Beneficial effects of sunitinib on tumor microenvironment and immunotherapy targeting death receptor5

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ABSTRACT
Tumor-associated blood vessels and lymphatics are abnormal and dysfunctional. These are hallmarks of the tumor microenvironment, which has an immunosuppressive nature, such as through hypoxia. Treatment with anti-death receptor5 (DR5) monoclonal antibody MD5-1, which induces tumor cell death, is a potent anti-tumor immunotherapy. Generally, MD5-1 induces cell death mainly via antigen presenting cells (APCs) and generates tumor-specific effector T cells. To date, the effects of a simultaneous functional improvement of abnormal blood vessels and lymphatics on the immune microenvironment are largely unknown. A combination therapy using sunitinib, vascular endothelial growth factor (VEGF) and platelet-derived growth factor receptor inhibitor, and MD5-1 substantially inhibited tumor growth. Sunitinib improved pericyte coverage on endothelial cells and the expression levels of regulator of G-protein signaling 5, suggesting blood vessel normalization. Sunitinib also increased lymph flow from tumors to central lymph nodes, suggesting improved lymphatic function. In concordance with improved vasculature functions, sunitinib alleviated the tumor hypoxia, suggesting an improved tumor microenvironment. Indeed, the combination therapy induced strong activation of CD8+ T cells and dendritic cells in draining lymph nodes. The combination therapy reduced the ratio of immune-suppressive T regulatory cells in the tumors and draining lymph nodes. The combination therapy enhanced the numbers and activation of tumor-infiltrating CD8+ T cells. CD4 and/or CD8 depletion, or APC inhibiting experiments showed the contribution of CD8+ T cells and APCs to the combination therapy. These findings suggest that targeting blood vessels and lymphatics may have potential benefits for immunotherapy mediated by CD8+ T cells and APCs.

Introduction
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the tumor necrosis factor family. Death receptor5 (DR5) is a TRAIL receptor. Its agonistic monoclonal antibody (mAb) MD5-1 exerted potent anti-tumor effects in mice, and DR5 targeting mAbs is now under clinical trials. MD5-1 induces apoptosis in TRAIL-sensitive tumor cells when crosslinked. For crosslinking, Fc receptor-expressing immune cells, including natural killer (NK) cells, macrophages, and dendritic cells (DCs), are required. APCs, including macrophages and DCs, play a central role in the crosslinking of MD5-1. After the induction of apoptosis, APCs such as DCs incorporate apoptotic cells coated by MD5-1. These APCs migrate to draining lymph nodes (LNs) via lymphatics. In LNs, APCs develop tumor-specific cytotoxic T lymphocytes. Thus, in addition to inducing apoptosis in tumor cells, MD5-1 induces tumor-specific effector and memory T cells. The anti-tumor effects of MD5-1 were largely APC and T cell dependent. To promptly induce tumor-specific cytotoxic T lymphocytes, the activation of APCs is necessary. The activated DCs enhance the expression of co-stimulatory molecules such as CD80 and CD86. Activated T cells enhance the expression of CD69.

Tumor blood vessels are structurally and functionally abnormal. The vessels are variable in size, irregularly shaped, and tortuous. Pericytes on tumor vessels have abnormal shapes and are loosely attached to endothelial cells. These abnormalities result in the tumor microenvironment, such as hypoxia, which has an immunosuppressive nature. Anti-angiogenic therapies induce blood vessel normalization and improve the pericyte coverage on endothelial cells and the irregular shape of blood vessels.

Lymphatics form a one-way conduit for tissue fluid drainage and leukocyte migration. Among lymphangiogenic factors, signaling via vascular endothelial growth factor-C/D
(VEGF-C/D) and VEGF receptor-3 (VEGFR-3) is the central pathway.\textsuperscript{17,18} Lymphatics in tumors were suggested to be dysfunctional.\textsuperscript{19} For normalization of the tumor microenvironment, it has been suggested that normalization of the blood vessels is insufficient and normalization of other factors such as lymphatics is necessary.\textsuperscript{19}

Lymphatic drainage has been mapped using dyes such as Evans blue in rodents.\textsuperscript{20} The mapping showed that lymph in peripheral tissues such as footpads flows to popliteal LNs. The lymph in popliteal LN drains centrally to iliac LNs,\textsuperscript{21,22} and finally into blood in the lymphovenous junction at the subclavian vein.\textsuperscript{23} The presence of tumors in the footpads increased the lymph flow from the footpad to popliteal LNs compared to footpads without tumors.\textsuperscript{24} As for the role of lymphatics in leukocyte migration, peripherally activated DCs migrate to the draining LNs via the lymphatics.\textsuperscript{25}

Suppressive mechanisms in the immune response induce tolerance and allow tumor growth. CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3\textsuperscript{+} regulatory T cells (Tregs) are an important population of immune suppressors.\textsuperscript{4} Tumor hypoxia promotes immune tolerance by Tregs.\textsuperscript{26} The immunosuppressive tumor microenvironment is suggested to be converted to an immunosupportive one by the alleviation of hypoxia.\textsuperscript{10,19} Sunitinib inhibits receptor tyrosine kinases including VEGFRs, platelet-derived growth factor (PDGF) receptors, c-Kit, and macrophage colony-stimulating factor receptor. Sunitinib therapy reduced the numbers or percentages of immunosuppressive cells in the tumors, LNs, and peripheral blood.\textsuperscript{27–30} One study reported that sunitinib therapy reduced lymphatic endothelial growth and lymph node metastasis,\textsuperscript{31} whereas other studies reported comparable\textsuperscript{32} or greater lymph node metastasis, possibly by inducing VEGF-C in tumors.\textsuperscript{33,34}

Recent studies have suggested that vascular normalization by anti-angiogenic therapies may enhance the effects of cancer immunotherapies.\textsuperscript{11,12} Cancer immunotherapy by IL-12 and granulocyte macrophage colony-stimulating factor, by adaptively transferred T cells combined with anti-angiogenic therapy, or IL-2 and vascular targeting anti-CD40 antibody exerted better therapeutical effects on tumors.\textsuperscript{35–37} Very recent studies suggested the beneficial effects of combining anti-angiogenic therapy with immunotherapy mediated by immune checkpoint blockade.\textsuperscript{38–40} As for lymphatics, their immunomodulatory roles in cancer immunotherapy have been suggested.\textsuperscript{41} One study suggested that tumor-associated lymphatics and VEGF-C can impair anti-tumor T cell responses,\textsuperscript{42} whereas another study suggested the favorable effects of VEGF-C on cancer immunotherapy.\textsuperscript{43} Overall, simultaneous analysis of blood vessels and lymphatics after anti-vascular therapy is rare. Moreover, the anti-tumor effects of combining anti-vascular therapy with immunotherapy mediated by both T cells and APCs are largely unknown.

In this study, we employed sunitinib as an anti-vascular therapy and MD5-1 as an immunotherapy. We examined the effects of sunitinib on blood vessels, lymphatics and the tumor microenvironment using a footpad tumor model, which has a clear route and sequence of lymph flow from tumors to central LNs. We evaluated the favorable effects of the improved functions of the tumor-associated vasculature and tumor microenvironment on anti-tumor immunity. Since MD5-1 exerts its effects primarily via effector T cells and APCs, we were able to analyze the effects of anti-vasculature therapy not only on T cells but also on APCs in this study.

**Results**

**Effects of sunitinib and MD5-1 combination therapy on tumor growth**

We inoculated 4T1 or CT26 tumor cells into the hind flank of mice to observe the tumor growth rate. These tumor cells express DR5 and are sensitive to MD5-1 therapy.\textsuperscript{3} Sunitinib or MD5-1 monotherapy moderately impaired the growth of 4T1 mammary carcinoma tumors in the hind flank compared to the control on day 21 (Figure 1a). The combination of sunitinib and MD5-1 therapies exhibited greater tumor growth inhibition as compared to either monotherapy alone (Figure 1a). We observed similar effects of the combination therapy on the growth of CT26 colon

![Figure 1a](image1.png)  
**4T1**

![Figure 1b](image2.png)  
**CT26**

**Mice were subcutaneously inoculated with 4T1 cells (a) or CT26 cells (b) on day 0 and treated with sunitinib and/or MD5-1. Sunitinib or MD5-1 monotherapy moderately impaired the tumor growth when compared to controls. Sunitinib and MD5-1 combination therapy substantially inhibited tumor growth when compared to each monotherapy. n = 6–10 in each group, \( p < 0.05 \) vs control, \( p < 0.05 \) vs MD5-1 monotherapy, \( p < 0.05 \) vs sunitinib monotherapy.**

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carcinoma tumors (Figure 1b). These results suggest the potential benefits of combining MD5-1 therapy with sunitinib therapy.

Effects of sunitinib monotherapy on blood vessel normalization in footpad tumors

We speculated that blood vessel normalization and improved functions of the tumor-associated lymphatics by sunitinib therapy would exert beneficial effects in combination with MD5-1 therapy. We hypothesized that the tumor associated vasculature as conduits of cell trafficking and tissue liquid flow between tumors and draining LNs, and its function would be improved by sunitinib. Footpad tumors have a clear hierarchy of lymphatic drainage to their draining popliteal LNs and to further central iliac LNs. To precisely analyze the circulation between tumors and their draining LNs, we subcutaneously (s.c.) inoculated 4T1 cells into footpads. First, we analyzed blood vessels by immunohistochemistry for endothelial cell marker CD31 and pericyte marker α-smooth muscle actin (α-SMA). The immunohistochemistry of control tumors showed that the blood vessels had a tortuous structure with a loose attachment of pericytes to endothelial cells (Figure 2a-b). Sunitinib monotherapy reduced the CD31 and α-SMA area densities in the tumors (Figure 2c-d). The extensive pericyte coverage on blood endothelial cells by sunitinib monotherapy suggested normalization of the blood vessels (Figure 2e). To further confirm this result, we measured the expression levels of regulator of G-protein signaling 5 (RGS5), the loss of which results in tumor blood vessel normalization. Sunitinib monotherapy reduced the expression levels of RGS5 in the tumors compared to controls (Figure 2f). MD5-1 monotherapy did not affect tumor vascularity, pericyte density, pericyte coverage, or the expression levels of RGS-5 (Figure 2a-f). Overall, these results suggest that sunitinib monotherapy normalized the blood vessels in footpad tumors but MD5-1 monotherapy did not.

Effects of sunitinib monotherapy on lymphatic drainage from the tumors to draining and central LNs, and tumor hypoxia

Next, we evaluated the effect of sunitinib on functions of lymphatics by measuring the lymphatic drainage from the tumors to the central LNs. We injected Evans blue into tumors and quantified their amounts in the tumors and the draining popliteal LNs. Five minutes after the injection, draining popliteal LNs from control and sunitinib monotherapy showed comparable amounts of Evans blue (Figure 3a). In the tumors, controls showed a trend toward containing greater amounts of Evans blue than the sunitinib monotherapy group (Figure 3b). Thirty minutes after the injection, both in the popliteal LNs and the tumors, the controls showed greater amounts of Evans blue than the sunitinib monotherapy group (Figure 3c-d). We hypothesized that this was due to improvement in the central lymphatic drainage from popliteal LNs. Indeed, in the iliac LNs, the central LN of popliteal LN (Figure 3e), the amount of Evans blue was greater in the sunitinib monotherapy group than in controls (Figure 3f). To further evaluate the improved lymphatic drainage by sunitinib monotherapy, we labeled tumors for hyaluronan (HA) using biotinylated HA-binding protein (Figure 3g). HA is a ubiquitously distributed extracellular matrix glycosaminoglycan whose turnover occurs through lymphatics by their uptake through lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). Functional lymphatics reduced the amount of HA in tissues. The quantification of HA immunostaining showed that sunitinib monotherapy reduced the amount of HA in tumors compared to controls (Figure 3h). These results suggest sunitinib monotherapy improved the lymphatic drainage not only to draining LNs but also to further central LNs. We morphologically analyzed the lymphatics associated with tumors and in the popliteal LNs by immunostaining for LYVE-1. We could not find clear morphological changes in the lymphatics associated with tumors or inside popliteal LNs between controls and a sunitinib treated group (Supplementary Figure 1a-b). This result suggests that sunitinib monotherapy did not disrupt the lymphatics associated with tumors or in draining LNs. Hematoxylin and eosin staining of popliteal LNs showed that sunitinib monotherapy did not promote lymph node metastasis (Supplementary Figure 1c), whereas it enhanced the expression levels of VEGF-C in tumors (Supplementary Figure 1d). The central cause of immune suppression in tumor microenvironments is hypoxia. Thus, we evaluated the effects of sunitinib monotherapy on the tumor microenvironment by measuring tumor hypoxia. We visualized tumor hypoxia by pimonidazole staining and found that sunitinib monotherapy improved the oxygen supply, whereas MD5-1 monotherapy did not (Figure 3i-j). These findings suggest sunitinib monotherapy normalized blood vessels, improved the lymphatic function, and increased the oxygen supply to tumor microenvironments.

Beneficial effects of sunitinib and MD5-1 combination therapy on tumor infiltration by immune cells

Normalized blood vessels with improved lymphatic drainage and reduced hypoxia may induce a supportive tumor microenvironment for immunotherapy. Indeed, immunohistochemistry for CD8+ T cells in the footpad tumors showed that sunitinib monotherapy, and sunitinib and MD5-1 combination therapy induced the infiltration of greater numbers of effector CD8+ T cells than in controls (Figure 4a and c). Immunohistochemistry for effector CD4+ Foxp3+ T cells showed that sunitinib monotherapy and the combination therapy induced the infiltration of substantial numbers of effector CD4+ Foxp3+ T cells (Figure 4b and d). The numbers of tumor-infiltrating immune-suppressive CD4+ Foxp3+ Tregs were comparable among the treatment groups (Figure 4e). Sunitinib monotherapy and the combination therapy induced a greater effector/regulatory CD4+ T cell ratio in tumors than in controls (Figure 4f). MD5-1 monotherapy induces tumor-specific T cell immunity, but
the anti-tumor effect of MD5-1 on primary tumors is also mediated by APCs such as DCs and macrophages. To confirm the recruitment of APCs into tumors, we examined the DC marker CD11c by immunohistochemistry. DCs infiltrated into the tumor edge in all the groups (Figure 4g). Immunohistochemistry for macrophage marker F4/80 showed substantial numbers of F4/80+ mononuclear cells in the tumor edge (Figure 4h). Collectively, these data suggest that sunitinib monotherapy and the combination therapy enhance the tumor infiltration of effector T cells and, in all the groups, the presence of APCs in the tumor edge.

**Figure 2.** Effects of sunitinib or MD5-1 monotherapy on endothelial cells, pericytes, and RGS5 expression levels in footpad tumors.

(a and b) Images of tumor sections immunostained for endothelial cells (CD31, green) and pericytes (α-SMA, red) at low (a) and high (b) magnification, isolated from control (left panels), sunitinib monotherapy (center panels), or MD5-1 monotherapy (right panels) group. (c and d) Fractional areas of CD31 (c) or α-SMA (d) after sunitinib or MD5-1 monotherapy. (e) Sunitinib monotherapy increased the relative ratio of pericyte-covered blood endothelial cells. (f) Quantitative real-time PCR of RGS5 using mRNA isolated from total tumors from control, sunitinib monotherapy, or MD5-1 monotherapy group. n = 6 in each group, *p < 0.05 vs control. Scale bar: 50 µm in A, 25 µm in B.

**Effects of sunitinib and MD5-1 combination therapy on the activation of immune cells in draining LNs**

Since sunitinib monotherapy and the combination therapy enhanced the tumor infiltration of effector T cells, we speculated that these therapies activated immune cells in draining LNs. The ratio of CD8+ T cells among live cells in draining LNs was comparable between the therapies (Figure 5a). Activated CD8+ T cells express CD69 on their cell surface. MD5-1 monotherapy and the combination therapy induced greater ratios of CD69 expressing CD8+ T cells than controls or sunitinib monotherapy,
suggesting the enhanced activation of CD8$^+$ T cells (Figure 5b). Furthermore, the combination therapy induced a greater ratio of CD8$^+$ T cells that produce IFN-γ than the other therapies (Figure 5c). As for immune-suppressive Tregs, MD5-1 monotherapy and
the combination therapy reduced the ratio of CD4+ Foxp3− T cells compared to controls or sunitinib monotherapy (Figure 5d). Given the major role of APCs in the anti-tumor effect of MD5-1, we assessed CD11c+ DCs in draining popliteal LNs. We examined expression levels of their activation markers CD80 and CD86 on CD11c+ DCs. MD5-1 monotherapy and the

Figure 4. Effects of sunitinib and/or MD5-1 therapy on tumor infiltration of immune cells. (a) Comparison of blood vessels (CD31, green) and CD8+ T cells (CD8, red) in footpad tumors or (b) comparison of effector CD4+ Foxp3− T cells (CD4, green; Foxp3; red) and CD4+ Foxp3+ Tregs in tumors isolated from control (upper left panel), sunitinib monotherapy (upper right panel), MD5-1 monotherapy (lower left panel), or sunitinib and MD5-1 combination therapy (lower right panel) group. (c) Quantification of tumor infiltrating CD8+ T cells. (d and e) Quantification of tumor infiltrating effector CD4+ Foxp3− T cells (d) and immune-suppressive CD4+ Foxp3+ Tregs (e) in tumors isolated from control, sunitinib monotherapy, MD5-1 monotherapy, or sunitinib and MD5-1 combination therapy group. (f) The ratio of effector/regulatory CD4+ T cells treated with sunitinib monotherapy, MD5-1 monotherapy, or sunitinib and MD5-1 combination therapy group. (g) Presence of CD11c+ DCs (CD11c, red) and blood vessels (CD31, green) in tumor edge isolated from tumors treated as indicated. (h) Presence of substantial numbers of F4/80+ mononuclear cells (F4/80, red) and blood vessels (CD31, green) in tumor edge isolated from tumors treated as indicated. n = 6 in each group, *p < 0.05 vs control, ¶ not significantly different from sunitinib monotherapy. Scale bar: 20 µm.
combination therapy substantially increased the ratio of CD80 or CD86 expressing DCs compared to controls (Figure 5e-f). CD80 positive cells were 23.8% in control and 54.3% in the combination therapy group. The expression levels of CD80 and CD86 on DCs were comparable between sunitinib monotherapy and controls (Figure 5e-f). These results suggest that the combination therapy...
and, to lesser extent, MD5-1 monotherapy enhanced the activation of T cells and DCs in draining LNs, whereas sunitinib monotherapy did not.

**Effects of the combination therapy on the infiltration and activation of immune cells in the hind flank tumors**

We next analyzed immune cells in the hind flank tumors. Sunitinib monotherapy and the combination therapy enhanced the tumor infiltration of CD8⁺ T cells compared to controls (Supplementary Figure 2a-b). MD5-1 monotherapy and the combination therapy enhanced the expression levels of CD69 on CD8⁺ T cells compared to controls, suggesting greater activation of CD8⁺ T cells (Supplementary Figure 2c). Sunitinib monotherapy and the combination therapy enhanced the infiltration of effector CD4⁺ Foxp3⁻ T cells compared to controls (Supplementary Figure 2d-e). Sunitinib and the combination therapies reduced the infiltration of immune-suppressive CD4⁺ Foxp3⁺ Tregs into tumors (Supplementary Figure 2f). Sunitinib monotherapy and the combination therapy induced a greater effector CD4⁺/regulatory CD4⁺ T cell ratio than that in controls (Supplementary Figure 2g). These results suggest that the combination therapy enhanced the infiltration of effector T cells, which were greatly activated, in the hind flank tumors.

**Effects of the combination therapy on CT26 tumors**

We analyzed the effects of the therapies in another tumor model, CT26 hind flank tumors. Immunohistochemistry for blood vessels showed that the combination therapy improved the tortuous structure of the vessels and the loose attachment of pericytes (Supplementary Figure 3a-b). The combination therapy reduced the CD31 and α-SMA area densities (Supplementary Figure 3c-d) and improved the pericyte coverage on blood endothelial cells (Supplementary Figure 3e). The combination therapy enhanced the tumor infiltration of CD8⁺ T cells (Supplementary Figure 3f-g).

**Differential contribution of immune cells to the anti-tumor effects of the combination therapy**

The above findings suggest the beneficial effects of the combination therapy on tumor infiltration and the activation levels of effector immune cells. Thus, we further analyzed the role of T cells by depleting CD8⁺ and/or CD4⁺ T cells in the combination therapy. The depletion of CD8⁺, or CD8⁺ and CD4⁺ T cells in the combination therapy reduced the tumor size by 52.3% or 52.5%, respectively (Figure 6a). CD4⁺ T cell depletion did not impair the anti-tumor effect of the combination therapy (Figure 6a). This effect of CD4⁺ T cell depletion was probably caused by depleting both immune-suppressive CD4⁺ Foxp3⁺ and effector CD4⁺ Foxp3⁻ T cells. These results suggest a partial contribution of the CD8⁺ T cells to the anti-tumor effects of the combination therapy. In a previous study, inhibiting the recruitment of APCs by the administration of anti-CD11b mAb abrogated the effects of MD5-1 on primary tumor growth. In the current study, APCs were present in tumors (Figure 4g-h), and the activation levels of DCs in draining LNs were augmented by the combination therapy (Figure 5e-f). To analyze the contribution of APCs to the anti-tumor effects of the combination therapy, we administered anti-CD11b mAb. Anti-CD11b mAb treatment could not abrogate but it impaired the anti-tumor effect of the combination therapy (Figure 6b). The combination therapy reduced the tumor size by 56.3%, whereas the combination therapy with anti-CD11b mAb treatment reduced the tumor size by 31.4% (Figure 6b). This result suggests the substantial contribution of APCs to the anti-tumor effect of the combination therapy. Overall, these observations suggest the involvement of CD8⁺ T cells and APCs in the anti-tumor effects of the combination therapy, and the beneficial effects of sunitinib monotherapy on both types of immune cells.

**Figure 6.** Contribution of T cells and APCs to the anti-tumor effects of the combination therapy. BALB/c mice were inoculated with 4T1 cells and treated with the combination therapy. The mice were then treated with additional anti-CD4 and/or anti-CD8 mAb (a) or anti-CD11b mAb (b). n = 6 in each group, *p < 0.05 vs control, †p < 0.05 vs MD5-1 and sunitinib group.
Discussion

In this study, sunitinib monotherapy normalized the tumor blood vessels, enhanced lymphatic drainage from the tumors to the LNs, and generated better oxygenation in the tumors. These data suggest sunitinib monotherapy improved the tumor microenvironment. In the draining LNs, MD5-1 monotherapy enhanced the activation of T cells and DCs. Thus, each monotherapy had different targets of action and the combination therapy enhanced the tumor infiltration of effector T cells, which were greatly activated. As a result, the combination therapy substantially inhibited tumor growth. Moreover, the administration of anti-CD4/8 or CD11b mAb to the combination therapy suggests that sunitinib therapy is beneficial for immunotherapy involving T cells and APCs.

Our data suggest that sunitinib normalized the blood vessels and improved the lymphatic function. In addition, sunitinib improved tumor hypoxia. Overall, above findings may enhance the intra-tumoral infiltration of immune cells. MD5-1 monotherapy activated immune cells in the draining LNs and tumors. Moreover, previous studies have shown that MD5-1 induces tumor cell death. Improvement in the tumor microenvironment by sunitinib may enhance the above-mentioned immune responses by MD5-1. Overall, under an improved tumor microenvironment, the extensive tumor infiltration of activated immune cells accompanied by the induction of tumor cell death by the combination therapy resulted in substantial anti-tumor effects.

In addition to the sunitinib or MD5-1 monotherapy, we assessed the effects of the combination therapy on tumor blood vessel normalization (data not shown). Immunohistochemistry for CD31 and α-SMA showed that the combination therapy reduced the CD31 and α-SMA area densities in the tumors. The combination therapy induced greater pericyte coverage on blood endothelial cells than in controls. In conclusion, these data suggest that the combination therapy normalized the tumor blood vessels.

In this study, we evaluated the effect of sunitinib on lymphatics by functional analysis using Evans blue drainage and immunoreactivity to HA. However, compared to the commonly applied methods to evaluate blood vessel normalization, this analysis might be insufficient to evaluate the effect of sunitinib on lymphatics. Thus, we tried to identify morphological changes in lymphatics associated with tumors or in tumor draining LNs. We counted the numbers of lymphatic branches in LNs, but we could not obtain clear differences. Consequently, this result suggests the importance of developing a quantitative method for evaluating the morphological changes of lymphatics in tumor studies. Moreover, these results suggest the need for establishing common methods to evaluate tumor associated lymphatics.

The dose of sunitinib in this study was 62% the dose used in other studies as an anti-angiogenic reagent. The moderate dose of sunitinib in this study might be beneficial for blood vessel normalization and improving the lymphatic function rather than disrupting tumor-associated blood vessels and lymphatics. DR5 activation on tumor endothelial cells by the oligomeric form of TRAIL disrupted the vasculature. However, in this study, MD5-1 did not affect the tumor vasculature.

Among immune cells, we analyzed the roles of APCs and T cells in this study. Previous studies have shown that these cells can regulate tumor angiogenesis. Among APCs, DCs enhanced tumor angiogenesis by the production of angiogenic cytokines such as tumor necrosis factor α and IL-4. A subpopulation of DCs, plasmacytoid DCs, promoted tumor angiogenesis, whereas another subpopulation, myeloid DCs, suppressed tumor angiogenesis. Furthermore, DC precursors promoted tumor vasculogenesis, which suggested their role as endothelial progenitor cells. Macrophages also promote tumor angiogenesis by producing angiogenic cytokines and angiogenesis modulating enzymes.

Depending on the cell types, T cells can positively or negatively regulate tumor angiogenesis. Effector CD8+ T cells and CD4+ T cells produce IFN-γ, which negatively regulates tumor angiogenesis. On the other hand, Tregs secrete VEGF-A and promote tumor angiogenesis. Moreover, a recent report showed the importance of type 1 T helper cells, which secrete IFN-γ in tumor blood vessel normalization. In this study, the combination therapy induced greater infiltration of effector CD8+ T cells and CD4+ T cells, and reduced the infiltration of Tregs into the tumors. Therefore, not only the sunitinib but also these immune cells might have had potential effects on the tumor vasculature in this study.

In some parts of the tumors, F4/80+ mononuclear cells massively infiltrated together with the formation of clusters, whereas, at other parts, they scarcely infiltrated. We found such unequal distributions of F4/80+ mononuclear cells in tumors isolated from all the groups. Since the massively infiltrating cells overlapped with other cells, we could not precisely count the numbers of F4/80+ mononuclear cells in tumors. CD11c+ mononuclear cells also distributed unequally in tumors isolated from all the groups. Thus, we also could not count the numbers of CD11c+ mononuclear cells. We examined the expression levels of F4/80 or CD11b mRNA in a whole tumor by quantitative real-time PCR, but, the data were not convincing. Overall, in this study, we were able to show the presence of APCs in tumors, and these may play a key role in the antitumor effect of the combination therapy, as shown by the anti-CD11b mAb treatment experiment.

Hypoxia causes the tumor microenvironment to become immunosuppressive. In addition, the tumor microenvironment is acidic. Under a hypoxic and acidic microenvironment, T cells lost their antitumor function and became anergic and apoptotic, while Tregs blocked the activity of effector T-cells. Hypoxia in tumors induces the production of angiogenic cytokines such as VEGF and CXC chemokine CXCL12. Pathologic concentrations of VEGF and CXCL12 synergistically and sufficiently induced tumor angiogenesis. Moreover, CXCL12 recruited plasmacytoid DC precursors into tumors and protected them from apoptosis. In addition to promoting tumor angiogenesis, plasmacytoid DCs impair tumor immunity. In this study, sunitinib therapy improved the hypoxia in tumors. Indeed, the combination therapy strongly induced the infiltration of effector T cells into tumors and, moreover, infiltrated CD8+ T cells into tumors.
were potently activated. We detected strong activation of CD8$^+$ T cells in the hind flank tumors. Footpad tumors were too small to isolate sufficient numbers of CD8$^+$ T cells to determine the activation levels of the cells by flow cytometric analysis.

Anti-angiogenic therapy improved the expression levels of adhesion molecules. 55 Therefore, we analyzed the expression levels of adhesion molecules in the tumors by quantitative real-time PCR using mRNA isolated from the whole tumor. However, we could not detect differences in the expression levels between the therapies. Improvements in the procedure, such as isolating mRNA from tumor endothelial cells, might be required for the precise analysis of adhesion molecules.

Sunitinib monotherapy accelerated tumor metastasis in the mouse tumor models. 32 Another study showed that short term sunitinib monotherapy accelerated tumor metastasis, whereas sustained sunitinib monotherapy delayed the metastasis. 56 In the same study, in immune-deficient mice, the anti-tumor effects of sunitinib were not consistent. 56 Collectively, this study suggested that anti-tumor effects of sunitinib were dependent on the administration protocol of sunitinib. 56 On the other hand, previous studies suggested the beneficial effects of sunitinib on tumor immune responses, which might explain the inconsistency in the anti-tumor effects of sunitinib in immune deficient mice. 27–30,57 Thus, the anti-tumor effects of sunitinib may depend on the experimental conditions such as immunity of the host, in addition to the experimental protocols. In this study, using immuno-competent mice, sunitinib monotherapy did not accelerate the tumor metastasis to draining LNs. Overall, the experimental protocol of sunitinib monotherapy in this study showed beneficial effects on DR5 targeting immunotherapy.

Sunitinib monotherapy provided beneficial therapeutic effects on immunotherapy mediated by effector T cells and APCs. Blood vessel normalization and improved lymphatic function may contribute to this effects. This combination therapy induced no apparent toxicity in the mouse tumor models. Immune checkpoint blockade can activate effector T cells. Further studies should be undertaken to improve the efficacy of this combination therapy, such as through the addition of multiple immune checkpoint blockades.

Materials and methods

Mice

Male BALB/c mice and BALB/c nude mice at 7 to 9 week of age were from Charles River Japan (Kanagawa, Japan). All mice were maintained under a specific pathogen-free condition. Mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine and xylazine. The Laboratory Animal Committee at Tohoku University approved all experimental procedures.

mAbs and tumors

Agonistic anti-mouse DR5 mAb (MD5-1), anti-mouse CD4 mAb (GK1.5), anti-mouse CD8 mAb (53–6.7), anti-mouse CD11b mAb (5C6) were prepared as previously described. 4 The colon 26 (CT26) carcinoma was provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, in 2016. CT26 was passaged less than 10 times after the initial revival from frozen stocks. TRAIL-sensitive 4T1 mammary carcinoma cells (obtained from Dr. Hideo Yagita in 2012, Juntendo University, Tokyo, Japan) and CT26 were prepared as previously described. 4

Therapy of transplanted tumors

Mice were inoculated with $2 \times 10^5$ 4T1 tumor cells s.c. in the footpads. Sunitinib (S1042, Selleckchem, USA) was dissolved in corn oil (10 mg/ml). Mice were gavaged with 35 mg/kg sunitinib 5 days a week and/or administered i.p. with 100 μg MD5-1 on days 4, 8, 12. Mice were anesthetized and sacrificed on day 14 for tumor, LN, and Evans blue analysis. For the hind flank experiments, mice were subcutaneously inoculated with $2 \times 10^5$ 4T1 tumor cells or $1 \times 10^7$ CT26 tumor cells. Mice were gavaged with sunitinib 5 days a week and/or administered i.p. with 100 μg MD5-1 every 4 day. Tumor size was measured daily and quantified as width$^2 \times$ length$ \times 0.52$. 58 We isolated 4T1 tumors on day 21 and CT26 tumors on 28. Some groups of mice were administered i.p. with 100 μg anti-CD4 and/or anti-CD8 mAb on day −1, 3, 7, 11, 15, 19, or 300μg anti-CD11b mAb on day −1,4,9,13. The effective depletion of CD4$^+$ and/or CD8$^+$ T cells was verified by flow cytometry. 2

Immunohistochemistry

Mice were anesthetized and tumors were isolated as described previously. 59 Tumor cryostat sections of 30 μm thickness were stained with the following primary antibodies. CD31/PECAM-1 (hamster; MA3105, Thermo Fisher Scientific, USA), Cy3 conjugated α-SMA (mouse; G6198, Sigma, USA), LYVE-1 (rabbit; 11–034, Angiobio, USA), CD4 (rat; 14–0042–85, eBioscience, USA), CD8 (rat; 14–0081–85, eBioscience), F4/80 (Rat; MF68000, Bio-Rad, UK), phycoerythrin (PE) conjugated CD11c (hamster; 561044, BD Biosciences, NJ), biotin conjugated Foxp3 (rat; 13–5773–82, eBioscience), hyaluronic acid binding protein biotin bovine (H9910, Sigma). Species-specific secondary antibodies were labelled with Cy3, fluorescein isothiocyanate (FITC), Dylight (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) or, Alexa Fluor 555 Conjugated Streptavidin (S32355, Invitrogen, USA). For biotin-conjugated antibody staining, Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories, USA) was used according to the manufacturer’s instructions. 4 To identify hypoxic regions, mice were injected with 1.5 mg pimonidazole hydrochloride (HP2-100, Hypoxprobe Inc, USA) intravenously (i.v.) 1 hour before the perfusion on day 14. 60 Specimens were examined with a Carl Zeiss LSM780 confocal microscope using ZEN 2011 software.
RNA isolation and quantitative real-time PCR

Total RNA was isolated from the tumors using TRIzol Reagent (15596026, Thermo). Quantitative real-time PCR was performed as previously described using the following primers: β-actin forward, 5′- CTTGACATCCGTAAGACCTC –3′; β-actin reverse, 5′- AGCCACCCGATCCACAGA –3′; RGS5 forward, 5′-GGGTTGCTTGTAAGATTACA –3′; RGS5 reverse, 5′- TGAAGTGCTAAGTTCACCTCT –3′; VEGF-C forward, 5′- GCTGCGGCTGTCGCCAGTCC –3′; VEGF-C reverse, 5′- CAAAAGCCTTGACCTCGCCCCC –3′.61

Morphometric measurements

Samples were examined with an Olympus BX51 fluorescence microscope equipped with a DP70 Olympus digital camera (Tokyo, Japan) using Lumina Vision software (Mitani Corporation, Tokyo, Japan). The area density of CD31, α-SMA, pimonidazole hydrochloride, and HA were measured with Image J software (http://rsbweb.nih.gov/ij/) on fluorescence images taken at a magnification of ×200, 10 images per sample, using empirically determined threshold values.62 The area density was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold.62 The pericyte coverage on blood endothelial cells was defined as CD31 and α-SMA double fluorescent positive signals, divided by the total blood endothelial cell CD31 positive signals in the tumors. Images were taken at a magnification of ×200, 10 images per sample. Double positive signals were calculated by using the AND operation of image calculator in the image J software. A positive signal was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold. The numbers of tumor infiltrating CD8+, CD4+Foxp3+, and CD4+Foxp3+ cells were quantified using fluorescence images taken at a magnification of ×200 for the hind flank and ×400 for the footpad tumors, 10 images per sample.6

Evans blue measurement

On day 14, Evans blue (0.75 mg/50 μl, 056–04061, Wako, Osaka, Japan) was injected into the footpad tumors. Five or 30 minutes after the injection, the tumors and the lymph nodes were isolated and incubated in formamide overnight at 67 °C. Extracted Evans blue was measured as described previously.62 Color images were taken using a digital camera, Canon EOS Kiss X7 (Canon, Tokyo, Japan).

Flow cytometric analysis

Cell isolation, immunofluorescent staining, and flow cytometric analyses were performed as previously described with minor modifications.6,63 Briefly, the LNs and tumors were minced and digested in Hank’s balanced salt solution supplemented with 2 % FBS (10270, Life Technologies, USA), 1 mg/ml collagenase A (10103578001, Roche Applied Science, USA), and 20 μg/ml Dnase (11284932001, Sigma) at 37 °C for 35 minutes for the LNs or 2 hours for the tumors. After being filtered through a 70 μm cell strainer, erythrocyte lysis was performed for tumors using ACK Lysing Buffer (A1049201, Life Technologies). Approximately 1 × 10⁶ cells were stained. After the blocking, the samples were incubated with the following fluorescence-conjugated mAbs; all the antibodies were from Tonbo Bioscience, USA: FITC or PE anti-CD3e (clone 145-2C11; 35–0031), allopoxycyanin anti-CD4 (clone RM4-5; 20–0042), allopoxycyanin anti-CD8 (clone 53–6:7; 20–0081), PE anti-Foxp3 (clone 3G3; 50–5773), FITC anti-CD69 (clone H1.2F3; 35–0691), PE anti-IFN-γ (clone XMG1.2; 50–7311), FITC anti-CD11c (clone N418; 35–0114), PE anti-CD80 (clone 16-10A1;50-0801), PE anti-CD86 (clone GL-1; 50–0862). For IFN-γ and Foxp3 incubation, Foxp3/Transcription Factor Staining Buffer Kit (TNB-1020-L050, Tonbo Bioscience) was used according to the manufacturer’s instructions. The samples were analyzed on FACSComp (BD Biosciences) with FlowJo ver.10 software (TreeStar, Ashland, OS, USA).

Statistics

All data were presented as means ± SEM. All statistical analyses were performed using the GraphPad Prism 6. Two tailed unpaired student’s t-test was used to compare the statistical difference between two groups. ANOVA followed by Tukey’s multiple comparison test was used for multiple comparisons. P values less than 0.05 were considered significant.

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References

1. Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. Oncogene. 2007;26:3745–3757. doi:10.1038/sj.onc.1210374.
2. Takeda K, Yamaguchi N, Akiba H, Kojima Y, Hayakawa Y, Tanner JE, Sayers TJ, Seki N, Okumura K, Yagita H, et al. Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. J Exp Med. 2004;199:437–448. doi:10.1084/jem.20031457.

3. Uno T, Takeda K, Kojima Y, Yoshizawa H, Akiba H, Mittler RS, Gejyo F, Okumura K, Yagita H, Smyth MJ. Eradication of established tumors in mice by a combination antibody-based therapy. Nat Med. 2006;12:693–698. doi:10.1038/nm1405.

4. Takeda K, Kojima Y, Uno T, Hayakawa Y, Teng MW, Yoshizawa H, Yagita H, Gejyo F, Okumura K, Smyth MJ. Combination therapy of established tumors by antibodies targeting immune activating and suppressing molecules. J Immunol. 2010;184:5493–5501. doi:10.4049/jimmunol.0903033.

5. Reck M, Krzakowski M, Chmielowska E, Sebastian M, Hadler D, Fox T, Wang Q, Greenberg J, Beckman RA, von Pawel J. A randomized, double-blind, placebo-controlled phase 2 study of tigatuzumab (CS-1008) in combination with carboplatin/paclitaxel in patients with chemotherapy-naive metastatic/unresectable non-small cell lung cancer. Lung Cancer. 2013;82:441–448. doi:10.1016/j.lungcan.2013.09.014.

6. Inaba K, Inaba M, Witmer-Pack M, Hatchcock K, Hodes R, Steinman RM. Expression of B7 costimulator molecules on mouse dendritic cells. Adv Exp Med Biol. 1995;378:65–70.

7. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol. 2004;4:941–952. doi:10.1038/nri1498.

8. Okazaki T, Nakao A, Nakano H, Takahashi F, Takahashi K, Shimotozo O, Takeda K, Yagita H, Okumura K. Impairment of bleomycin-induced lung fibrosis in CD28-deficient mice. J Immunol. 2001;167:1977–1981.

9. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev. 2005;15:102–111. doi:10.1016/j.gde.2004.12.005.

10. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. Cancer Cell. 2014;26:605–622. doi:10.1016/j.ccell.2014.10.006.

11. Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. Cancer Res. 2013;73:2943–2948. doi:10.1158/0008-5472.CAN-12-4354.

12. Motz GT, Cokous G. The parallel lives of angiogenesis and immunosuppression: cancer and other tales. Nat Rev Immunol. 2011;11:702–711. doi:10.1038/nri3064.

13. Tartour E, Pere H, Mailleire B, Terme M, Merillon N, Taieb J, Sandoval F, Quintin-Colonna F, Lacerda K, Karadimou A, et al. Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. Cancer Metastasis Rev. 2011;30:83–95. doi:10.1007/s10555-011-9281-4.

14. Falcon BL, Hashizume H, Koumoutsakos P, Chou J, Bready JV, Coxon A, Oliner JD, McDonald DM. Contrasting actions of selective inhibitors of angiopoietin-1 and angiopoietin-2 on the normalization of tumor blood vessels. Am J Pathol. 2009;175:2159–2170. doi:10.2353/ajpath.2009.090391.

15. Altitado K. The lymphatic vasculature in disease. Nat Med. 2011;17:1371–1380. doi:10.1038/nm.2545.

16. Kim H, Kataru RP, Koh GY. Inflammation-associated lymphangiogenesis: a double-edged sword? J Clin Invest. 2014;124:936–947. doi:10.1172/JCI71607.

17. Podgrabinska S, Skobe M. Role of lymphatic vasculature in regional and distant metastases. Microvasc Res. 2014;95:46–52. doi:10.1016/j.mvr.2014.07.004.

18. Zheng W, Aspelund A, Altitado K. Lymphangiogenic factors, mechanisms, and applications. J Clin Invest. 2014;124:878–887. doi:10.1172/JCI71603.

19. Jain RK. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J Clin Oncol. 2013;31:2205–2218. doi:10.1200/JCO.2012.46.3635.
