ORIGINAL RESEARCH

An epitope-specific novel anti-EMMPRIN polyclonal antibody inhibits tumor progression

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ABSTRACT
Extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) mediates tumor cell–macrophage interactions, and has been shown to induce both matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF). However, the epitope responsible for MMP induction is controversial, and the epitope responsible for VEGF induction is yet unknown. We generated a novel anti-EMMPRIN antibody directed against a specific epitope that successfully inhibited the production of both MMP-9 and VEGF in tumor cell–macrophage \textit{in vitro} co-culture systems, exhibiting a U-shaped dose response. Furthermore, this antibody efficiently inhibited \textit{in vivo} tumor progression in both the RENCA renal cell carcinoma and CT26 colon carcinoma subcutaneous tumor models, and reduced tumor size and number of metastatic foci in the 4T1 orthotopic model. This was achieved by inhibiting angiogenesis as assessed by immunohistochimical staining for the endothelial marker CD31, by inhibiting tumor cell proliferation as assessed by the staining for Ki-67, and by enhancing tumor cell apoptosis as assessed in the TUNEL assay. Moreover, administration of the antibody recruited more macrophages into the tumor, and skewed the tumor microenvironment for angiogenesis and metastasis.7 Last, recent studies show that elevated EMMPRIN expression is associated with greater resistance to radiotherapy.14,15 Thus, EMMPRIN functions as a central hub with multiple activities, positioning it as a potential target for cancer therapy, with potential profound beneficial consequences on tumor cell metabolism, viability, proliferation, angiogenesis and metastasis.7

Introduction
Angiogenesis, which provides oxygen and nutrients, is a necessary process that enables both tumor expansion and metastasis. This process is promoted by the secretion of pro-angiogenic factors, such as the potent VEGF that drives angiogenesis and metastasis.1 Angiogenesis and metastasis.1 Angiogenesis and metastasis.

EMMPRIN is a glycoprotein belonging to the Ig superfamily, with two heavily glycosylated extracellular domains. EMMPRIN is weakly or moderately expressed on many cell types (including monocytes, T cells, and some epithelial cells), but its expression is greatly increased on many types of malignant cells in positive correlation with higher grade and stage of tumors, and often together with increased invasiveness and poor prognosis.6,7 EMMPRIN is mostly known as an inducer of MMPs, including MMP-9, but it is also capable of inducing stromal VEGF, and thus is implicated in angiogenesis and invasiveness.8-10 EMMPRIN’s ability to induce MMPs was generally mapped to a region of 20 amino acids in the first of the two highly glycosylated extracellular domains (EC-I),11-13 but the precise epitope regulating VEGF induction remained unknown.

In addition to its pro-angiogenic activities, EMMPRIN is a multifunctional protein that can interact with different proteins to mediate multiple activities that are important in the tumoral context. For example, it can bind with extracellular cyclophilin A (CypA) to achieve a chemotactic activity that helps recruit leukocytes to the tumor; it is involved in the multidrug resistance phenotype via interactions with P-gp/MDR-1; and it can chaperon the lactate transporters MCT-1 and MCT-4, to facilitate lactate transport out of the cells to maintain pH and tumor cell viability (reviewed in8-7). Last, recent studies show that elevated EMMPRIN expression is associated with greater resistance to radiotherapy.14,15 Thus, EMMPRIN functions as a central hub with multiple activities, positioning it as a potential target for cancer therapy, with potential profound beneficial consequences on tumor cell metabolism, viability, proliferation, angiogenesis and metastasis.7

Although several anti-EMMPRIN antibodies are already available commercially, they were usually elicited against the entire extracellular domain of the protein, and the specific epitope that they target remained unknown. Here, we
report on a novel anti-EMMPRIN antibody (designated henceforth 161-Ab) that specifically recognizes and inhibits an epitope that is responsible for the induction of both MMP-9 and VEGF, and can also inhibit tumor progression in vivo.

**Results**

**Peptide design**

In planning the immunogen to be used to elicit an anti-EMMPRIN polyclonal antibody, we used the published sequence of the mouse EMMPRIN protein (accession number NP_001070652.1), with three main considerations in mind: high immunogenicity of the peptide, maximal homology with the human EMMPRIN sequence (accession number NP_940991), and no homology with any mouse protein. High immunogenicity is usually determined in hydrophilic areas that are exposed on the surface of the protein, and we used the EMBOSS and the Open Biosystem algorithms to identify these regions. Based on the BLAST search, the entire mouse and human EMMPRIN sequences are only 58% identical, making it very difficult to identify peptides that are completely homologous to both mouse and human protein sequences. However, we identified two candidate peptides (designated 161 and 162, Fig. 1A), that demonstrated 73% and 60% identical amino acids, with three and four amino acids difference, respectively. To avoid any possible cross-reactivity, the peptides were tested by BLAST analysis and were shown not to be homologous with other mouse proteins, especially the proteins neuroplastin and embigin that belong to the same family.

**The 161-Ab specifically recognizes EMMPRIN**

Both peptides were synthesized, conjugated to KLH and injected in three boost injections to two rabbits each, to raise two polyclonal antibodies (designated accordingly as 161-Ab and 162-Ab). Both the 161-Ab and the 162-Ab immune sera were first shown to be able to bind each to its respective immunogenic peptides with high titers (1:1,312,500 or 1:1,560,000), but not to BSA that served as a negative control (Table 1). To show that the antibodies recognize the peptide sequence within the context of the entire EMMPRIN protein, we evaluated the ability of each immune serum to bind to the protein in its

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Table 1. 161-Ab and 162-Ab specifically recognize their immunizing peptides.

| Titer/coating peptide | 161 peptide | BSA | 162 peptide | BSA |
|-----------------------|-------------|-----|-------------|-----|
| 1:500                 | 3.184 ± 0.146 | 0.014 ± 0.001 | 3.715 ± 0.117 | 0.025 ± 0.006 |
| 1:2,500               | 1.051 ± 0.107 | 0.004 ± 0.001 | 2.663 ± 0.207 | 0.004 ± 0.001 |
| 1:12,500              | 0.234 ± 0.025 | 0.002 ± 0.001 | 1.081 ± 0.119 | 0.001 ± 0.0001 |
| 1:62,500              | 0.146 ± 0.006 | 0.002 ± 0.001 | 0.281 ± 0.035 | 0.0001 ± 0.0001 |
| 1:1,562,500           | 0.009 ± 0.001 | 0.001 ± 0.001 | 0.056 ± 0.008 | 0.0001 ± 0.0001 |

*aOptical density values of the binding of each antibody to its respective peptides are indicated (n = 6).*

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Figure 1. EMMPRIN structure and peptide design, and specificity of 161-Ab: (A) Partial amino acid sequence of the murine EMMPRIN EC-I and EC-II domains compared to human sequence, with the 161-peptide (position 52-63) and the 162-peptide (position 136–145) indicated. (B) Mouse recombinant EMMPRIN (200 ng/lane) was loaded onto a 10% SDS-PAGE, separated and transferred onto a nitrocellulose membrane. The membrane was cut into strips and each strip was probed with the rabbit anti-mouse immune (I) or pre-immune serum (P) serum diluted 1:1,000. One strip was probed with the commercial rat anti-mouse EMMPRIN and served as positive control (P.C). Representative images of EMMPRIN identification in RENCA tumor sections by immunohistochemistry using (C) the pre-immune serum, (D) the immune serum (161-Ab), or (E) a commercial anti-EMMPRIN antibody.
To screen for an inhibitory activity of the 161-Ab co-cultures, 161-Ab inhibits secretion of MMP-9 and VEGF in induced by 15-folds.[p<0.001] (single cultures, MMP-9 concentrations were synergistically was achieved after tumor cells and macrophage-like cell lines.

To establish if 161-Ab can cross-react with human EMMPRIN, as the human epitope sequence differs in only three amino acids from the mouse sequence, we next examined its ability to inhibit VEGF and MMP-9 secretion in a human co-culture system. We used two human tumor cell lines (the renal cell carcinoma A498 and the breast carcinoma MCF-7) and incubated each of them in the presence of a human monocyte-like cell line (U937) for 48 h with the addition of TNFα (1 ng/mL), as these conditions yield maximal levels of MMP-9 and VEGF,16 and with the addition of the optimal dilutions of 161-Ab previously determined for the mouse cultures. In both co-cultures, MMP-9 secretion was selectively inhibited by the addition of the immune, but not the pre-immune serum by 43% at dilution 1:312,500 (p < 0.05 for the MCF-7 co-culture), although in the A498 co-culture this did not reach significance (Fig. 2C). Likewise, the 161-Ab immune serum, but not the pre-immune serum, inhibited VEGF secretion by 31% and 25% for the A498 and MCF-7 co-cultures (p < 0.05 for both), respectively, at dilution 1:312,500.

| Antibody | Immune serum | Pre-Immune serum | BSA | P valuea |
|----------|--------------|-----------------|-----|----------|
| 161-Ab   | 0.235 ± 0.033 | 0.064 ± 0.008   | 0.031 ± 0.003 | 0.0002 (** ) |
| 162-Ab   | 0.076 ± 0.007 | 0.091 ± 0.002   | 0.021 ± 0.004 | 0.9538 (ns)  |

aOptical density values of the binding of 161-Ab or 162-Ab to the mouse recombinant EMMPRIN protein are indicated (n = 4).

161-Ab inhibits secretion of MMP-9 and VEGF in co-cultures

To screen for an inhibitory activity of the 161-Ab in vitro, we first determined that maximal MMP-9 and VEGF secretion was achieved after tumor cells and macrophage-like cell lines were incubated in co-culture (Fig. 2A). Relative to each of the single cultures, MMP-9 concentrations were synergistically induced by 15-folds (p < 0.0001) and 5-folds (p < 0.01) for the CT26 and TRAMP-C2, respectively, that were co-cultured with RAW 264.7 cells. Likewise, VEGF concentrations were induced by 2–3-folds in both co-culture systems (p < 0.001). Similar results were also observed in two additional tumor cell lines (the mouse renal cell carcinoma RENCA and the prostate cell carcinoma TRAMP-C1) that were co-cultured with RAW 264.7 cells, or when all four tumor cell lines were incubated with primary thioglycollate-elicited peritoneal macrophages (data not shown). These results suggest that the interaction between tumor cells and macrophages, even without the addition of any other stimulus, is necessary for the induction of high amounts of pro-angiogenic factors, and can serve as an in vitro screening platform for the identification of an antibody with the ability to inhibit MMP-9 and VEGF secretion.

We next examined the ability of both immune sera to inhibit secretion of MMP-9 and VEGF using the established screening platform. Both pre-immune and immune sera were 5-fold serially diluted (Fig. 2B), and added to the co-culture systems and after 48 h supernatants were collected and concentrations of VEGF and MMP-9 were evaluated. Whereas the 162-Ab did not inhibit MMP-9 or VEGF in any of the dilutions tested in both co-culture systems (data not shown), the immune serum containing the 161-Ab, but not the pre-immune serum, was effective in inhibiting both MMP-9 and VEGF in the two screening platforms of CT26 or TRAMP-C2 co-cultured with RAW 264.7 cells, resulting in a U-shaped curve (Fig. 2B). In the CT26 co-culture system, serial dilutions of 1:312,500 and 1:1,562,500 of the immune serum resulted in maximal inhibition of 69% and 60% in MMP-9 secretion (p < 0.05), whereas in the TRAMP-C2 co-culture system lower dilutions of 1:12,500 and 1:62,500 inhibited MMP-9 secretion by 75% and 83% (p < 0.05). VEGF was inhibited by 53% at dilution 1:1,562,500 in the CT26 co-culture system and by 62–67% at dilutions 1:312,500 and 1:1,562,500 in the TRAMP-C2 co-culture system (p < 0.05). Thus, the optimal range for inhibiting both MMP-9 and VGEF was found to be between 1:312,500 and 1:1,562,500, suggesting that our epitope is responsible for the induction of both these proteins in the EMMPRIN protein.

161-Ab inhibits tumor progression

After the specificity of the 161-Ab to EMMPRIN was determined, and its ability to inhibit the pro-angiogenic MMP-9 and VEGF proteins was established, we examined its ability to inhibit tumor progression in vivo. Thus, we first injected the RENCA or CT26 tumor cells to the flanks of BALB/c mice, and only after tumors were palpable (around day 12), we i.p. injected the 161-Ab every 7 d in different concentrations (25 μg, 50 μg or 100 μg in 2 mL saline). We injected either saline or used the 162-Ab which did not show any specificity to EMMPRIN, in the control groups. At the end of the experiment (day 39 for RENCA tumors and day 30 for CT26 tumors), differences between the control groups and all doses of the 161-Ab groups were visible. In RENCA tumors, the dose of 50 μg, and to a greater extent the dose of 25 μg, inhibited tumor progression by 90% (p < 0.01) or 99% (p < 0.05), respectively (Fig. 3A). Likewise, for the CT26 tumors, the 161-Ab significantly inhibited tumor growth in all doses used, where the 100 μg dose inhibited tumor growth by 58% (p < 0.01), the 50 μg dose inhibited by 85% (p < 0.001) and the 25 μg dose inhibited tumor growth by 97% (p < 0.001, Fig. 3B).
We next examined the effect of the 161-pAb in the 4T1 mammary gland orthotopic model, as tumor behavior and response to anticancerous agents have been shown to differ between subcutaneous and orthotopic tumor models.\textsuperscript{17,18} Tumor cells were injected into the mammary fat pad of BALB/c female mice, and when the tumors were well established (with an average size of 0.19 cm\(^3\)) we injected three boost injections every 7 d of either the 161-pAb or the control 162-pAb to mice in the two groups. At the end of the experiment (day 36), the 161-pAb inhibited tumor progression by 37\% (\(p < 0.001\)) relative to the negative control group (Fig. 3C). Moreover, the control group developed about 2-folds more metastatic foci than the treated group (means of 60 and 33 foci, respectively, \(p < 0.005\), relative to the co-cultures).

### Mechanisms of 161-Ab activity

We next evaluated the effects of the administration of 161-Ab on different aspects of tumor progression. Of note, since the lower dose of 161-Ab markedly inhibited RENCA tumor growth, no material was available for evaluation, and therefore, we used tumors harvested after treatment with the 50 \(\mu\)g dose. To demonstrate that the 161-Ab inhibited the pro-angiogenic activity of EMMPRIN, we looked at the blood vessel densities, measured by the staining of tumor tissues with anti-CD31 directed against the endothelial cell marker. The blood vessels in the control sections (162-Ab or saline) were long, continuous and dense (Fig. 4A). In contrast, blood vessels in tumors treated with the 161-Ab were short and discontinuous, with bigger gaps between them. This was clearly reflected in the significant reduction of the vessel surface area by about 2.5-folds (\(p < 0.0001\)) in both RENCA and CT26 tumors. Furthermore, the concentrations of the pro-angiogenic factors VEGF and MMP-9 were also altered by the antibody in a dose-dependent manner (Fig. 4B). The use of 50 \(\mu\)g 161-Ab in RENCA tumors or 25 \(\mu\)g 161-Ab in CT26 tumors inhibited MMP-9 production by 4.5 (\(p < 0.05\)) and 4-folds (\(p < 0.01\)), and VEGF production was inhibited by 6- and 5-folds (\(p < 0.05\)), respectively.

To estimate the effects of the 161-Ab on the rate of proliferation of the tumor cells, we evaluated the proliferation index by...
Ki-67 staining in the tumor sections (Fig. 5A). Control sections demonstrated high proliferation rate that was reduced by 3.6-folds for the RENCA tumors \((p = 0.0011)\), and by 3.25-folds for the CT26 tumors \((p = 0.0042)\) by the 161-Ab. In contrast, the number of apoptotic cells (Fig. 5B) was increased by 161-Ab treatment by 46-folds for the RENCA tumors \((p = 0.0019)\) and by 3.5-folds \((p = 0.0005)\). The ability of the 161-Ab to increase apoptotic death was further corroborated by the determination of the concentration of activated caspase 3 in the tumor lysates (Fig. 5C). In comparison to the control groups, activated caspases 3 was elevated by 8.8-folds \((p = 0.0005)\), and by 3.25-folds \((p = 0.0011)\) relative to all groups in the CT26 tumors \((n = 5\) in each group). (C) 4T1 tumor cells \(\left(5 \times 10^5\right)\) were orthotopically injected into the mammary fat pad. When tumors became well established at day 14, mice were assigned to one of the two groups and received an i.p. injection of the 161-pAb \((n = 6)\) or the control 162-Ab \((n = 5)\) at the concentration indicated, and additional two boost injections every 7 d (black arrows). Tumor volumes were monitored as before. *: \(p < 0.05\) and **: \(p < 0.001\). At day 36, mice were sacrificed and (D) the number of metastatic foci in their lungs was counted.

Changes in the cytokine microenvironment may suggest that in vivo tumor-associated macrophages (TAMs) could shift toward M1-activation in order to mediate tumor cell death. These changes were indeed demonstrated (Fig. 6C). In untreated RENCA and CT26 tumors, the pro-inflammatory cytokines TNFα and IL-1β were found in small, almost negligible amounts in the tumor lysates. However, after administration of 50 μg of 161-Ab in RENCA tumors, these cytokines were increased to 21.6 pg/mL/μg \((p < 0.05)\), and 2.3 pg/mL/μg protein \((p < 0.001)\), respectively. Similarly, administration of 25 μg of the 161-Ab in the CT26 tumors increased the pro-inflammatory cytokines by 4- and 11-folds \((p < 0.05)\), respectively. The anti-inflammatory cytokine IL-10 also exhibited negligible amounts in untreated tumors in both RENCA and CT26 tumors, and addition of 50 μg or 25 μg of the 161-Ab resulted in 160-folds and 15-folds increase in IL-10 \((p < 0.01)\).
for the RENCA and CT26 tumors, respectively. The concentrations of the anti-inflammatory cytokine TGFβ in the tumor lysates reflected a mirror image to that described above for the pro-inflammatory cytokines. In control tumors, injected with the irrelevant 162-Ab or with saline, TGFβ concentrations were higher than those of the pro-inflammatory cytokines by at least two orders of magnitude. Administration of 50 μg or 25 μg of 161-Ab reduced those levels by about 11-folds (p < 0.01), in RENCA and CT26 tumors, respectively.

**Discussion**

We show here a novel, epitope-specific antibody directed against EMMPRIN, that inhibited secretion of the pro-angiogenic factors MMP-9 and VEGF in four in vitro co-culture systems of mouse and human tumor cells and macrophages, and that significantly reduced tumor size and its progression in two in vivo mouse model systems. This was achieved by inhibiting angiogenesis, attenuating tumor cell proliferation while enhancing their apoptosis, and by altering the microenvironment, so that more macrophages infiltrated the tumor and were then encountered by a relatively less immunosuppressive microenvironment, allowing them to become tumoricidal.

Sequences similar or adjacent to our epitope have been mentioned before in other studies as important for the ability of EMMPRIN to induce MMPs. In one study, addition of a peptide with the sequence SLNDSATEVTGHRWLK to the co-culture of human fibroblasts and cervical carcinoma SKG-II cells, interfered in a dose-dependent manner with the secretion of MMP-1 and caused up to 60% inhibition. The same, but slightly more elongated peptide (with the sequence SLNDSATEVTGHRWLVGGVV) was used in another study, and inhibited EMMPRIN-mediated production of MMP-2 in a dose-dependent manner by up to 80% in a co-culture of human fibroblasts and cervical carcinoma SKG-II cells.
fibroblasts and different tumor cell lines.\textsuperscript{13} Both studies included part, or almost all of our epitope, and showed that the sequences chosen could inhibit MMP induction, possibly through interference with the homophilic EMMPRIN interactions. However, when the peptide was truncated into two fragments (peptides with the sequences SLND$\text{S}$ATEVT and GHRW$L$KG$G$VV, the latter including almost all of our epitope), no inhibition was detected, even when high concentrations of the peptides were used.\textsuperscript{13} Thus, the entire sequence of 16 amino acids could be considered crucial for the activity of MMP induction. In support of this conclusion is the study that elicited a novel anti-EMMPRIN antibody directed against the epitope TCSLND$\text{S}$ATEVTGHRW (including only four amino acids of our epitope), which inhibited T cell proliferation and reduced MMP-9 production, reduced T cell cytotoxicity to neurons and successfully reduced the clinical score in an EAE mouse model.\textsuperscript{19} However, in contrast to these findings, another study found that the sequence AAGTVFTTEDLGSKILLTCSLND$\text{S}$ATEV (positions 22–50 in the human EMMPRIN sequence), which does not include our epitope at all, was responsible for the EMMPRIN MMP induction activity and association with tumor invasion.\textsuperscript{20} We suggest that because we used an antibody to target a specific epitope of 11 amino acids, we could use a shorter sequence than the two previous studies that used peptides to directly generate a steric hindrance or to compete with the homophilic binding to EMMPRIN. More importantly, all of these epitope-mapping studies focused only on the MMP-induction activity of EMMPRIN, and showed that it might span over a stretch of about 40 amino acids, but no data so far revealed the epitope responsible for the VEGF-induction activity of the protein. Here we show for the first time, that our epitope is responsible not only for the MMP-induction activity of EMMPRIN, but also for its ability to induce VEGF. The ability to induce both of these proteins at the same time, using the same epitope, suggests a common signaling pathway that is evoked and affects both proteins, but this remains to be further studied.

Figure 5. 161-Ab reduces proliferation but increases apoptotic death. RENCA and CT26 tumor sections were prepared and analyzed for proliferation (Ki-67) and apoptosis (TUNEL). (A) Representative images of immunohistochemical staining for Ki-67 (Bar size for RENCA tumors is 100 μm and for CT26 tumor is 25 μm), and Determination of the Ki-67 positive fraction of cells (n = 4); (B) Representative images of immunohistochemical staining for apoptosis (TUNEL, bar size for RENCA tumors is 50 μm and for CT26 tumor is 25 μm), and quantification of the number of apoptotic cells per mm² (n = 4-5); (C) Determination of the concentration of activated caspase 3 in tumor lysates by ELISA (n = 4–5).
Specificity of the antibody was demonstrated by the binding of 161-Ab to the peptide and to the recombinant EMMPRIN protein in protein gel blot analysis and in direct ELISA in vitro, as well as by the ability to recognize EMMPRIN in tumor sections, whereas the pre-immune serum showed no binding activity. Of note, differences in the binding intensity between the non-purified 161-pAb serum and the purified monoclonal commercial antibody to the denatured EMMPRIN protein in the protein gel blots could be explained by the different concentrations of the relative antibodies. In contrast, the opposite response observed in the staining of the tissue sections, where the 161-pAb more strongly stained tumor tissues may suggest that in the tumoral context, 161-pAb better recognizes an exposed epitope. We have previously demonstrated that targeting EMMPRIN in a human in vitro co-culture system using a commercial antibody or siRNA, inhibited the secretion of both MMP-9 and VEGF, and placed EMMPRIN as a key regulator of the interactions between tumor cells and macrophages that promote tumor angiogenesis and invasiveness. Based on these results, we show here similar data in two mouse in vitro co-culture systems. First, MMP-9 and VEGF secretion are maximal when tumor cells and macrophages are co-cultured, even without any additional stimulus. This allowed us to use these co-culture systems as screening platforms to identify the ability of the 161-Ab to inhibit MMP-9 and VEGF secretion. Indeed, 161-Ab, but not its pre-immune serum or the 162-Ab, could specifically inhibit both mouse and human MMP-9 and VEGF secretion, suggesting that the 161-Ab has cross-reactivity with the human EMMPRIN.

In the mouse in vitro co-culture systems, the antibody demonstrated hormetic dose-response manifested as a U-shaped curve for the inhibition of MMP-9 and VEGF secretion. Similarly, in the in vivo setting the administration of the antibody was most effective in the lower dose, rather than the higher dose, also suggesting a U-shape curve. Although the mechanisms underlying hormesis are currently unknown, this dose-response is often characteristic of anti-angiogenic agents, such as different VEGF pathway inhibitors, RGD-mimetic integrin

Figure 6. 161-Ab increases macrophage infiltration, mediates tumor cell cytotoxicity, and changes tumor microenvironment. (A) Representative images of immunohistochemical staining for F4/80 in RENCA and CT26 tumor sections. Bar size for RENCA and CT26 tumors is 50 μm. Number of macrophages per mm² was quantified (n = 4–5). (B) CT26 cells (5 x 10⁶ cells) were labeled with Cell Tracker, incubated in vitro with 1.28 ng/mL of the 161-Ab for 6 h, in the presence of complement (diluted 1:50), RAW 264.7 cells at a ratio of 2:1, or both. Percent cytotoxicity were determined as described in the methods (n = 4). (C) Cytokine concentration in tumor lysates were determined by ELISA, and normalized to the amount of total protein in each sample (n = 4–5).
inhibitors, endostatin and ATN-161 (reviewed in21). One possible explanation, at least for the in vivo setting, may be the development of neutralizing anti-idiotypic antibodies that may have resulted in downregulation of the 161-Ab effects at the higher concentrations used. However, this phenomenon should be further investigated.

We show that the 161-pAb works effectively to reduce tumor size both in subcutaneous and orthotopic models, even when used on well-established tumors, and also to attenuate development of metastases in the orthotopic model, thereby increasing its attractiveness as a potential therapy and opening a wide window of opportunity to use it. Monoclonal antibodies gain increasing importance as novel therapeutic tools against cancerous diseases. However, their therapeutic mode of action remains unclear. Depending on their antigen specificity, antibodies can directly induce apoptosis and inhibit proliferation by neutralizing growth factors or blocking their receptors (e.g. by anti-VEGF, anti-EGFR). Indirectly, antibodies can induce complement-dependent cytotoxicity (CDC) through classical activation of the complement, or ADCC, which is based on the expression of different Fcy receptors on NK cells and macrophages.22 In fact, different studies that involved macrophage depletion or use of different FcyR deficient mice demonstrated that infiltrating monocytes and macrophages are the important cell population mediating antibody-dependent tumor cell killing (reviewed in23). Here, we show that our 161-Ab may act in several ways to reduce tumor progression. First, 161-Ab directly targets EMMPRIN, and so reduces angiogenesis. EMMPRIN is pro-angiogenic, as it regulates tumor cell–macrophage interactions, and induces the expression and secretion of MMPs, including MMP-9, and VEGF from macrophages. Here, using the effective low dose of the 161-Ab, we show that angiogenesis, manifested by mean vessel density (MVD) is markedly reduced, as is the tumor concentrations of MMP-9 and VEGF. Secondly, we show that proliferation is reduced while apoptosis is increased. Apoptotic death can be the direct result of disruption of EMMPRIN interactions with MCT-1 or MCT-4, which leads to accumulation of lactate in the tumor cells and increased acidosis. Additionally, it could be indirectly increased by immune-mediated cell death. In fact, we show an increase in macrophage infiltration into the tumors. We demonstrate in vitro that presence of macrophages, complement and the 161-Ab increase tumor cell killing, suggesting that though some degree of CDC may be mediated by the 161-Ab, a bigger effect might be due to the presence of macrophages with their different FcyRs that enhance ADCC. Additionally, although NK cells are usually associated with ADCC, we could not find increased infiltration of these cells upon 161-Ab administration (data not shown). Last, it has been reported that binding of antibodies to TAMs can shift their activation toward cytotoxic M1-activated macrophages (reviewed in24). Here, we find evidence to support this premise, as the administration of 161-Ab shifts the TAMs toward M1-activation and change the tumor microenvironment. This may be due to a direct effect of the antibody that targets EMMPRIN and disrupts the tumor cell–macrophage interaction that leads to M2-activation of the macrophages, or to indirect effects that rely on the macrophages FcyR expression that upon engagement trigger M1-activation of the macrophages, and this phenomenon should be further investigated. Pro-inflammatory cytokines like TNFα and IL-1β are increased in a reverse dose-dependent manner, while the anti-inflammatory cytokine TGFβ is markedly reduced, in agreement with the hormetic effects of the antibody. In addition, the concentrations of nitrates are increased in the treated tumor lysates. We have shown before that RENCA tumor cells cannot express iNOS or produce NO due to elevated levels of microRNA-146a,25 and similar findings in CT26 tumor cells are now also available in our lab (unpublished data). Thus, most NO, if not all, are the product of infiltrating macrophages, and as NO production is the hallmark of M1-activated macrophages, we interpret these data to point to an in vivo shift in macrophage activation. Furthermore, the relative concentrations of the cytokines in the tumor lysates indicate that the dominant immunosuppressive cytokine is TGFβ, rather than IL-10, and that excess over the pro-inflammatory cytokines in the absence of the 161-Ab, dictates an immunosuppressive microenvironment. However, once the 161-Ab is administered, the microenvironment becomes much less immunosuppressive and more pro-inflammatory, allowing macrophages to successfully mediate tumor cell cytotoxicity.

In conclusion, we believe that EMMPRIN represents a good target for cancer therapy, as its expression is elevated on tumor cells in many types of cancerous diseases. Furthermore, it is an attractive target, because of its critical functions in tumor cell metabolism and proliferation, as well as its pro-angiogenic and pro-metastatic properties. Indeed, several attempts have already been made to inhibit EMMPRIN expression in tumors. these were achieved by either transfecting cell lines with EMMPRIN siRNA in vitro,26 or by using monoclonal antibodies that were raised against the entire extracellular fragment of EMMPRIN both in vitro27 and in vivo.28,29 All of these strategies resulted in decreased MMPs secretion, as well as in inhibition of tumor invasiveness. In fact, the F(ab’)2 fragment of an anti-EMMPRIN monoclonal antibody called Licartin or Metuximab, is now being used to delay recurrence of hepatoma after transplantation in human patients.30 Our antibody, which must now be further developed into a monoclonal antibody, joins these previous strategies for cancer immunotherapy, and has demonstrated its effectiveness in inhibiting angiogenesis, proliferation, apoptosis and alteration of the inflammatory tumoral microenvironment, all of which cumulatively inhibit tumor growth, in line with the central role of EMMPRIN in tumor progression.

Methods

Cells

The human renal carcinoma A498 (ATCC HTB-44), breast carcinoma MCF-7 (ATCC HTB-22) and U937 monocyte-like cells (ATCC CRL-1593) were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS), 1% L-glutamine and antibiotics. The tumorigenic mouse RENCA renal carcinoma (kind gift of Dr. Bernhard Hemmerlein, Georg-August University Hospital, Göttingen, Germany), and the CT26 colon carcinoma (ATCC CRL-2638) were cultured in RPMI-1640 medium, 10% FCS, 1% L-Glutamine and antibiotics, with addition of 100 mM HEPES buffer (pH 7.4) for the RENCA cells, and 1%
sodium pyruvate for the CT26 cells. The RAW 264.7 macrophage-like (ATCC TIB-71) cell line and the mouse TRAMP-C2 prostate cancer cell line (ATCC CRL-2731) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), 1% L-glutamine and antibiotics, with addition of 1% sodium pyruvate for the RAW 264.7 cells and 5 μg/mL Insulin and 10^-8 mol/L Dihydrotestosteron (Perkin-Elmer, Waltham, MA) for the TRAMP-C2 cells. Tumor cells and macrophages were co-cultured using the tumor cell line medium. To avoid possible masking of signals by exogenous stimulii, tumor cells (10^6 cells) were plated in 24-well plates in a serum-free medium for 48 h, alone in or co-culture with macrophages at a 2:1 ratio. Cell viability was determined using the XTT kit (Biological industries, Beit-Haemek, Israel). All cell lines were regularly tested for morphological changes and presence of mycoplasma. RAW 264.7 cells were identified as macrophages by their ability to phagocytose zymosan particles, and tumor cells were tested as cells of epithelial origin by their cytokeratin 18 expression.

**ELISA**

The mouse MMP-9 and VEGF concentrations were determined using ELISA kits according to the manufacturer’s instructions (R&D systems, Minneapolis, MN), and the supernatant samples were diluted 1:100 according to previous calibration experiments. The concentrations of TNFα, IL-1β, IL-10 and TGFβ in tumor lysates were determined using ELISA kits (R&D systems), and normalized per 1 μg of total protein that was determined by Bradford reagent.

**Peptide conjugation, immunization and affinity purification of polyclonal antibodies**

We identified an 11 amino acid peptide in the mouse EMMPRIN protein that exhibited high homology between the human and the mouse sequences, and showed no homology to other mouse proteins, including neuroplastin and embigin which belong to the same family of proteins. This sequence is located in the extracellular domain 1 (EC-I) of the protein (GHRWMRRGGKVL, position 52–63, Fig. 1). As a control peptide we chose a different 10-amino acid sequence, located in the extracellular domain 2 (EC-II) of the protein (position 136–145 TDWFVKFSTSD). The generation of the polyclonal antibody was carried out by Adar Biotech, LTD (Rehovot, Israel). Briefly, the company synthesized the requested peptides and added a cysteine residue to each peptide to facilitate its binding, conjugated them to the Keyhole Limpet Hemocyanin (KLH) carrier protein, immunized two rabbits with each peptide and performed bleeding before (pre-immune sera) and after immunization following additional boost vaccinations. To establish an immune response, the company performed an ELISA test, where the titer of the antibodies was determined. The sera were collected after the final boost injection and were sent to us, or were further purified by affinity chromatography. The **in vitro** assays determining specificity of the antibodies were carried out using the crude sera, whereas the **in vivo** experiments determining the effects of the antibodies were carried out with the purified antibodies.

**Protein gel blot**

Mouse recombinant EMMPRIN (200 ng/ lane, R&D systems) was loaded onto a 10% SDS-PAGE, electrophoretically separated and transferred onto a nitrocellulose membrane. The membrane was cut into strips and each strip was blocked with 20% skimmed milk and 1% BSA in TBST (0.1% Tween-20, 10mM Tris pH 8.0, 150mM NaCl) at room temperature overnight, and then probed for 1 h with immune or pre-immune serum (both diluted 1:1000), washed and incubated with 1:5,000 diluted HRP-conjugated donkey anti-rabbit IgG (711–035–152, Jackson ImmunoReserch Laboratories, West Grove, PA). One strip was probed with the 1:1,000 diluted commercial rat anti-mouse EMMPRIN (MAB772, R&D systems) and then with the 1:5,000 diluted HRP-conjugated goat anti-rat IgG (112–035–062, Jackson) and served as positive control (P.C). The enhanced chemiluminescence (ECL) system (Biological industries) was used for detection.

**Direct ELISA**

To determine if our polyclonal antibodies specifically recognize EMMPRIN in its native form, we developed a direct ELISA assay. The wells in a 96-well plate were coated with the mouse recombinant EMMPRIN (200 ng/mL, R&D systems) overnight at 4°C. The plate was washed three times with wash buffer (0.05% Tween-20 in PBS), and blocked with blocking buffer (1% BSA in PBS) for 2 h at room temperature. Strips in the plate were incubated with 100 μL of the primary antibodies (immune or pre-immune sera) diluted 1:1,000 for 2 h at room temperature, and then washed four times and incubated with 100 μL of the biotinylated donkey anti-rabbit (BAF109, R&D systems) secondary antibody diluted 1:10,000. After three more washes the streptavidin-HRP was diluted 1:200 and added for 1 h. After three additional washes, the TMB solution was added for 5 min, and the reaction was stopped with 100 μL of stop solution. The absorbance of each well was measured at 450 nm and 540 nm. The assay was repeated four times in duplicates.

**In vivo mouse model**

BALB/c mice (female, 8 wk old) were purchased from Harlan Laboratories (Jerusalem, Israel), and were kept with a 12 h light/dark cycle and access to food and water *ad libitum*. Mice were cared for in accordance with the procedures approved by the Supervision of Animal Experiments Committee at the Technion, and outlined in the NIH Guideline for the Care and Use of laboratory Animals. Tumors were generated by subcutaneously injecting 2 × 10^6 RENCA or CT26 cells suspended in matrigel in a total volume of 200 μL into the flank of BALB/c mice. After tumors became palpable (around day 12), mice were randomly assigned to five groups: the control groups were i.p. injected three times with 100 μL saline or an irrelevant antibody (162-Ab, another polyclonal anti-EMMPRIN antibody that had no effect during the screening process), and the other groups were i.p. injected with different concentrations of the 161-Ab (25, 50 and 100 μg per 25 gr body weight) in a volume of 2 mL, in 3 boosts, every 7 d. Similarly, 5 × 10^4 T1 cells were injected directly to the mammary fat pad, and a single
dose (20 μg per 25 g body weight) of the 161-pAb or the 162-pAb negative control were injected i.p. three times as described above. Tumors were measured every 3–4 d and their volume was calculated for each mouse (length × width × 0.5 cm³). At the end of the experiment or when tumors were greater than 1.0 cm³, mice were euthanized. Parts of the tumor were freshly frozen for evaluation of cytokine concentrations, while other parts were fixed in 4% neutrally buffered formalin and embedded in paraffin for immunohistochemical staining.

**Immunohistochemistry**

Four μm thick paraffin embedded tissue sections of CT26 subcutaneous tumors were mounted on a glass slide and deparaffinized with xylene substitute K-Clear Plus (Kaltex, Padova, Italy) and rehydrated with decreasing ethanol immersions. Antigen retrieval for EMMPRIN was carried out by microwave heating for 15 min in Tris-EDTA buffer pH 9.0, for Ki-67 and F4/80 by microwave heating in citrate buffer pH 6.0, for CD31 by immersing the slides in 42 mg/mL Proteinase XXIV solution (Sigma) for 10 min at 37°C, or in 20 mg/mL of Proteinase K in Tris buffer, pH 7.4–8.0 for the TUNEL kit. Endogenous peroxidase was quenched in 3% H₂O₂ solution for 10 min, then the slides were blocked with 5% BSA and incubated overnight at 4°C with the following primary antibodies: the 161-Ab, the rabbit monoclonal anti-CD147 (ab108317, Abcam, Cambridge, United Kingdom) diluted 1:400, rat monoclonal anti-CD31 (BM4086, Acris Antibodies, Herford, Germany) diluted 1:50, rabbit monoclonal anti-Ki67 (ab16667, Abcam) diluted 1:140, rat monoclonal anti-F4/80 (ab6640, Abcam) diluted 1:200. After washing, the antibodies were detected with HRP-Polymer anti-rabbit (ZUC032–006, Zytomed, Berlin, Germany) or with the N-Histofine Simple Stain Mouse MAX PO (Rat) (414311F, Nichirei Bioscience, Tokyo, Japan) for 1 h and the DAB substrate Kit (Zytomed). All sections were counterstained with hematoxylin (Sigma) and coverslips were applied using Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden). TUNEL staining was performed using the *in situ* death detection kit POD (Roche Life Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. All sections were viewed under the Olympus BX-60 bright field trinocular microscope equipped with a Sony DXC-950P digital camera. Images were acquired using the GrabBee X video grabber (VideoHome Technology Corp., Taipei, Taiwan). Vessel densities were acquired using the GrabBee X video grabber (VideoHome Technology Corp., Taipei, Taiwan). Vessel densities were calculated for each mouse (length × width × 0.5 cm³). At the end of the experiment or when tumors were greater than 1.0 cm³, mice were euthanized. Parts of the tumor were freshly frozen for evaluation of cytokine concentrations, while other parts were fixed in 4% neutrally buffered formalin and embedded in paraffin for immunohistochemical staining.

**In vitro cytotoxicity assay**

Target cells (5 × 10⁴ CT26 cells) were labeled with 5 μM of Cell Tracker orange™ (Molecular Probes, Invitrogen) and incubated for 6 h with 1.28 μg/mL of the purified 161-Ab, with addition of RAW 264.7 cells (2.5 × 10⁴ cells) stimulated for 24 h with IFNγ (100 U/mL, R&D systems) and LPS (1 μg/mL, Sigma), or with mouse complement diluted 1:50 (MP biomedicals, Solon, OH), or with both. Release of the fluorescent stain to the supernatants indicated cell death. Percent cytotoxicity of target cells was calculated by the formula:

\[
\text{Cytotoxicity(\%) = \frac{\text{RFU} - \text{Spontaneous RFU release}}{\text{Maximal RFU} - \text{Spontaneous RFU release}} \times 100}
\]

where RFU are the relative fluorescent units, spontaneous release (0% cytotoxicity) was measured from CT26 cells incubated alone, and maximal RFU (100% cytotoxicity) was measured from CT26 cells incubated with 5% Triton X-100.

**Statistical analyses**

All values are presented as means±SE. Significance between two groups was determined using the two-tailed unpaired t test. Differences between three or more experimental groups were analyzed using analysis of variance (ANOVA) and the post-hoc Bonferroni’s multiple comparison tests. p values exceeding 0.05 were not considered significant.

**Disclosure of potential conflicts of interest**

E.S. and V.B. have declared that no conflict of interest exists. M.W., N.L., H.B. and M.A.R. are the inventors of a pending patent application related to the research described in the manuscript.

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