CXCR4 engagement triggers CD47 internalization and antitumor immunization in a mouse model of mesothelioma

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Abstract

Boosting antitumor immunity has emerged as a powerful strategy in cancer treatment. While releasing T-cell brakes has received most attention, tumor recognition by T cells is a pre-requisite. Radiotherapy and certain cytotoxic drugs induce the release of damage-associated molecular patterns, which promote tumor antigen cross-presentation and T-cell priming. Antibodies against damage-associated molecular patterns, which promote tumor progression and metastasis, also flags tumor cells to immune recognition. Both CXCL12, the natural CXCR4 ligand, and BoxA, a fragment of HMGB1, promote the release of DAMPs and the internalization of CD47, leading to protective antitumor immunity. We designate as Immunogenic Surrender the process by which CXCR4 turns in tumor cells to macrophages, thereby subjecting a rapidly growing tissue to immunological scrutiny. Importantly, while CXCL12 promotes tumor cell proliferation, BoxA reduces it, and might be exploited for the treatment of malignant mesothelioma and a variety of other tumors.

Keywords CD47; CXCR4; HMGB1; immunogenic cell death; mesothelioma

Subject Categories Cancer; Immunology

Introduction

Chronic inflammation and the presence of an unfavorable inflammatory microenvironment can promote tumor development. A common example is colon carcinoma (Terzić et al, 2010), but as representative is malignant mesothelioma (MM), a tumor that is associated with asbestos exposure and comprises a large inflammatory component, in particular macrophages (Lievense et al, 2013). We previously showed that both MM cells and macrophages secrete High Mobility Group Box 1 protein (HMGB1) (Yang et al, 2010; Jube et al, 2012), an alarmin that alerts the innate and adaptive immune systems to tissue damage and cell stress (Bianchi et al, 2017). HMGB1 plays a central role in tissue regeneration, in part by recruiting monocytes/macrophages via the CXCR4 receptor and directing them toward a tissue-healing phenotype (Tirone et al, 2018). In MM, secreted HMGB1 sustains chronic inflammation initially caused by asbestos and supports disease progression (Jube et al, 2012; Xue et al, 2020). HMGB1 has several receptors, among which TLR4, RAGE, and CXCR4 are the most well-known (Bianchi et al, 2017). BoxA is a fragment of HMGB1 that corresponds to its first HMG-box.
domain and competes with HMGB1 for binding to the RAGE and TLR4 without activating them (Venereau et al., 2016; He et al., 2018). We previously reported that targeting HMGB1 with monoclonal antibodies or BoxA extends the survival of mice xenografted with human MM cells by interfering with tumor cell proliferation (Yang et al., 2015). However, extracellular HMGB1 also primes antigen recognition (Rovere-Querini et al., 2004) and is involved in immunogenic cell death (ICD). ICD is induced by certain chemotherapeutics or radiotherapy and increases the processing of apoptotic tumor cells by dendritic cells (DCs), enhances their immunogenicity, and elicits an efficient antitumor immune response and immunological memory (Galluzzi et al., 2020). The mechanism of ICD involves the apoptosis of tumor cells, preceded by endoplasmic reticulum (ER) stress, with concomitant induction of the unfolded protein response (UPR) and the release of HMGB1, ATP, and calreticulin (Kroemer et al., 2012). Calreticulin is an abundant ER-resident protein that becomes an “eat me” signal once relocated to the cell surface (ecto-calreticulin).

To test whether targeting HMGB1 is beneficial or detrimental in immunocompetent tumor-bearing hosts, we set up a syngeneic model of MM, where mouse AB1 malignant mesothelioma cells are grafted into the peritoneum of syngeneic BALB/c mice (Mezzapelle et al., 2016). Surprisingly, we found that BoxA, besides being antiproliferative, also promotes protective antitumor immunity responsible for MM rejection and long-term survival in a large fraction of mice.

Exploration of the mode of action of BoxA revealed that it acts via CXCR4. CXCR4 is a G-protein coupled receptor that induces cell migration upon binding its main ligand CXCL12 (also known as SDF-1) (Teicher & Fricker, 2010; Bianchi & Mezzapelle, 2020). CXCR4 is also involved in metastatization, and in many types of tumors upregulation of CXCR4 and of its ligand CXCL12 are predictive of short disease-free survival (Guo et al., 2016). Robust upregulation of CXCR4 was reported in human mesothelioma cell lines and in mesothelioma tissues (Li et al., 2011). However, in MM cells engagement of CXCR4 by BoxA does not promote cell growth but rather induces the surface exposure of calreticulin and the depletion of surface CD47, tilting the balance of “eat me” and “don’t eat me” signals, and promoting tumor cell phagocytosis by macrophages. CD47 is a ubiquitous transmembrane protein that prevents the phagocytosis of functionally fit cells by interacting with its ligand SIRPα2 (signal regulatory protein 1α) on the surface of macrophages and DCs (Barclay & van den Berg, 2014). Lack of CD47 on erythrocytes, platelets, and lymphohematopoietic cells results in rapid clearance of these cells by macrophages (Blazar et al., 2001). CD47 is expressed at increased level on the cell surface by a variety of malignant cells (Willingham et al., 2012); its blockade with monoclonal antibodies allows the efficient phagocytosis of cancer cells and leads to tumor rejection and development of antitumor immunity (Liu et al., 2015). CD47 blockade has remarkable therapeutic efficacy in various preclinical models of bladder, colon and breast cancer, glioblastoma, lymphoma, and acute lymphocytic leukemia (Jaiswal et al., 2009; Willingham et al., 2012; Liu et al., 2015). The published studies involve the masking of CD47 by antibodies that prevent its interaction with SIRPα2; whether and how CD47 exposure is modulated in response to the microenvironment is still unknown. Here, we show that CXCR4 engagement promotes the internalization of CD47 and the downstream antitumor responses, both when triggered by BoxA or CXCL12. Thus, we argue that the CXCL12/CXCR4 axis activates immunosurveillance via a mechanism (which we name Immunogenic Surrender) that allows tumor identification by innate cells and tumor-specific T-cell priming.

Results

BoxA promotes tumor rejection and the development of protective antitumor immune memory

HMGB1 promotes human MM cell survival and proliferation via RAGE (Jube et al., 2012), whereas BoxA, its N-terminal fragment, acts as an HMGB1 competitor and antagonist on RAGE and TLR4 receptors (Venereau et al., 2016). Accordingly, BoxA was found to reduce tumor growth and extend mice survival in a model where human MM cells were injected into immunodeficient mice (Jube et al., 2012; Yang et al., 2015). However, HMGB1 plays a key role in inducing ICD (Kroemer et al., 2012), and therefore, targeting HMGB1 might reduce antitumor immune responses. To investigate the antitumor potential of BoxA in immune-competent mice and possible underlying mechanisms, we exploited the syngeneic mouse model of MM we previously developed, where mouse AB1-B/c MM cells are engrafted in the peritoneum of BALB/c mice (Mezzapelle et al., 2016). Inoculation of 7 × 10⁶ MM cells produced MM tumors (Fig 1A) that were highly infiltrated by inflammatory cells, mostly represented by CD206⁺ CD86⁺ macrophages, and few CD3⁺ T and B cells (Fig EV1A and B). HMGB1 was highly expressed both in the nucleus and in the cytosol of tumor cells. This pattern is very similar to that of human mesothelioma (Fig EV1C).

In a first small-scale experiment, we inoculated AB1-B/c mouse MM cells in the peritoneum of 12 BALB/c mice, and 3 days later, we started treatment with 0, 200, 400, and 800 µg BoxA, three times a week. After 22 days, all control mice had developed tumor lesions, while mice treated with 800 µg BoxA had no discernible lesions, and mice treated with smaller doses of BoxA had an intermediate incidence (Fig 1B). Thus, BoxA was not toxic at the highest dose (800 µg per injection) and showed antitumor effects also in immunocompetent mice.

To follow tumor development in longitudinal analysis by BioLuminescence Imaging (BLI), we adopted AB1 cells expressing luciferase (AB1-B/c-Luc, henceforth called MM cells). Mice received i.p. delivery of BoxA or PBS (control) 2 days after MM injection (Fig 1A). At day 6, the high level of abdominal BLI signal (Fig 1C) indicated the engraftment of MM cells in both control and BoxA-treated mice. In the following weeks, most control mice experienced an increase of the BLI signal and had to be sacrificed. Notably, 4 control mice (20%) showed a tenfold decrease of the BLI signal relative to the first measurement, but then experienced remission and survived beyond the end of the 3-week treatment (Fig 1C and D). In contrast, 18 BoxA-treated mice showed a decrease of the BLI signal after day 6, in some cases to 10-fold below the initial measurement, and 15 (75%) survived after the end of the treatment. At day 75, the difference in survival curves between control mice and BoxA-treated mice was highly significant (P < 0.0001) (Fig 1D). We sacrificed two of the surviving mice per group (control and BoxA treated) and we could not identify any tumor mass, either in the abdomen or elsewhere; this difference in tumor rejection was highly significant (4/20 versus 15/20, P = 0.0012 Fisher’s test).
Figure 1.
These results indicate that BoxA treatment can extend the survival of model MM mice, but most of all increases the fraction of mice that reject the tumor. Notably, the efficacy of BoxA appears strikingly higher in immunocompetent mice compared to immunodeficient ones (Yang et al., 2015), suggesting that the immune system is an active player in the activity of BoxA.

To test whether surviving mice had developed immunological memory against the tumor, we re-challenged them with MM cells. All mice showed a high level of bioluminescence soon after the re-challenge, but only background levels 7 days later; all of them survived for several weeks without signs of disease (Fig 1E).

We then repeated the experiment described in Fig 1B and tested for the presence of tumor-specific CD8+ T memory cells, exploiting luciferase as surrogate tumor-associated antigen (Limberis et al., 2009). We recovered splenocytes from 4 groups of mice: (i) naïve (not injected with MM cells, not treated), (ii) injected with MM cells and surviving after being treated with control (PBS), (iii) injected with MM cells and sacrificed because of tumor progression despite being treated with BoxA (BoxA not cured), and (iv) injected with MM cells and surviving after being treated with BoxA (BoxA cured). The splenocytes were cultured for 5 days in the presence of the luciferase peptide GFQSMYTFV to expand LUC-specific T cells and then stimulated (pulsed) or not (un pulsed) for 4 h with the Luc peptide. Splenocytes from BoxA-cured mice contained a significantly higher percentage of CD8+ CD44hi IFNγ-producing T cells upon peptide stimulation than splenocytes from mice of the other treatment groups (Fig 1F and G; P < 0.005, Kruskal–Wallis test). This experiment indicates that BoxA promotes T-cell responses to a surrogate tumor-associated antigen. In spontaneously surviving mice (not treated with BoxA), the absence of detectable populations of LUC-specific T cells may reflect sub-optimal priming and a relative paucity of antitumor T-cell clones.

We also tested the requirement for T cells in BoxA-dependent antitumor responses. We depleted mice of CD8+ T cells (Appendix Fig S1) prior to the inoculation of MM cells, and then we treated them with either BoxA or PBS (Fig 2A). All CD8-depleted mice developed tumors regardless of treatment (BoxA or PBS) and were sacrificed after 2 weeks. In contrast, some of the non-depleted control mice survived longer than their control counterpart, and this was further promoted by BoxA (Fig 2B and C).

Overall our results show that transplantation of MM cells evokes a spontaneous protective immune response, which can lead to tumor rejection and immunological memory in a small fraction of mice. BoxA boosts immune-mediated recognition, increases the number of T cells that recognize tumor-associated antigens, and increases the fraction of mice that develop long-term antitumor immunity.

**BoxA induces the relocation of calreticulin without causing cell death**

We had expected that BoxA might interfere with ICD, but the results reported in the previous section showed that BoxA favors antitumor immune responses and immunization. We then investigated whether BoxA might instead promote ICD.

BoxA induced an increase in the surface exposure of calreticulin to an extent comparable to the well-known ICD inducer mitoxantrone (MTX) (Figs 3A and EV2A). Tumor masses explanted from mice treated with BoxA displayed calreticulin on the surface of cells, contrary to tumors from untreated mice (Fig 3B). BoxA also induced the release of HMGB1 (Fig 3C), although less efficiently than MTX, and the phosphorylation of eIF2α (Fig 3D), which is pathognomonic for ICD (Bezu et al., 2018). However, we detected a transient and dose-dependent activation of each of the three branches of the UPR (Fig EV2B–D), whereas MTX activated only the PERK-eIF2α arm (Fig 3D), in line with data from the literature (Panaretakis et al., 2009).

BoxA inhibited MM cell proliferation (Fig 3E); however, it induced no apoptosis, detected as caspase-3 cleavage (Fig 3F and G). Mice engrafted with MM cells that were pretreated or not with 800 nM BoxA for 16 h had comparable tumor growth and overlapping survival curves (Fig 3H and I), which is in keeping with the fact that BoxA does not induce MM cell death. In stark contrast, tumor cells treated with classical ICD inducers before inoculation confer antitumor immunization (Apetoh et al., 2007).

These data show that BoxA has a direct antiproliferative effect on tumor cells, where it induces stress and release of DAMPs, but does not cause apoptotic cell death, which is a hallmark of ICD. We thus inferred that tumor eradication by BoxA exploits a pathway different from ICD.

**BoxA promotes tumor cell phagocytosis by macrophages**

We showed that BoxA induces cell stress but no apoptosis; thus, we investigated a non-cell autonomous death mechanism that would provide tumor antigens for cross-presentation. Macrophages play a significant role in recognition and clearance of foreign, aged, and damaged cells, and their role in immunosurveillance has been reported (Jaiswal et al., 2009; Willingham et al., 2012). We therefore tested whether macrophages can ingest MM cells treated with BoxA.
Indeed, time-lapse imaging (Fig 4A and Movie EV1) of MM cells expressing GFP (GFP+ MM cells) co-incubated with bone marrow-derived macrophages indicated that BoxA increased the engulfment of tumor cells in a dose-dependent manner, from < 1% to almost 20% (Fig 4B).

Phagocytosis by macrophages depends on the balance of “eat me” signals, such as calreticulin, and “don’t eat me” signals, such as CD47. A decrease of about one-third in surface CD47 was previously reported to be effective in promoting tumor cell phagocytosis otherwise reported to be effective in promoting tumor cell phagocytosis.
by macrophages (Chao et al, 2010). Indeed, flow cytometry indicated that MM cells do express CD47 and that increasing concentrations of BoxA significantly reduced its surface exposure after overnight treatment (Fig 4C and D). CD47 was internalized (Fig 4E, immunofluorescence imaging) and did not appear to be degraded or shed into the medium (Fig EV3A and B).

Together, these results indicate that BoxA induces the internalization of CD47 and thus the unbalancing of “don’t eat me” and
“eat me” signals, which is sufficient to allow MM cell phagocytosis by macrophages.

**BoxA exerts therapeutic effects in a model of colon cancer**

Given the widespread expression of surface CD47 in tumors (Chao et al, 2011), we tested if other tumor cell lines respond to BoxA with a decrease in CD47 surface exposure. Cell lines MC38 (mouse colon cancer), B16 (mouse melanoma) and U87 (human glioblastoma) exposed to 800 nM BoxA also reduced surface CD47 after 24 or 48 h (Fig EV4A). We also extensively tested another human cell line—colorectal adenocarcinoma LoVo—and confirmed that BoxA-induced CD47 surface downregulation is associated with the exposure of calreticulin on the plasma membrane, the release HMGB1 in
Figure 5.
the medium, and the activation of the three arms of the UPR (Fig EV4B–E).

We then set up a syngeneic tumor model of colorectal carcinoma (CC), which is among the most common cancers worldwide in terms of incidence and mortality, and is a typical example of an inflammatory tumor. First, we investigated the effect of BoxA on the mouse colon adenocarcinoma cell line CT26: It induced calreticulin exposure (Figs 5A and EV5A), HMGB1 release (Fig 5B), the activation of the three arms of the UPR (Figs 5C and EV5B and C), and the depletion of surface CD47 (Fig 5D), without inducing cell death but merely inhibiting cell growth (Figs 5E and F, and EV5D).

We then injected different numbers (2 × 10^4, 5 × 10^4 and 10^5) of CT26 cells expressing LUC (CT26-LUC) in the peritoneum of BALB/c mice and we followed tumor growth by BLI (Fig EV5E). Most mice died within 16 days, suggesting that this model is far more aggressive compared to the AB1 mesothelioma model. Mice injected with 3.5 × 10^5 CT26-LUC cells developed a large number of small tumor masses (around 50) in the abdomen in about 2 weeks (Fig EV5F).

These tumors showed very high expression of HMGB1 in the nuclei and in the cytosol and were infiltrated by macrophages and T cells (Fig EV5G).

To test BoxA in the CT26 model, we treated the mice as indicated in Fig 5G. All control mice died within 19 days (Fig 5H); BoxA-treated mice survived significantly longer, and one eventually showed no BLI signal (Fig 5H and I). The surviving mouse was then re-challenged with 3.5 × 10^5 CT26-LUC cells, which it rejected (Fig 5H and J); at necropsy, we could not find any residual tumor mass. In contrast, three control naïve mice injected at the same time developed sizeable tumor masses (Fig 5J) and were sacrificed for severe illness after 14 and 18 days.

CXCR4 engagement promotes CD47 internalization

We next investigated which receptor mediates the immunotherapeutic effects of BoxA. BoxA was previously shown to interact with TLR4, RAGE, and CXCR4 receptors (Tirone et al., 2018), and MM cells express transcripts for all of them (Fig EV6A). Proximity Ligand Assays (PLA) (Soderberg, 2006) executed on CT26 and CXCR4 gave a strong signal on the plasma membrane of resting MM cells, whereas PLA executed with CT26-LUC cells and treated with either 800 µg BoxA or PBS three times a week, 10 times in total. Yellow arrows represent BLI imaging.

Data information: In panels (A–F), bars and error bars represent mean ± SD; statistics: One-way ANOVA with Dunnett’s post-test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Source data are available online for this figure.
the CD47/CXCR4 PLA signal from the cell surface, whereas it persisted in the cytoplasm (Fig 6A). BoxA also caused a modest decrease of the weak CD47/RAGE and CD47/TLR4 PLA signals on the plasma membrane, consistent with a general decrease of CXCR4 on the cell surface and thus of its availability to contact other surface molecules. These results suggest that CXCR4 cointernalizes with CD47 upon binding BoxA. Indeed, MM cells where CXCR4 expression was reduced by stable silencing (Fig 6B) were not responsive to BoxA: it did not reduce their proliferation (Fig 6C) nor did it cause CD47 depletion from their surface (Fig 6D). Moreover, AMD3100 (Plerixafor), a specific inhibitor of CXCR4 that hinders its internalization (Hitchinson et al., 2017), interfered with BoxA-induced depletion of surface CD47 (Fig 6E).

We then asked whether CXCL12, the natural CXCR4 ligand, would have effects similar to those of BoxA. Indeed, exposure of MM cells to 10–30 nM CXCL12 induced eIF2α phosphorylation (Fig 7A), the release of HMGB1 (Fig 7B), and internalization of surface CD47 (Fig 7C and D). Accordingly, 10 nM CXCL12 also
caused tumor cell phagocytosis in co-cultures of MM cells and macrophages (Fig 7E).

Overall, these results show that CXCR4 engagement, whether triggered by BoxA or CXCL12, causes its co-internalization with CD47.

Discussion

In this study, we demonstrate that the engagement on the surface of tumor cells of the CXCR4 receptor induces the depletion of surface CD47, the molecule that physiologically prevents phagocytosis by macrophages. As a consequence of CXCR4 engagement, in two different tumor models, mice develop an antitumor immune response that depends on CD8\(^+\) T cells and acquire antitumor immune memory. We designate this mechanism “Immunogenic Surrender” because the tumor cells surrender to macrophages in response to CXCR4 activation, which generally promotes their growth and dissemination.

We performed most of our experiments on a mouse mesothelioma model using BoxA, a fragment of HMGB1. We found that BoxA triggers the activation of the three arms of the UPR, the release of HMGB1, the exposure of the “eat me” signal ecto-calreticulin and the internalization of the “don’t eat me” signal CD47. Several of these events and molecular actors are also associated with immunogenic cell death; notably, both ICD and Immunogenic
Surrender cause the release of DAMPs from tumor cells. However, Immunogenic Surrender activates all branches of the UPR, whereas ICD activates one (Panaretakis et al, 2009) and does not promote apoptosis, which is functionally replaced by cell phagocytosis via depletion of surface CD47.

We envision Immunogenic Surrender as an antitumor immunosurveillance mechanism that occurs spontaneously as consequence of CXCR4 activation by CXCL12 or by the HMGB1-CXCL12 heterocomplex. Indeed, in our MM model a small number of mice spontaneously reject MM cells and develop antitumor immunity. In fact, we found that CD47 internalization is also induced upon binding of CXCR4 by CXCL12. It is no coincidence that BoxA, the molecule that we found to induce Immunogenic Surrender, is a fragment of HMGB1 and behaves in part like HMGB1 and its heterocomplex with CXCL12.

A role of CXCR4 in antitumor responses is unexpected, since the CXCL12-CXCR4 axis has been so far correlated with tumor initiation and progression (Guo et al, 2016). Indeed, both HMGB1 and CXCL12 promote tumor cell growth, survival, and invasion (Teicher & Fricker, 2010; Jube et al, 2012). In fact, the HMGB1-CXCL12-CXCR4 axis is involved in tissue repair and regrowth after damage (Tirone et al, 2018) and may be promoting tumor cell growth in similar ways (Bianchi & Mezzapelle, 2020). In this context, Immunogenic Surrender can be seen as a counterbalancing activity of the HMGB1-CXCL12-CXCR4 axis, one that induces the repairing/growing tissue to submit itself to immunological scrutiny. We speculate that such a check-and-balance should provide a definite evolutionary advantage.

We suggest that several tumor types activate Immunogenic Surrender during their development. We verified that BoxA promotes UPR, DAMPs release, and CD47 surface depletion in several mouse and human tumor cell lines; most importantly, BoxA induces tumor rejection and immunization also in mice inoculated with colon carcinoma CT26 cells. We note that BoxA has immunotherapeutic effects similar to the blockade of CD47 with monoclonal antibodies; in fact, antibody targeting of CD47 can be seen as an extrinsic therapeutic intervention that mimics and exploits the CD47 surface depletion that occurs in Immunogenic Surrender.

Critically, in our work we unveiled Immunogenic Surrender using BoxA, which acts as an antagonist on TLR4 and RAGE, and is a partial activator of CXCR4. BoxA suppresses tumor cell growth while, in contrast, CXCL12 promotes tumor cell growth (Guo et al, 2016). These contrasting effects might be due to the remarkable plasticity of G-coupled protein receptors in the activation of downstream signaling pathways after binding to different yet related agonists—a process called biased signaling (Violin et al, 2014; Hitchinson et al, 2017). The difference in cell growth induced by BoxA and CXCL12 is critical from a translational point of view: Even if both were equally potent in inducing Immunogenic Surrender, injecting CXCL12 would also promote cancer growth, while our data indicate that injecting BoxA does restrain it. BoxA is not toxic to mice, possibly because it may be more effective on tumor cells than on normal cells. In the perspective of drug development, BoxA can be considered a hit molecule that enabled the discovery of Immunogenic Surrender; such a hit might be improved by design or entirely replaced by small molecules that bind to CXCR4 and promote CD47 internalization, while not retaining the full agonist activity of CXCL12.

In conclusion, we have unveiled a previously unknown activity of CXCR4, which by co-internalizing CD47 exposes tumor cells to immunosurveillance. We have also identified a molecule, BoxA, which is not cytotoxic but can enhance the immunosurveillance-related activity of CXCR4 without promoting cell growth. We suggest that BoxA-instructed recognition of tumor antigens should be complementary to checkpoint inhibitors, which potentiate the response of immune cells toward already recognizable tumor antigens. The use of BoxA, or a small molecule mimicking its action on CXCR4, may hold promise for malignant mesothelioma, for which there are very few available therapeutic interventions, and more generally for the wide variety of tumors vulnerable to CD47 blockade.

Materials and Methods

Cell lines and drug compounds

AB1 mouse mesothelioma cells (Cell Bank Australia), CT26 mouse colon cancer cells, and LoVo human colon cancer cells (both obtained from ATCC, Manassas, VA, USA) were cultured at 37°C under 5% CO2 in RPMI 1640 (Life Technologies) supplemented with 5 or 10% v/v fetal bovine serum (Life Technologies), respectively, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10 mM HEPEs. Luciferase-expressing AB1-B/C-LUC cells were previously described (Mezzapelle et al, 2016). CT26-LUC cells were obtained by transfecting CT26 cells with the pLenti PKG V5-LUC Neo (w623-2) plasmid (Addgene #21471). MC38 cells were kindly provided by Mario Colombo (Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy), B16 cells by Matteo Bellone and U87 cells by Andrea Graziani (both IRCCS Ospedale San Raffaele, Milano).

All cell lines were passaged for no longer than 10 passages after thawing. Cell lines were routinely tested for mycoplasma contamination by PCR. To elicit the endoplasmic reticulum (ER) stress, cells were treated 1 μM Mitoxantrone (MTX, Sigma-Aldrich) for 24 and 48 h. AMD3100 was purchased from Sigma-Aldrich, BoxA, and CXCL12 (LPS-free) from HMGBiotech (Milano, Italy).

Mice

Animal experiments were approved by the Animal Care and Use Committee (IACUC #839) of Ospedale San Raffaele in accordance with the Italian law. Eight-week-old male BALB/c mice were purchased from Charles River Laboratories (Calco, Italy). Animals were housed under specific pathogen-free conditions and allowed access to food and water ad libitum. Mice were inoculated intraperitoneally (i.p.) with 7 × 10^6 MM or with 3.5 × 10^6 CT26-LUC cells. Cell engraftment was confirmed by bioluminescence imaging (BLI). Two days after inoculation, mice were randomized into experimental groups and treatments started. BoxA (800 μg) was administered i.p. three times a week for 3 weeks (10 times in total). Control mice were injected i.p. with saline. Tumor growth was assessed weekly by BLI. Mice were monitored daily and were sacrificed when severely distressed (BLI signal > 10^9 photons/s).

In vivo BioLuminescence optical imaging (BLI)

BLI was performed on mice after the i.p. injection of 7 × 10^4 MM cells or 3.5 × 10^6 CT26-LUC using an IVIS SpectrumCT Preclinical In Vivo Imaging System (Perkin Elmer). The system is equipped with a low noise, back-thinned, back-illuminated CCD camera cooled at −90°C.
(quantum efficiency in the visible range above 85%). Before BLI, each mouse received an intra-peritoneal injection of 6 g luciferin/kg body weight. During image acquisition, the animals were kept at 37°C and under gaseous anesthesia (2–3% isoflurane and 1 lt/min O2). After luciferin injection, dynamic BLI was performed from 0 to 30 min by acquiring an image every 2 min (exposure time = auto, binning = 8, f = 1 and a field of view equal to 13 cm (field C)) in order to detect the highest BLI signal. BLI image analysis was performed by measuring the total light flux (photons/s) in a uniform region of interest (ROI) placed over the animal abdomen. Images were acquired and analyzed using Living Image 4.4 (Perkin Elmer).

**Tumor re-challenge**

BALB/c mice that rejected the first tumor challenge and naïve BALB/c mice were inoculated i.p. with 7 × 10⁶ MM cells for the mesothelioma model and with 3.5 × 10⁶ CT26-LUC cells for the colon carcinoma model. Tumor growth was followed by BLI. Mice were sacrificed when the bioluminescent signal remained at background levels for three consecutive scans.

**Histology and immunofluorescence**

**Mouse samples**

Tumor masses were explanted from each sacrificed mouse, fixed in zinc formalin for 24 h, processed with Leica TP1020, embedded in paraffin, and slices were cut. Briefly, the sections (3 μm) were deparaffinized in xylene and rehydrated in graded alcohol. Sections were stained with hematoxalin and eosin. Immunohistochemical staining was performed using the following antibodies: anti-F4/80 (clone A3-1 Bio-Rad), anti-CD68 (clone SP7 Abcam), anti CD45R/B220 (clone RA3-6B2 BD Biosciences), anti-HMGB1 (#18256 Abcam), and anti-calreticulin (#2907 Abcam). Slides were counterstained with hematoxalin and mounted. For immunofluorescent staining, 6-μm-thick serial cryostat sections from mice tumor samples, immediately snap-frozen in OCT after removal, were fixed with cold acetone for 10 min and co-stained with the following Ab: rabbit anti-mouse mAb to F4/80 (clone A3-1 Bio-Rad); rat anti-mouse mAb to CD206 (clone 1:100 miap301, Bioxcell), rat anti-mouse mAb to CD86 (clone PO.3, 1:100, #04-1527 Rad), and rat anti-mouse mAb to CD206 (clone A3-1 Bio-Rad), anti-CD3 (clone SP7 Abcam), anti CD45R/B220 (clone RA3-6B2 BD Biosciences), anti-HMGB1 (#18256 Abcam), and anti-calreticulin (#2907 Abcam). Images were analyzed by confocal microscopy and acquired with a 63× objective using a confocal microscope (TCS SP5 AOBS Leica LSM; Leica Microsystems).

**Patients’ samples**

The study involved mesothelioma patients admitted to the Thoracic Surgery Unit of San Raffaele Hospital (Milano, Italy) and Maggiore della Carità Hospital (Novara, Italy) between 2015 and 2017. Diagnosis of mesothelioma was based on standard histological and immunohistochemical criteria, including positivity to calretinin, vimentin, and cytoketones 5 and 6, and negativity to carcinoembryonic antigen, thyroid transcription factor 1, and Ber Epy 4. Pleural biopsies were collected and fixed in formalin for 24 h, embedded in paraffin, and processed.

**Image acquisition and analysis**

All images were scanned using the Aperio Scanscope C2 system (Leica Biosystems).

**RNA extraction and real-time PCR analysis**

Total RNA was extracted from cells and tumors using Nucleospin RNA (Macherey-Nagel) and treated with DNase I. The amount of total RNA was determined by UV spectrophotometry using a Nanodrop Spectrophotometer (Thermo Fisher Scientific). Next, 1 μg of total RNA was reversed transcribed using the Superscript III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer’s protocol. PCR analysis was carried out using AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific).

Receptor expression was evaluated using the following primers:

- **CXCR4** Forward: 5’ TAGAGCAGTGTTGCAAATGG 3’; Reverse 5’ TGAAGTACGAGTCGTCGACG 3’.
- **RAGE** Forward 5’ TCCTCAGGGTCAGTGTGG 3’; Reverse 5’ TGTTGACCCGTAGTCGACCAG 3’.
- **TLR4** Forward 5’ CAGTGTGCTAGTGTGATTG 3’; Reverse 5’ TCTCCGGATGATGTCGCCG 3’.
- **β-actin** Forward: 5’AGA CGG GGT CAT CAC CCA CAC TGT GCC CAT CTA 3’; Reverse 5’ CTA GAA GCA CTT CCG GTG CAT GAT GGA GGG 3’.

**Western blotting**

Protein extracts from different cell lines were prepared as follows. Cells were lysed by an ice-cold lysis buffer containing 50 mM...
Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 % NP-40, 0.1 % SDS supplemented with a cocktail of protease inhibitors (Roche). Samples were sonicated prior to mixing with reducing SDS–PAGE sample buffer and heating (5 min, 90°C). After transferring proteins to PVDF or nitrocellulose, the membrane was blocked with 5% BSA or 5% non-fat dry milk. Antibodies to eIF2α (#9722), p-eIF2α (#3597), and cleaved caspase-3 (#9664) were from Cell Signaling Technology, ATF6 (ab37149), IRE1 (ab37073), HMGB1 (ab18256), and CD47 (ab215616) were from Abcam; β-actin (F-3022) and α-tubulin (T-5168) from Sigma-Aldrich. Anti-mouse or anti-rabbit antibodies, conjugated with horseradish peroxidase, were used as secondary antibodies. An ECL chromogenic substrate was used to visualize the bands. In some experiments, Ponceau S staining was used for assessing equivalent protein loading. To detect extracellular HMGB1, the culture medium was collected and concentrated via centrifugation (5,000 g-force for 1 h) through microconcentrators (Centricon plus 10 KDa filter Amicon Ultra, Millipore). Proteins were then analyzed by Western blotting. Some blots were cut and probed with different antibodies for different proteins, including β-actin. In some cases, to examine proteins of similar molecular weight, the PVDF membranes were subjected to a mild stripping protocol, as recommended by Abcam. Western blot bands were quantified using Fiji or Image Lab software.

The efficiency of CD8 T-cell depletion in vivo was determined by flow cytometry in peripheral blood at day 5 after MM cell inoculation and in the spleen. Blood and spleen cells were stained with APC-Cy7 conjugated anti-CD3 (1:50, #130-102-306, Miltenyi Biotec) and PerCP-Cy5.5 conjugated anti-CD8 (1:400, clone 53-6.7, BioLegend) antibodies. The stained cells were analyzed by BD FACS Canto II (BD Biosciences). Data were analyzed using the FCS Express 6 software.

**Generation of macrophages**

Bone marrow cells were isolated from the femurs of 8-week-old male BALB/c mice. Macrophages were obtained by culturing bone marrow cells in DMEM containing 10% FCS supplemented with macrophage colony-stimulating factor (M-CSF, 20 ng/ml) for 6 days. At day 4, the medium was replaced with fresh M-CSF, and at day 6, adherent macrophages were harvested.

**In vitro phagocytosis assay**

GFP-positive AB1 mesothelioma cells (12.5 × 10³) were incubated with 3 × 10⁶ mouse macrophages in 48-well plates in the presence or not of BoxA (100, 200, 400, and 800 nM) or CXCL12 (10 nM) for 16 h. Cells were observed under a confocal microscope (Leica TCS SP5) using a 20× dry objective (0.7 numerical aperture). The confocal microscope is equipped with an incubation system to maintain the cells at 37°C in 5% CO2. GFP-positive AB1 cells were imaged with low-intensity 488 nm Argon laser, while macrophages were observed in the transmission channel. Images were acquired in 16-bit format (1,024 × 1,024 pixels) every 5 min for 16 h.

**Long-term culture of splenocytes and IFNγ intracellular staining**

Single-cell suspensions from mouse spleens were obtained by mechanical dissociation; 3 × 10⁷ splenocytes were cultured for 5 days in RPMI medium plus 10% FCS supplemented with a firefly luciferase-derived peptide (1 μM, peptide sequence NH2-GFOSMYTFV-COOH, Primm srl) (Limberis et al., 2009). After 5 days, vital lymphocytes were isolated using Lympholyte Cell Separation media (Cedratane Labs) and 5 × 10⁵ lymphocytes were stimulated with low-intensity 488 nm Argon laser, while macrophages were observed in the transmission channel. Images were acquired in 16-bit format (1,024 × 1,024 pixels) every 5 min for 16 h. The stained cells were analyzed by BD FACS Canto II, and data were analyzed using the FCS Express 6 software.

**In vivo CD8 depletion**

Two hundred μg of either anti-mouse CD8 monoclonal antibody (Clone 2.43, BioXcell) or IgG control were injected i.p. at day −3,
The paper explained

Problem
Malignant mesothelioma (MM) is a tumor arising from asbestos-induced chronic inflammation and for which few therapeutic options are available. High Mobility Group Box 1 (HMGB1) protein favors the onset and progression of MM. We tested the therapeutic potential of BoxA, a fragment of HMGB1 that competes with the intact protein, in an immune-competent mouse model of MM.

Results
We find that BoxA induces MM remission and antitumor immunization in a large fraction of mice. The binding of BoxA to the CXCR4 receptor induces DAMPs release and CD47 internalization, leading to tumor cell phagocytosis by macrophages. CXCL12, the natural ligand of CXCR4, also promotes CD47 internalization.

Impact
Our study indicates that the CXCL12/CXCR4 axis, which is known to promote cancer progression, also promotes a counterbalancing antitumor response. BoxA is non-toxic and, contrary to CXCL12, inhibits tumor cell growth. Thus, BoxA, by shifting the balance from tumor growth to antitumor immunization, might hold promise as first-in-class antitumor drug that should be synergic with checkpoint inhibitors. Furthermore, synthetic ligands that act like BoxA may be as effective as anti-CD47 antibodies, which are in advanced clinical development.

Proximity ligation assay (PLA)

2x10^6 MM cells were seeded on glass coverslips and the following day treated overnight with either BoxA (400 nM) or CXCL12 (10 nM) and PBS (control). Following treatment, cells were fixed with 4% paraformaldehyde in PHEM buffer for 10 min at RT, washed twice with 1% BSA in PBS for 5 min, and then blocked with 4% BSA and 10% goat serum in PBS. Cells were overlaid with the primary antibodies: rabbit monoclonal anti-CD47 (1:100, EPR21794, Abcam #AB218810); mouse monoclonal anti-CD47 (1:50, B6H12, Santa Cruz #sc12730) either alone or in combination or goat polyclonal anti-CXCR4 (1:100, Abcam #AB1670), or rabbit monoclonal anti-TLR4 (1:50, Cell signaling #14358) and or rabbit polyclonal anti-CXCR4 (1:100, Invitrogen #PA1-075) or mouse monoclonal anti-CD47 (1:50, B6H12, Santa Cruz #sc12730) either alone or in combination or goat polyclonal anti-CXCR-12 (10 nM) and PBS (control). Following treatment, cells were fixed with 4% paraformaldehyde in PHEM buffer for 10 min at RT, washed twice with 1% BSA in PBS for 5 min, and then blocked with 4% BSA and 10% goat serum in PBS. Cells were overlaid with the primary antibodies:

PLA quantification

Custom-made MATLAB routines were used to quantify the intensity of the PLA signal for each cell, available upon request. In short, to segment the nuclei, we used the signal from the Hoechst channel. Nuclear masking was performed using as a threshold the mean image intensity plus twice the standard deviation, while segmentation was performed after a watershed transformation allowing to segment most of the overlapping nuclei. The resulting segmented nuclei were filtered by size to exclude artifacts or improperly segmented clusters of nuclei. To estimate the signal intensity per cell, a ring of 100 pixels (8.5 μm) around each segmented nucleus was applied, able to cover most of the cell’s surface and of the signal coming from each cell. We then estimated the intensity of the signal per cell as the total intensity of the nucleus plus the intensity of the ring around it, after background subtraction. When two or more rings did overlap in a given region, the intensity of such region was evenly divided between the cells involved.

Calreticulin IHC quantification

To estimate the calreticulin intensity from immunohistochemistry images, ad hoc MATLAB routines were developed (available upon request). Pixels of the RGB images have three coordinates (corresponding to the red, green, and blue intensities), and we classified them in different “colors” using a K-means clustering algorithm with K = 4 (K > 4 led to similar final results). As a result, this algorithm defines the color of calreticulin-positive pixels. The next step is to measure the distribution of calreticulin-positive pixels: If calreticulin is diffuse within the cytoplasm, a large number of contiguous pixels is positive, and calreticulin-negative pixels are few. Ecto-calreticulin, in contrast, corresponds to a lower number of positive pixels, and a larger number of calreticulin-negative pixels. The fraction of calreticulin-negative pixels, thus, is a descriptor of the location of calreticulin.

Silencing of CXCR4

AB1 (MM in the text) cells were transfected with a lentiviral expression vector containing either a short hairpin RNA directed against CXCR4 or a scramble control (Open Biosystems code v2MM_217115 SM 2566) using Lipofectamine 3000 (Invitrogen), according to the manufacturer’s instructions. Cells surviving puromycin selection were harvested as a pool and analyzed for CXCR4 expression. Total RNA was extracted from AB1 cells shCTR and AB1 shCXCR4 (pool 1 and 2) using NucleoSpin RNA (Macherey-Nagel) and treated with DNase I. The RNA amount was determined using the NanoDrop Spectrophotometer (NanoDrop Technology). Next, 1 μg of total RNA was reversed transcribed using the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer’s protocol. The cDNA reaction was used in triplicate SYBR-Green (Applied Biosystems) qPCR reactions containing primers specific for CXCR4: Forward: 5'-TCA GTG CCT GAC CTC CTC TT-3'; Reverse: 5'-CTT GCC CTC TGA CTG TTG GT-3'. β-actin Forward: 5'AGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3'; Reverse: 5'CTA GAA GCA CTT GGC GTG CAC CAT GGA GGG 3'. β-actin RNA was used for normalization.
Statistical analysis

We performed all experiments in duplicate, triplicate, or quadruplicate if we expected that nonparametric comparisons between two groups would be appropriate, in triplicate if we expected that parametric would be appropriate. Most experiments did not have a large enough sample size to test for normal distribution; we used parametric tests only where normality was expected a priori. Experiments that used multiple doses or multiple times were done in duplicate or triplicate. In general, we used the minimum number of replicates that can be analyzed statistically.

For animal studies, the sample size estimate was indicated in the application to obtain an IACUC. In our case, the number of animals was estimated assuming a survival \( \geq 0 \) in the control group and \( >30\% \) survival in the treatment group; to get a statistical significance in survival analysis with an 80% power and alpha error for 25%, we needed \( >6 \) animals/group, and we generally used 9 mice/group. In the experiment reported in Fig 1, the sample size was chosen as 20 per group because we expected no surviving animals, but only an extension of their survival; the actual result was unexpected. There were no pre-established criteria for animal exclusion and, in general, we did not exclude animals, save the ones where an error in handling was done, for example, a mouse that died during anesthesia. All animals were assigned a code and assigned to a group after random number generation. The investigator who performed treatments was not blinded, because it either made no sense or was impractical; the investigator who performed BLI measures was a different one and was blinded to which animals were treated or not. For flow cytometry analyses, which often require gating that to some extent is subjective, the technician who collected the data was unaware of which samples were which. Results were analyzed statistically without the option of reallocating or excluding samples.

Statistical analyses were performed with GraphPad Prism software, version 8.1.1 (GraphPad software, Inc.).

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions

Conceptualization: RM, AM, MPC, VC, MEB. Data curation: RM, SZ, VC, MEB. Formal analysis: RM, VC, MEB. Funding acquisition: LS, AM, VC, MEB. Investigation: RM, FDM, CP, ML, FB, FCo, PC, RE, MS, FCa, VB, FS, LS. Methodology: RM. Project Administration: MEB. Resources: MC, AP, RB, AC, OR. Software: SZ. Supervision: AM, VC, MEB. Writing: MR, AR, AM, MPC, VC, MEB.

Conflict of interest

MEB is founder and part owner of HMGBiotech, and MC and AP were partially supported by HMGBiotech. The other authors declare no conflict of interest.

For more information

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