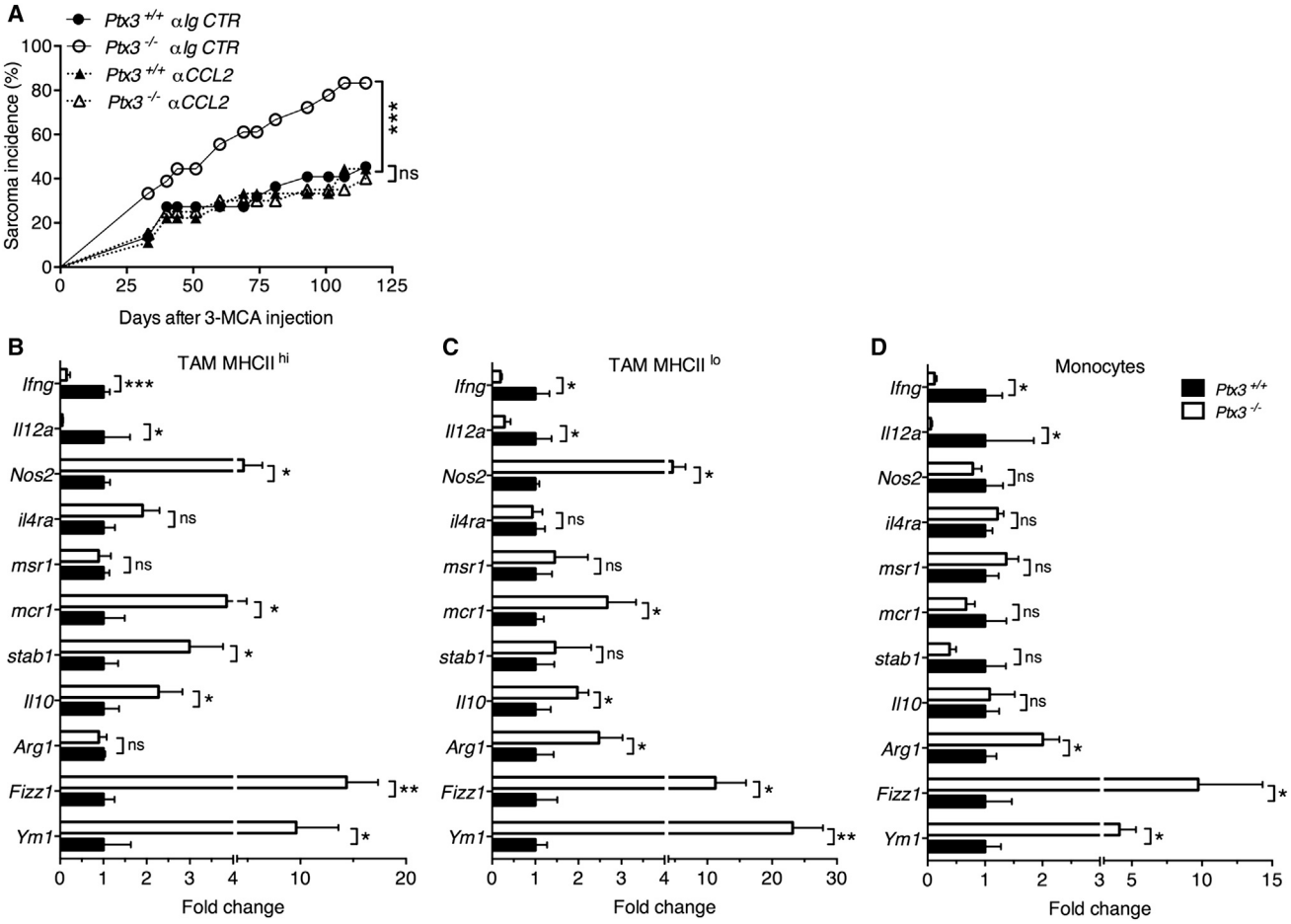

Figure 5. Role of CCL2 in Carcinogenesis and TAM Polarization in PTX3-Deficient Mice

(A) Incidence of 3-MCA-sarcoma in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice treated with anti-CCL2, or irrelevant mAb (n = 9–11) (100 μg/mouse three times/week) for the duration of the experiment. ***p < 0.001, paired Student's t test.

(B–D) Analysis by RT-PCR of selected M1 and M2 markers of macrophages (B and C) and monocytes (D) infiltrating 3-MCA-sarcoma in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student's t test. In (B), (C), and (D), data were relative to 18S expression and normalized versus the mean of wild-type and expressed as mean ± SEM.

See also Table S2.

abolished the difference in 8-OH-dG and γ-H2Ax immunoreactivity between *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ tissues (Figures 6D and 6E). These results suggest that increased CRI in PTX3-deficient mice is associated with increased genetic instability as assessed by the frequency of *Trp53* mutations, oxidative DNA damage, and expression of DDR markers.

Methylation of the PTX3 Gene in Human Cancer

We next investigated whether results obtained in the mouse are indeed relevant to human cancer. Studies in human esophageal squamous cell carcinoma had shown that the *PTX3* promoter is hypermethylated in this cancer and PTX3 expression is inhibited (Wang et al., 2011). In addition, in silico bioinformatics analysis in the Epigenomics database (http://www.ncbi.nlm.nih.gov/epigenomics) showed *PTX3* gene methylation in colorectal cancer (CRC) (File: GSM801957). We thus analyzed the methylation status of the promoter and of a CpG island located in a putative enhancer encompassing the second exon (Akhtar-Zaidi et al., 2012) in a series of mesenchymal and epithelial cancers. As shown in Figure 7A, both the promoter and the CpG island are

(J) Immunofluorescence analysis of C3 deposition on *Ptx3*$^{+/+}$ and *Ptx3*$^{-/-}$ cells, in the presence of recombinant PTX3, SRC19-20, or their combination. Arrows indicate faint C3 immunostaining in *Ptx3*$^{+/+}$ cells in basal conditions or after addition of PTX3 in the assay.

(K) Quantification of C3 deposition evaluated by immunofluorescence. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student's t test (A–F, H, and K), paired Student's t test (G and I). For (A), (B), (C), (E), (F), and (K), each dot represents the average of mean fluorescence intensity of ten regions of interest (ROI) per tumor (A–C, E, and F) or well (K) (mean ± SEM). Scale bars represent 150 μm.

See also Figure S4.

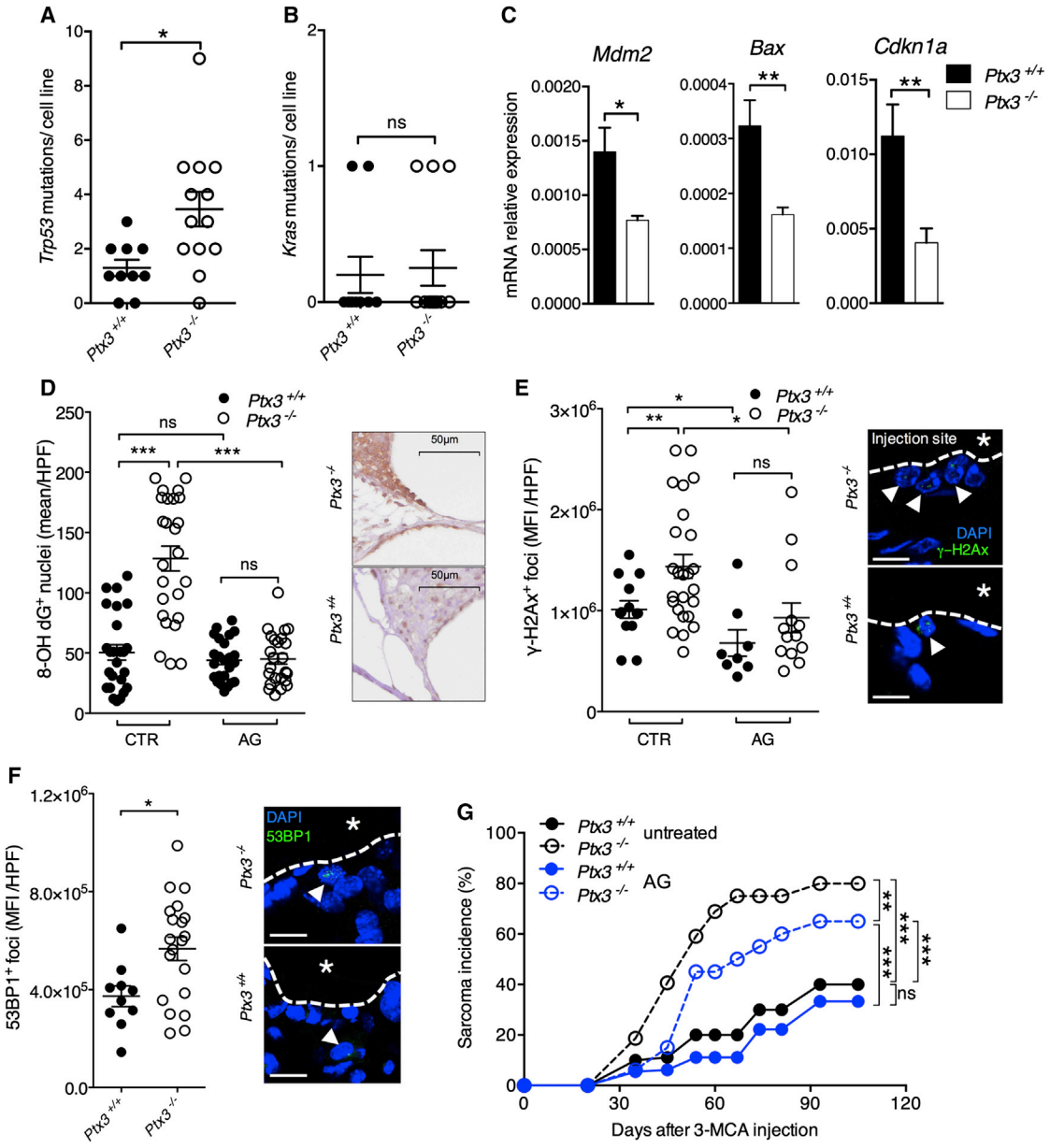

Figure 6. PTX3 Deficiency Is Associated to Increased Genetic Instability

(A and B) Number of *Trp53* (A) and *Kras* (B) mutations (mean ± SEM) in cell lines isolated from $Ptx3^{-/-}$ and $Ptx3^{+/+}$ tumors. See also Table S3.

(C) Analysis of the expression of p53-regulated genes by RT-PCR in cell lines isolated from $Ptx3^{-/-}$ and $Ptx3^{+/+}$ tumors. Data were relative to GAPDH expression and represented as mean ± SEM.

(D) Immunohistochemical analysis of 8-OH-dG 15 days after 3-MCA injection in $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice treated with AG. Left: data are reported as mean number ± SEM of 8-OH-dG^+ nuclei per field. Right: representative immunofluorescence images are shown.

(E and F) Immunofluorescence analysis of γ-H2Ax (E) and 53BP1 (F) 15 days after 3-MCA injection in $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice treated with AG (E). Left panels: data are reported as MFI of γ-H2Ax (E) and 53BP1 (F) positive foci per field and expressed as (mean ± SEM). Right panels: representative immunofluorescence images are shown.

(G) Sarcoma incidence in $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice treated with AG in the drinking water for the entire duration of the experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired (A-F) or paired (G) Student's t test.

highly methylated in leyomiosarcomas, desmoid tumors, CRC, and skin squamous cell carcinoma, in contrast with normal mesenchymal or epithelial tissues. We further investigated *PTX3* methylation and the functional consequences in CRC. We analyzed *PTX3* methylation in five patients with high-grade adenomas and 40 patients with microsatellite stable CRC at different stage (I–IV). As shown in Figure 7B, the methylation of the promoter region and of the CpG island progressively

increased from normal colon epithelium, to adenomas and to CRC independently of stage. The healthy tissue from CRC patients was more methylated than normal colon epithelium. Similar results were obtained in colorectal tumor cell lines (Figures 7C and S5A), in which treatment with the methylation inhibitor 5-Aza-2′-deoxycytidine (5-AZA-dC) significantly reduced the methylation of both the promoter and the CpG island. In the presence of 5-AZA-dC, the expression of PTX3 mRNA was significantly increased in five different cell lines analyzed, both under basal conditions and upon stimulation with TNFα or IL-1β (Figures 7D and S5B). Rescue of PTX3 protein expression was confirmed in 3 different cell lines (Figures 7D and S5C). In contrast, treatment with 5-AZA-dC did not increase PTX3 expression in normal endothelial or normal colon epithelial cells stimulated with TNFα (Figures S5D and S5E). In addition, chromatin immunoprecipitation (ChIP) analysis showed that histone modifications associated to transcriptional activation (H3K4me3, H3K27ac, and H3K9ac) were strongly increased in the *PTX3* promoter after treatment with 5-AZA-dC and TNFα, whereas trimethylation of H3K27 associated to gene repression was not modified (Figure S5F). Finally, the binding of NF-κB, c-Jun, and c-Fos to their consensus binding sites in the promoter and transcription factor binding sites where above the basal level only after cell treatment with 5-AZA-dC in association with a proinflammatory stimulus in CRC cells (Figure 7E). Collectively, these data suggest that the *PTX3* gene is silenced by methylation in selected human tumors including CRC.

DISCUSSION

The general objective of this investigation was to gain insight into the role of the humoral arm of innate immunity in CRI using the long pentraxin PTX3 as a paradigm (Bottazzi et al., 2010). We observed increased susceptibility to mesenchymal (3-MCA) and epithelial (DMBA/TPA) carcinogenesis in PTX3-deficient mice. The 3-MCA model was used for further analysis. This phenotype was associated with increased macrophage infiltration, cytokine production, angiogenesis, and genetic instability as revealed by an increased frequency of *Trp53* mutations, oxidative DNA damage, and expression of DDR markers in *Ptx3*$^{-/-}$ mice. Several lines of evidence suggest that PTX3 deficiency unleashes unrestrained Complement activation with production of C5a, CCL2 production, and enhanced recruitment of tumor-promoting macrophages. In selected human tumors (e.g., leiomyosarcomas and CRC) PTX3 expression was regulated epigenetically by methylation of the promoter region and of a putative enhancer. Thus, an essential component of the humoral arm of innate immunity and regulator of Complement activation acts as an extrinsic oncosuppressor by acting at the level of Complement-mediated, macrophage-sustained, tumor-promoting inflammation.

PTX3 is a humoral pattern recognition molecule essential for resistance against selected microorganisms including *A. fumigatus*, *P. aeruginosa* (Garlanda et al., 2002), *Klebsiella pneumonia* (Soares et al., 2006), and uropathogenic *Escherichia coli* (Jaillon et al., 2014). PTX3 is highly conserved in evolution and genetic evidence is consistent with a role of PTX3 in antimicrobial resistance in humans (Chiarini et al., 2010; Cunha et al., 2014; Olesen et al., 2007). PTX3 has antibody-like properties, including recognition of microbial moieties (Jeannin et al., 2005), opsonization via Fcγ receptors (Moalli et al., 2010), Complement activation and regulation (Deban et al., 2008; Inforzato et al., 2013), and regulation of inflammation (Deban et al., 2010; Lech et al., 2013; Norata et al., 2009; Salio et al., 2008; Soares et al., 2006). Given the fact that tumor-promoting inflammation is now recognized as an essential component of the tumor microenvironment (Coussens et al., 2013; Grivennikov et al., 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008), the increased susceptibility of PTX3-deficient mice to carcinogenesis is a reflection of its regulatory function on inflammation.

PTX3 binds P-selectin via its glycosidic component (Deban et al., 2010) and this accounts for the regulatory function on inflammation of PTX3 in selected P-selectin-dependent experimental models (Lech et al., 2013). It was therefore important to assess the relevance of the P-selectin pathway in the enhanced carcinogenesis observed in *Ptx3*$^{-/-}$ mice. P-selectin-deficient mice show no phenotype in terms of 3-MCA carcinogenesis. Moreover, PTX3/P-selectin-double-deficient mice showed enhanced 3-MCA carcinogenesis compared to controls. Thus, the P-selectin pathway is not involved in the enhanced susceptibility to carcinogenesis of PTX3-deficient mice.

PTX3 interacts with Complement at multiple levels (Inforzato et al., 2013). It binds C1q (Bottazzi et al., 1997) and activates or regulates (Nauta et al., 2003) the classic pathway of Complement activation. By interacting with ficolins and MBL it independently impinges upon the alternative pathway (Gout et al., 2011; Ma et al., 2009). PTX3 interacts and recruits Factor H by binding domains 19–20 and 7 through its N-terminal and glycosylated C-terminal domains, respectively, without interfering with Factor H capacity to negatively regulate the Complement cascade (Deban et al., 2008). We found an inverse relationship between C3 deposition and PTX3 presence in individual tumors. Conversely, the occurrence of Factor H was directly and significantly correlated with PTX3 deposition. In an effort to assess the actual significance of the Complement cascade in the onco-suppressive function of PTX3, double C3/PTX3-deficient mice were generated. Genetic inactivation of C3 strongly reduced, but did not completely ablate, the tumor-promoting function of PTX3 deficiency, suggesting that other mechanisms in addition to unleashed complement may contribute to increased CRI and susceptibility to cancer in PTX3-deficient mice.

A genetic approach to address the role of Factor H in vivo using double knockout mice is hindered by systemic C3 consumption and associated pathology in Factor H-deficient mice (Banda et al., 2013; Pickering et al., 2002). However, in an in vitro assay of C3 deposition on PTX3 competent and PTX3-incompetent cell lines, a recombinant Factor H inhibitor (SRC19-20) (Banda et al., 2013), which interferes with the binding between PTX3 and Factor H (Deban et al., 2008), increased C3 deposition on PTX3 producing cells and abolished the rescue of C3 deposition on *Ptx3*$^{-/-}$ cells obtained with recombinant PTX3. Thus, we infer that PTX3 deficiency unleashes Complement-dependent, C5a-mediated tumor-promoting inflammation, because of defective recruitment of the negative regulator Factor H.

Complement is not considered a canonical component of tumor-promoting inflammation (Coussens et al., 2013; Hanahan

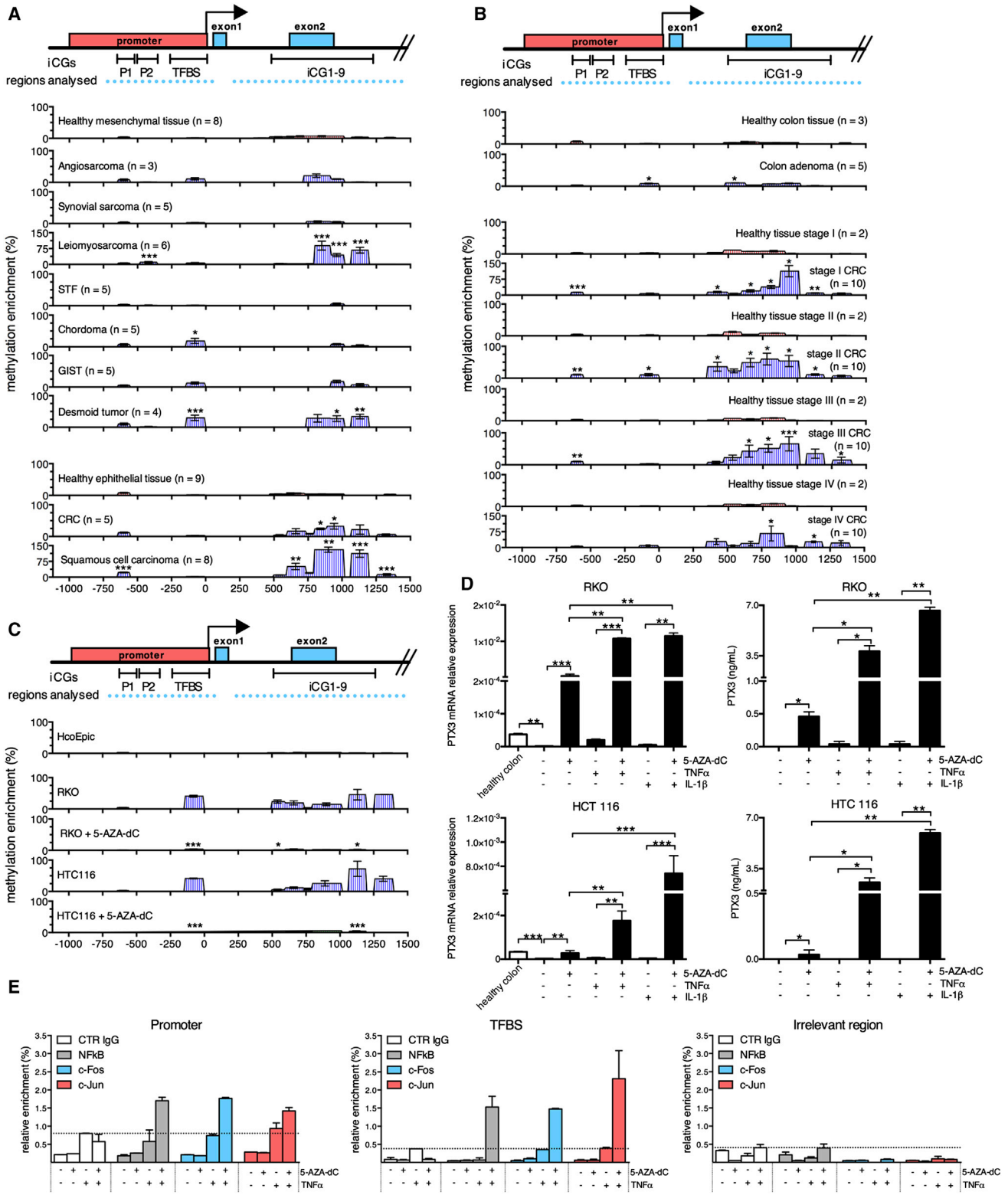

Figure 7. The *PTX3* Gene Is Epigenetically Modified in Human Cancers

(A) Analysis by methylated CpG island recovery assay (MIRA) of the percentage of methylation enrichment of the promoter region and of the *PTX3* CpG island in human mesenchymal and epithelial tumors. CpG rich regions (iCGs) (P1, P2, transcription factor binding site [TFBS], and CpG island [iCG1-9]) and surrounding

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and Weinberg, 2011; Mantovani et al., 2008). Complement can represent an effector mechanism against hematopoietic tumors activated by monoclonal antibodies (Golay et al., 2001). The generation of Complement C5a in a transplanted tumor enhanced growth by recruiting myeloid-derived suppressor cells and amplifying their T cell-directed suppressive abilities (Markiewski et al., 2008). However, no information based on genetic approaches concerning the role of Complement components in carcinogenesis is available. Here, we found that C3-deficient mice are strongly protected against 3-MCA (Figure 4G) and DMBA/TPA (Figure S4B) carcinogenesis, involving mesenchymal and epithelial cells, respectively. These results demonstrate that Complement is an essential component of tumor-promoting inflammation.

The Complement cascade can be activated by antibodies or by sensing tissue damage (Ricklin et al., 2010). Antibodies have been shown to orchestrate cancer-related inflammation in a model of epithelial carcinogenesis (HPV16 transgenic mice) by a remote control mechanism (Andreu et al., 2010). However, in that system the pathway driving tumor-promoting inflammation was Fcγ receptor-dependent and Complement-independent (Andreu et al., 2010; de Visser et al., 2004). It is therefore tempting to speculate that an antibody-independent pathway (e.g., interaction with senescent or apoptotic cells) is responsible for Complement-mediated orchestration of tumor-promoting inflammation in the two models of mesenchymal (3-MCA) and epithelial (DMBA/TPA) carcinogenesis used in the present study. Interestingly, in the present study, C3-deficient mice showed reduced susceptibility to DMBA/TPA skin carcinogenesis but were not protected against HPV16-driven squamous carcinoma development (Andreu et al., 2010; de Visser et al., 2004). CRI can differ considerably in its drivers and components in tumors originating in different organs and tumors (Ruffell et al., 2012). It is likely that Complement may play different roles in different carcinogenesis contexts.

Macrophages are an essential component of tumor-promoting inflammation (Qian and Pollard, 2010; Sica and Mantovani, 2012). Tumors originating in a PTX3-deficient context were characterized by an increased frequency of TAM throughout the natural history of the tumor. Increased macrophage accumulation was completely abolished in C3-deficient or C3/PTX3-double-deficient hosts. PTX3 deficiency was associated with increased C5a and CCL2. Both molecules are potent monocyte attractants. Interestingly at the end point (tumor volume 3 cm^3) macrophage, but not neutrophil, numbers were increased in PTX3-deficient hosts, consistent with a function of CCL2 in sustaining TAM recruitment and tumor-promoting inflammation. Macrophages in tumors usually acquire a skewed M2-like phenotype oriented to tumor promotion (Sica and Mantovani, 2012). Chemoattractants, and CCL2 in particular, have been shown to skew macrophage function in an M2-like direction (Roca et al., 2009). In PTX3-deficient hosts, TAM showed enhanced expression of molecules associated with M2-like polarization, including *Arg I*, *Fizz1*, and *Ym1*. Skewing of macrophage function with, for instance, increased immunosuppressive activity and may contribute to enhanced carcinogenesis in PTX3-deficient hosts. Antibodies directed against CCL2 reverted the phenotype of PTX3-deficient mice in terms of enhanced susceptibility to 3-MCA carcinogenesis but did not protect PTX3-competent mice, as observed in other murine models and clinical trials (Loberg et al., 2007; Pienta et al., 2013) suggesting that the treatment is effective only under conditions of exacerbated CCL2 production and M2-polarization. Chemoattractants including C5a induce chemokines (Sozzani et al., 1995). Thus, uncontrolled Complement activation with C5a production is likely to be upstream of CCL2 that in turn recruits tumor-promoting TAM in increased numbers, with skewed M2-like phenotype, in PTX3-deficient hosts. However, the strong reduction of TAM in PTX3/C3-double-deficient mice was not sufficient to completely rescue the susceptibility to carcinogenesis of *Ptx3*$^{-/-}$ mice, suggesting the involvement of other cell types or mechanisms in addition to increased TAM in the observed phenotype.

Inflammatory mediators have been suggested to contribute to genetic events that cause cancer and to the genetic instability of tumors, although there is little formal evidence for that (Colotta et al., 2009). PTX3 is an extracellular molecule whose only cellular receptors identified so far are Fcγ receptors (Lu et al., 2008; Moalli et al., 2010). PTX3 does not affect the growth and death of normal or neoplastic cells. PTX3-deficient mice showed increased susceptibility to carcinogenesis mediated by Complement-dependent tumor-promoting inflammation. Tumors developed in a PTX3-deficient context characterized by exacerbated inflammation had a higher frequency of *Trp53* mutations, increased DNA oxidative damage and higher expression of DDR markers (Figure 6). These findings indicate that exacerbated inflammation in the tumor microenvironment can indeed contribute to the genetic instability of cancer.

In an effort to assess the actual significance of the present findings to human cancer, supported by a bioinformatics analysis showing methylation of the PTX3 gene in CRC, we searched

regions analyzed by MIRA are shown below the structure of *PTX3* gene. Healthy epithelial tissues were from the normal counterpart of colon cancer patients (n = 3) and skin squamous carcinoma (n = 5); healthy mesenchymal tissues were from leiomyosarcoma (n = 1), angiosarcoma (n = 3), chordoma (n = 3), and desmoid tumor (n = 2) patients.

(B) Analysis by MIRA of the percentage of methylation enrichment of the same regions in healthy colon epithelium, high grade adenomas, CRC, and healthy counterparts for each CRC stage.

(C) Analysis by MIRA of the percentage of methylation enrichment of the same regions in one normal colon epithelial cell line (HcoEpic) and two CRC cell lines (RKO and HCT116) treated or not with the inhibitor of DNA methylation 5-AZA-dC.

(D) PTX3 mRNA expression and protein production by RKO and HCT116 cell lines treated or not with 5-AZA-dC in the presence of TNFα 20 ng/mL or IL-1β 20 ng/mL. Healthy colon: primary human intestinal epithelial crypt cells isolated from surgical biopsies.

(E) Analysis by ChIP of NF-κB, c-Fos, and c-Jun binding to the promoter, TFBS, and an irrelevant region in RKO cell line treated or not with 5-AZA-dC in the presence of TNFα 20 ng/ml. Data are expressed as percentage of enrichment relatively to input DNA normalized on a positive control and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired Student's t test.

See also Figure S5.

for epigenetic modifications of the *PTX3* gene in mesenchymal and epithelial tumors. In selected human tumors (e.g., leiomyosarcomas, CRC, and skin squamous cell carcinoma), we found that the *PTX3* gene was hypermethylated at the promoter region and in a CpG rich putative enhancer encompassing exon 2. The latter is indeed a functional enhancer (M.R., unpublished data). *PTX3* gene methylation was detected in CRC stages 1–4 as well as in adenomas, suggesting that this is an early event in the natural history of CRC (Vogelstein et al., 2013). Hypermethylation at the *PTX3* locus was also observed in CRC cell lines, where treatment with 5-AZA-dC rescued the PTX3 production in response to inflammatory cytokines. ChIP analysis showed that only after treatment with 5-AZA-dC and an inflammatory stimulus of CRC cells, histone modifications associated to transcriptional activation occurred and the transcription factors responsible of PTX3 expression (e.g., NF-κB, c-Jun, c-Fos) interacted with their binding sites in the *PTX3* promoter region. Thus the *PTX3* gene is silenced by hypermethylation in selected human tumors including CRC and this event occurs early in progression already at the level of adenomas.

The results presented here demonstrate that a regulatory component of the humoral arm of innate immunity, PTX3, acts as an extrinsic oncosuppressor gene in mouse and man. Although the connection between inflammation and cancer has long been perceived (Coussens et al., 2013; Grivennikov et al., 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008), we are not aware of a precedent for a humoral innate immunity effector molecule acting as a cancer gene. An additional general implication of the results reported here relates to Complement. The results obtained suggest that Complement is a key component of tumor-promoting cancer-related inflammation.

EXPERIMENTAL PROCEDURES

Cancer Models

Mice were injected s.c. with a single dose of 100 μg of 3-MCA (Sigma-Aldrich) dissolved in corn oil and assessed for tumor development over the course of 5 months. Skin carcinogenesis was performed as reported (Schioppa et al., 2011) in a two-step DMBA/TPA model. Procedures conformed to protocols approved by the Humanitas Clinical and Research Center in compliance with national and international law and policies. The study was approved by the Italian Ministry of Health.

Analysis of Complement Deposition

Deposition of Complement components was analyzed by confocal microscopy on tumor tissue or cell lines. C3 deposition on tumor cell lines was performed by incubating cells with 10% normal mouse serum in gelatin-containing Veronal buffer, recombinant PTX3, and/or 33 nM Factor H inhibitor peptide SRC19-20 (Braunschweig and Józsi, 2011).

Analysis of DNA Damage

Genomic DNA was extracted from 10 *Ptx3*$^{+/+}$ and 12 *Ptx3*$^{-/-}$ 3-MCA-induced sarcoma cell lines, and the direct sequencing of exons 5 to 8 of *Trp53* gene and of exons 1 and 2 of *Kras* gene was performed as previously reported (Watanabe et al., 1999). DNA damage marker (8-OH-dG) and DDR markers (γ-H2Ax and 53BP1) were analyzed by immunohistochemistry and immunofluorescence.

Patients

Paraffin-embedded tissues from oncologic Caucasian patients, who underwent resection surgery at Humanitas Clinical and Research Center, were examined for *PTX3* gene epigenetic modification by methylated CpG island recovery assay (MIRA). The Institutional Review Board of the Humanitas Clinical and Research Center approved this study (ICH-99/09).

Detailed procedures for in vivo studies, biochemical assays, FACS analysis, immunostaining and confocal analysis, quantitative PCR, statistical analysis, and all other methods are described in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.004.

AUTHOR CONTRIBUTIONS

E.B. designed and performed most experiments, analyzed the data, and drafted the manuscript. S.G. and M.R. performed experiments and analyzed data. R.P., P.K., E.Ba., and C.Gr. performed epigenetic studies and analyzed data. V.M., F.F., M.M., I.L., and M.R.G. performed experiments. J.D.L. provided reagents. A.D., S.T., F.P., and M.N. performed histological analysis and analyzed data. L.L., M.Ro., P.C., and G.B. provided human samples. S.J. contributed to the design of the experiments and data analyzation. C.G. and A.M. conceived the study, directed research, designed experiments, analyzed data, and wrote the manuscript.

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