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ORIGINAL RESEARCH

Local endothelial complement activation reverses endothelial quiescence, enabling t-cell homing, and tumor control during t-cell immunotherapy

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ABSTRACT

Cancer immunotherapy relies upon the ability of T cells to infiltrate tumors. The endothelium constitutes a barrier between the tumor and effector T cells, and the ability to manipulate local vascular permeability could be translated into effective immunotherapy. Here, we show that in the context of adoptive T cell therapy, antitumor T cells, delivered at high enough doses, can overcome the endothelial barrier and infiltrate tumors, a process that requires local production of C3, complement activation on tumor endothelium and release of C5a. C5a, in turn, acts on endothelial cells promoting the upregulation of adhesion molecules and T-cell homing. Genetic deletion of C3 or the C5a receptor 1 (C5aR1), and pharmacological blockade of C5aR1, impaired the ability of T cells to overcome the endothelial barrier, infiltrate tumors, and control tumor progression *in vivo*, while genetic chimera mice demonstrated that C3 and C5aR1 expression by tumor stroma, and not leukocytes, governs T cell homing, acting on the local endothelium. *In vitro*, endothelial C3 and C5a expressions were required for endothelial activation by type 1 cytokines. Our data indicate that effective immunotherapy is a consequence of successful homing of T cells in response to local complement activation, which disrupts the tumor endothelial barrier.

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Introduction

Evidence from basic and clinical research shows that tumors are frequently infiltrated by immune cells.[1] The presence of intratumoral $CD8^+$ T cells, in particular, appears to be associated with better clinical outcomes, indicating a role for these cells in the antitumor immune response.[1,2] This perception has led to the development of immunotherapy protocols aimed at augmenting tumor immune recognition and attack, with further clinical benefits for cancer patients.[3] Although promising, tumor immunotherapies, and particularly vaccines, have thus far shown only modest effects.[4,5] Experimental models suggest that the presence of circulating tumor-reactive T cells does not necessarily translate to effective T-cell homing and infiltration into the tumor bed. Tumor T-cell infiltration requires functionality with regard to the process of extravasation, including tethering, rolling, and transmigration by the infiltrating T cells and the expression of adhesion molecules such as intracellular cell adhesion molecule-1 (ICAM-1) and vasculature cell adhesion molecule-1 (VCAM-1) by the tumor endothelium.[6] Indeed, it has been shown that tumor-derived angiogenic growth factors such as vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1) downregulate the expression of adhesion molecules.[7] Similarly, tumor endothelial cells (TECs) can also express FasL, resulting in the apoptosis of Fas-expressing T cells,[8] while the expression of other immunosuppressive molecules such as PD-L1, PD-L2, IDO-1, IL-6, and IL-10 can directly inhibit T-cell function.[9,10] Supporting the concept that the tumor microenvironment induces endothelial quiescence, thereby establishing a barrier that prevents T cells from efficiently penetrating the tumor, we have previously reported that endothelial quiescence can be maintained by the endothelin B receptor (ET_BR),[7] which is engaged by ET-1, a paracrine ligand overexpressed by tumor cells.[11]

Notwithstanding the mechanisms mentioned above, adoptive lymphocyte therapy approaches have often produced objective tumor responses in humans.[12,13] Experimentally, the

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success of adoptive T-cell therapy in mouse models depends on the dose of tumor-reactive T cells,[14] suggesting that blood–tumor barrier mechanisms can perhaps be surmounted by T cells, if the appropriate conditions are met. In line with this idea, the efficacy of adoptive T-cell therapy has been shown to be enhanced by the co-administration of tumor-reactive $CD4^+$ cells, which facilitate the access of low-affinity $CD8^+$ cells to tumors.[15] Here, we have further investigated the mechanisms underlying the break of the endothelial barrier that could allow proper T-cell infiltration of tumors.

Our data show that complement activation in the tumor endothelium is a key determinant of reversing endothelial quiescence and permitting successful T-cell homing to tumors. We found that a critical mass of $CD4^+$ and $CD8^+$ tumor-reactive T cells is required to upregulate endothelial complement components through Th1 cytokines, to induce complement activation and local release of the C5a activation fragment. C5a, in turn, upregulates endothelial cell adhesion molecules (CAMs), reversing endothelial quiescence and promoting the adhesion, and extravasation of effector T cells, which lead to effective tumor control. Our findings indicate that the blood-tumor endothelial barrier is surmountable: local complement activation effectively counters this barrier to enable effective immune therapy.

Results

The number of tumor-reactive T cells is critical for overcoming the blood–tumor barrier

We have previously demonstrated that tumor-induced endothelial quiescence can be reversed by inhibiting ET_BR, thereby rescuing the infiltration of T cells into tumor sites after vaccination with Lm-LLO-E7, a DNA vaccine eliciting modest $CD8^+$ responses to the E7 protein of the human papilloma virus (HPV)16, or after adoptive transfer of Lm-LLO-E7 primed T cells in mice.[7] We asked whether the tumor's endothelial barrier could be overcome by the adoptive transfer of more effective T cells alone, without the need for ET_BR blockade. To this end, we identified a more immunogenic DNA vaccine to prime donor mice: The pConE6E7 vaccine used here is related to Lm-LLO-E7, and both are directed against the HPV16 gene E7,[16,17] but by comparison to Lm-LLO-E7, pConE6E7 is highly immunogenic. C57BL/6 mice were given three weekly injections of Lm-LLOE7 or pConE6E7, followed by evaluation of splenic immune responses. The pConE6E7 DNA vaccine elicited stronger E7-specific $CD8^+$ T-cell responses than did the Lm-LLO-E7 vaccine, as demonstrated by the increased number of E7 Tetramer positive cytotoxic T lymphocytes, and higher IFNγ-producing $CD8^+$ T cells recognizing the K^b-restricted E7 epitope RAHYNIVTF (Fig. S1A–C). Accordingly, the higher numbers of E7-specific T cells triggered by the pConE6E7 vaccine resulted in an increased release of IFNγ and TNF-α cytokines in the supernatant of splenocytes culture challenged *ex vivo* with the E7 peptide (Fig. S1D and E), as well as a higher proliferation index and increased T-cell cytotoxicity against target cells pulsed with E7 peptide (Fig S1F and G).

To determine whether a higher number of tumor-specific T cells could abrogate the tumor's endothelial barrier following adoptive T-cell therapy, we transferred 5×10^6 spleen $CD3^+$ cells isolated from Lm-LLO-E7- or pConE6E7-vaccinated mice (the latter henceforth referred to as "E7-primed T cells") into C57BL/6 mice bearing TC-1 tumors. Where indicated, the mice were also treated with the ET_BR antagonist BQ-788, which reverses endothelial dormancy.[7] As predicted, mice given E7-primed T cells from pConE6E7-immunized animals showed higher numbers of E7-specific T cells in the spleen when compared with their Lm-LLO-E7 counterparts (Fig. S2A). More interestingly, while only marginal numbers of E7-specific $CD8^+$ T cells were found in the tumors of mice receiving Lm-LLO-E7-derived T cells, ~40–50% of the $CD8^+$ T cells were E7-specific in the tumors of mice receiving pConE6E7-derived T cells (Fig. S2B). A stronger pConE6E7-induced T-cell response resulted in increased tumor infiltration and significant restriction of tumor growth, independent of BQ-788 administration (Fig. S2C), indicating that the tumor's endothelial barrier can be disrupted in response to a sufficiently large number of tumor-specific T cells.

Complement C3 is required for the homing of effective T cells and tumor suppression

To understand the mechanisms underlying successful T-cell infiltration into the tumor, we reassessed the earlier finding that complement C3 was the most highly upregulated transcript in TECs microdissected from tumors with brisk intratumoral T cells, when compared with TECs from tumors devoid of T cells.[7] The upregulation of C3 mRNA in TECs isolated from tumors containing tumor-infiltrating lymphocytes (TILs) was confirmed in new samples of purified TECs (Fig. 1A), suggesting a potential role for complement in the endothelial mechanisms regulating T-cell homing. C3, the central component required for the activation of all three complement pathways, is a well-appreciated modulator of inflammation and immune responses, determining the outcome of pathological conditions such as transplant rejection, autoimmunity, and cancer.[18,19] Notably, a significant increase in the deposition of the activation fragments C3b, iC3b, and C3c was detected in the tumor endothelium of mice receiving 5×10^6 of E7-primed T cells (henceforth referred to as "the effective dose") (Fig. 1B and Fig. S2D). Given the human and mouse data, we asked whether effective doses of T cells neutralize the endothelial barrier through increased complement activation.

To investigate whether T-cell infiltration into tumors requires complement activation, the infiltration of T cells into tumors after adoptive immunotherapy was compared in C3-sufficient ($c3^{+/+}$) and C3-deficient ($c3^{-/-}$)mice.[20] To specifically determine the role of C3 produced locally by the tumor stroma (connective tissue and vasculature) or tumor-associated leukocytes, chimeric mice (in which C3 was produced only by leukocytes, but not stromal cells) generated by transferring wild-type CD45.1 bone marrow (BM) into irradiated $c3^{-/-}$ mice; $c3^{+/+}$ recipients were used as controls. BM engraftment was followed by flow cytometry (Fig. S3A). $c3^{+/+}$ and $c3^{-/-}$ mice bearing wild-type leukocytes were inoculated subcutaneously with TC-1 tumors, followed by intravenous administration of an effective dose

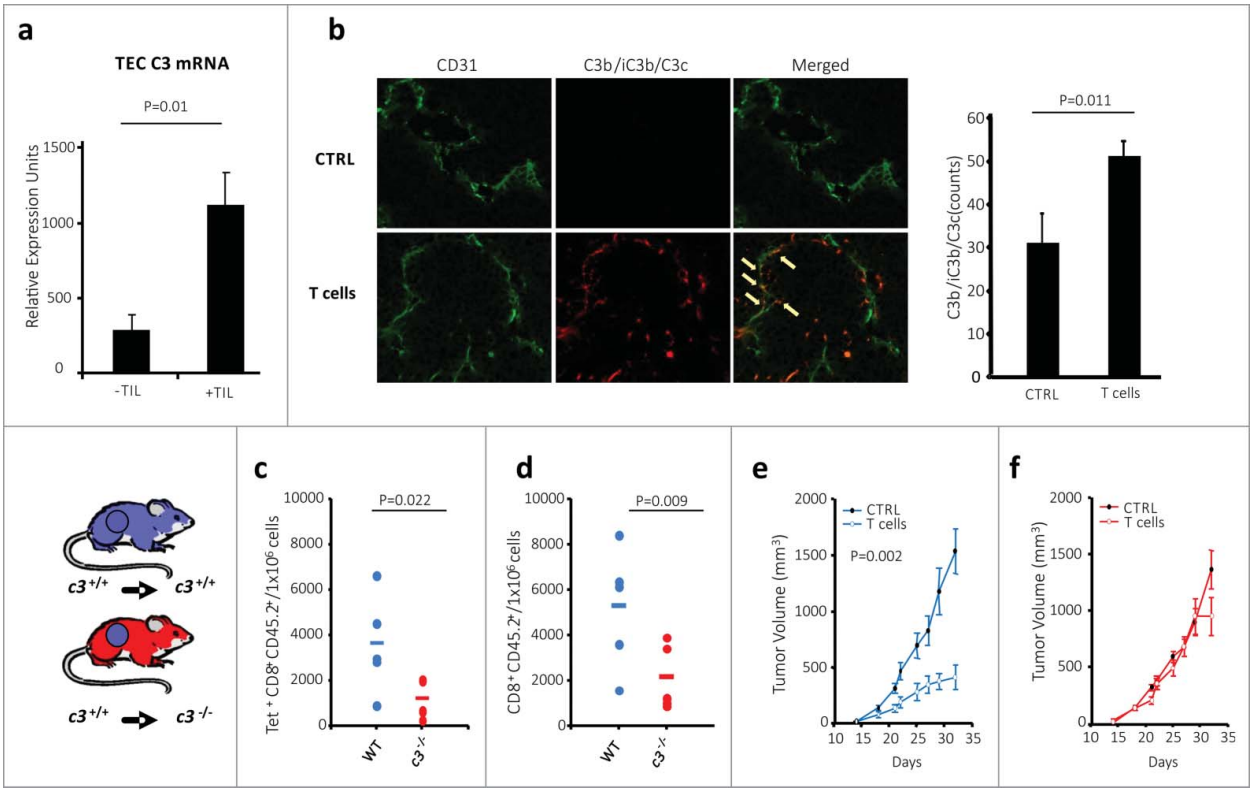

Figure 1. Complement C3 is required for the homing of effective (T) cells and tumor suppression. (A) C3 mRNA is significantly overexpressed in human tumor endothelial cells sorted from ovarian cancers with tumor-infiltrating lymphocytes (TILs), when compared with ovarian cancers lacking TILs ($n = 6$/group). (B) Detection of complement C3b/iC3b/C3c activation fragments (red) on tumor vasculature (CD31 in green) after adoptive transfer of 5×10^6 E7-primed $CD8^+$ T cells. Arrows indicate areas of juxtaposition of complement fragments and CD31. The right panel depicts the quantification of C3 fragments co-localized with CD31. (C–F) Mouse chimeras were generated by transferring wild-type bone marrow from B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice to lethally irradiated $c3^{+/+}$ and $c3^{-/-}$ mice. The chimeras were inoculated s.c with TC-1 tumors, followed by i.v. administration of 5×10^6 E7-primed CD45.2 $CD3^+$ T cells. (C and D) Flow cytometry analysis showing the number of donor E7-specific $CD8^+$ T cells and total $CD8^+$ T cells in the tumors. (E and F) TC-1 tumor growth in BM-transplanted $c3^{+/+}$ and $c3^{-/-}$ mice in the absence of treatment (CTRL) or after transfer of 5×10^6 E7-primed $CD3^+$ T-cells.

of E7-primed T cells. Tumor infiltration of E7-specific cells as well as total $CD8^+$ cells was significantly impaired in $c3^{-/-}$ mice as compared with $c3^{+/+}$ mice (Fig. 1C and D). Consistent with this finding, adoptive transfer of an effective dose of E7-primed T cells significantly restricted tumor growth in $c3^{+/+}$mice, but had no effect on the tumors in $c3^{-/-}$ mice (Fig. 1E and F); similar results were obtained in tumor-bearing $c3^{-/-}$ and $c3^{+/+}$ littermate mice that had no prior BM transfer (not shown).

We asked whether the lack of infiltration of adoptive T cells in $c3^{-/-}$ mice was associated with reduced deposition of the activation fragments C3b, iC3b, and C3c in the tumor endothelium. Importantly, we found no deposition of C3 fragments in the tumor endothelium in $c3^{-/-}$ hosts reconstituted with $c3^{+/+}$ BM after adoptive transfer (Fig. S3B). Collectively, the results described above indicate that upon adoptive transfer of a sufficiently large amount of tumor-reactive T cells, the endothelial barrier can be reversed, as long as endogenous complement activation can occur in proximity to the tumor endothelium. Our results also indicate that complement activation and C3 fragment deposition in the tumor endothelium, required for the homing of adoptive T cells, are independent of the expression of C3 by endogenous or exogenous leukocytes. Chimeric $c3^{-/-}$ mice bearing wild-type BM release sufficient amounts of C3 protein in the serum to maintain normal phenotypes.[20] Although we cannot exclude the participation of systemic C3, our results collectively suggest that complement activation in the tumor endothelium depends on local expression by tumor stromal cells, presumably tumor endothelium.

Triggering of the C5a-C5aR1 axis is required for T- cell extravasation and tumor suppression

When complement is activated, C3 is cleaved into the fragments C3a and C3b, with consequent formation of the C5 convertase and release of the C5a fragment.[18] C5a is a pro-inflammatory molecule that signals through the G-coupled receptor C5a receptor 1 (C5aR1)[21] and has been previously implicated in tumor growth.[22-27] Given the results obtained with the *c3* chimera, we hypothesized that C5a mediates some of the effects of complement activation on the tumor endothelium and, consequently, that generation of C5a is required for T-cell infiltration into tumors. Indeed, pharmacologic blockade of the C5aR1 with an antagonist peptide (C5aR1A) resulted in diminished infiltration of E7-specific $CD8^+$ as well as total $CD3^+$ cells into the tumors and an attenuated efficacy of the T-cell therapy in wild-type mice (Fig. 2A–C). Conversely, as already reported by others,[22] we found that treatment with the C5aR1A alone in the absence of adoptive T-cell transfer resulted in decreased tumor growth when compared with control mice that had not received T cells (Fig. 2C).

Expression of C5aR1 on T cells has been reported previously.[28-31] However, we found that pharmacologic blockade of the receptor did not compromise the systemic expansion of total or E7-specific $CD8^+$ T cells *in vivo* after adoptive transfer (Fig. S4A–C), nor did it affect the ability of the T cells to produce IFNγ *in vitro* after E7-specific re-stimulation (Fig. S4D). To further ascertain, whether C5aR1 signaling might directly affect T-cell expansion of effector function following adoptive transfer, we generated T cells in donor *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ littermate mice. Whereas *c5ar1*$^{-/-}$ mice have previously exhibited dampened responses to other vaccination approaches,[32] vaccination with plasmid DNA was quite effective and resulted in the generation of similar E7-primed T cells in *c5ar1*$^{-/-}$ mice and their *c5ar1*$^{+/+}$ littermates (Fig. S4E), which were then used for adoptive therapy. Importantly, the antitumor properties of adoptively transferred *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ E7-primed T cells into WT tumor-bearing recipients were comparable (Fig. S4F). Collectively, these data indicate that although C5a/C5aR1 axis activation is required for T-cell homing to tumors, this activation does not act by supporting the peripheral expansion or effector function of T cells.

To ensure that our findings were not strictly correlated with the experimental protocols of adoptive T-cell therapy, we used vaccination to determine whether the requirement for complement activation during T-cell tumor infiltration was reproducible. Wild-type mice bearing subcutaneous TC-1 tumors were first vaccinated with pConE6E7 and then treated with the C5aR1A or control peptide for 2 weeks. Post-vaccine C5aR1 blockade significantly reduced the number of E7-specific $CD8^+$ TILs and attenuated the efficacy of

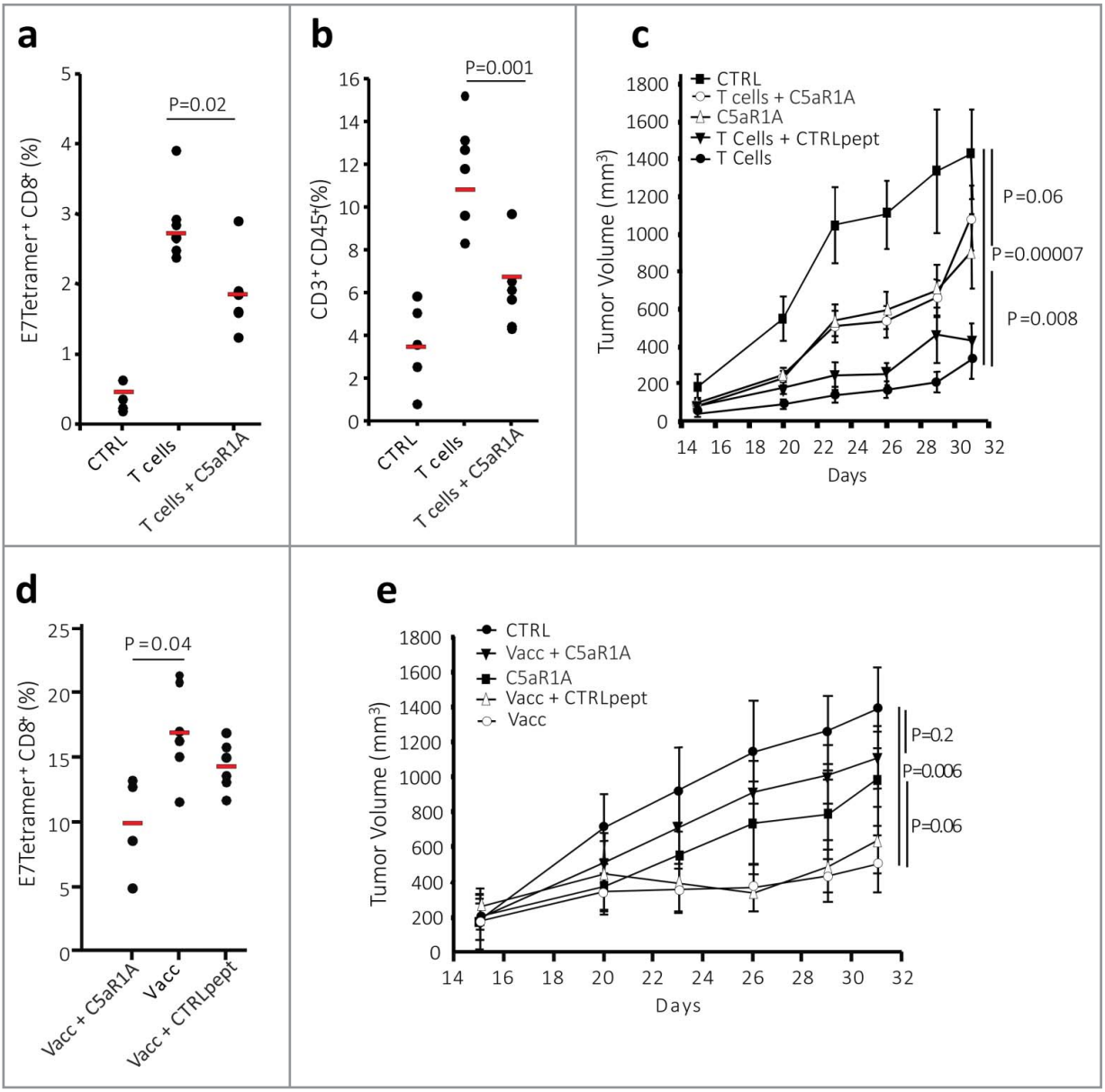

Figure 2. Triggering of the C5a-C5aR1 axis is required for T-cell extravasation and tumor suppression. Tumor-bearing mice received an adoptive transfer of 5×10^6 E7-primed T cells, and were then treated with the C5aR1 antagonist (C5aR1A) or a control peptide (CTRLpept). (A and B) The number of E7-specific $CD8^+$ T cells and total $CD3^+CD45^+$ T cells recruited to the tumor was determined by flow cytometry. (C) TC-1 tumor growth was measured over time. (D and E) Tumor-bearing mice were vaccinated with pConE6E7, followed by treatment with C5aR1A or control peptide. (D) The number of E7-specific $CD8^+$ T cells recruited to the tumor was determined by flow cytometry. (E) TC-1 tumor growth was measured over time.

the vaccine when compared with the control peptide (Fig. 2D and E), indicating that complement activation and C5a generation are required for the infiltration of effector T cells and tumor suppression.

C5aR1-mediated signaling in the tumor stroma is required for effective T-cell infiltration

Next, to validate the results obtained with pharmacological inhibition of the C5a-C5aR1 axis, we performed adoptive cell transfer in *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ tumor-bearing mice. Adoptive transfer of 5 × 10^6 E7-primed T cells from WT mice resulted in similar systemic cell expansion in *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ mice (Fig. 3A) and comparable T-cell functionality after *ex vivo* E7-specific stimulation (Fig. S4G). In contrast, tumor infiltration of total and E7-specific CD8$^+$ T cells was significantly impaired in *c5ar1*$^{-/-}$ mice relative to *c5ar1*$^{+/+}$ mice (Fig. 3B and C). Furthermore, adoptive lymphocyte transfer restricted tumor growth in *c5ar1*$^{+/+}$ mice, but not in *c5ar1*$^{-/-}$ mice (Fig. 3D and E). To dissect which cell population was affected by C5a-mediated signaling, we generated BM chimeras, transferring wild-type CD45.1 BM into *c5ar1*$^{-/-}$ or *c5ar1*$^{+/+}$ CD45.2 animals, which were then inoculated with TC-1 tumors. This approach allowed the production of host mice that expressed the C5aR1 on their leukocytes but lacked C5aR1 on the tumor stroma (including the endothelium). Mice then received an effective dose of E7-primed T cells. In spite of having *c5ar1*-sufficient BM, there was a significantly reduced accumulation of total and E7-specific CD8$^+$ T cells in the tumors of the *c5ar1*$^{-/-}$ mice when compared with the *c5ar1*$^{+/+}$ hosts (Fig. 3F and G). In addition, T-cell immunotherapy was not capable of restricting tumor growth in *c5ar1*$^{-/-}$ hosts (Fig. 3H and I), indicating that C5a-mediated infiltration of T cells and tumor suppression are independent of the expression of C5aR1 by leukocytes. Thus, the accumulation of T cells in tumors requires C5a, but the host cells responsible for promoting T-cell infiltration are tumor stromal cells, and not leukocytes.

Activation of the C5a-C5aR1 axis reverses endothelial cell quiescence

In light of the *in vivo* data showing enhanced deposition of C3 activation fragments on tumor vascular endothelium after adoptive transfer of an effective dose of T cells (Fig. 1B), and given the critical role of the endothelium in T-cell extravasation and tumor infiltration, we next investigated the impact of C5a on the activation of endothelial cells. The expression of the C5aR1 by endothelial cells is well appreciated,[33] and in line with this observation, treatment of human umbilical vein endothelial cells (HUVECs) with synthetic C5a increased T-cell adhesion *in vitro*, and this adhesion was restored to baseline levels in the presence of the C5aR1A antagonist but not a control peptide (Fig. 4A). We observed that the T-cell adhesion was mediated by ICAM-1 and VCAM-1, since the endothelial response to C5a was attenuated by neutralizing antibodies against the CAMs (Fig. 4A). In addition, we tested whether C5a could also reverse the endothelial quiescence induced by ET_BR signaling. For this purpose, we exposed HUVECs

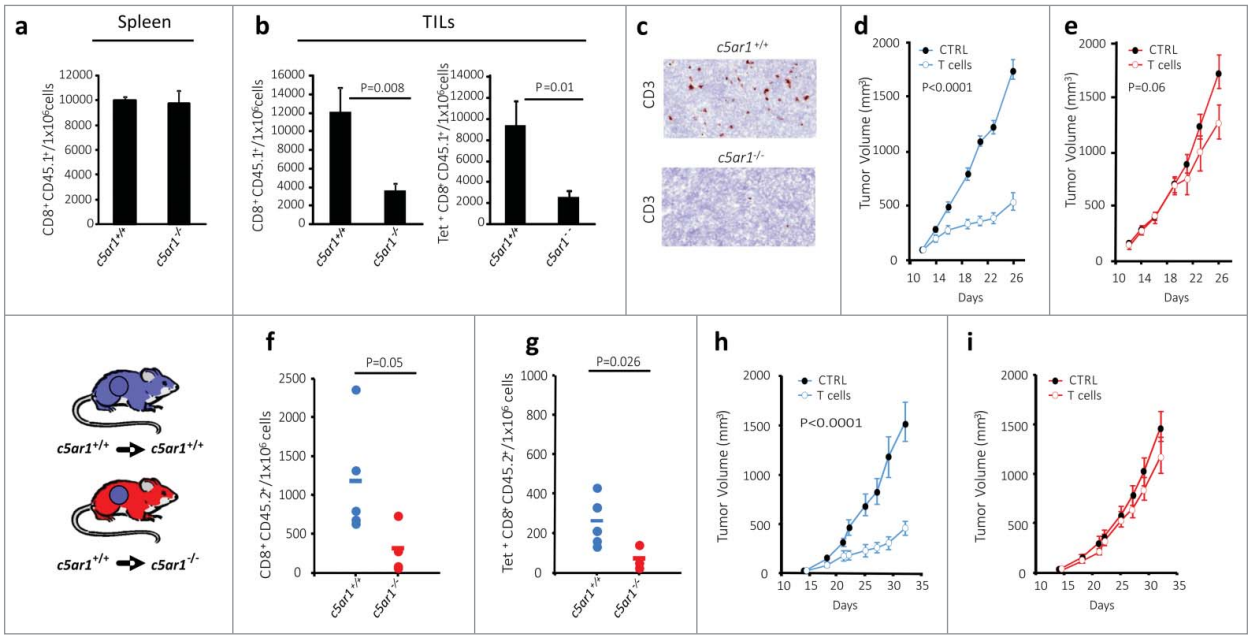

Figure 3. C5aR1-mediated signaling in the tumor stroma is required for effective T-cell infiltration. (A–E) Tumor-bearing C5aR1-deficient (*c5ar1*$^{-/-}$)mice and C5aR1-sufficient littermate controls (*c5ar1*$^{+/+}$) were given an effective dose (5 × 10^6) of E7-primed T cells. (A and B) Flow cytometry showing spleen expansion and tumor recruitment of total and E7-specific donor CD8$^+$ cells. (C) Immunohistochemical staining for CD3 of TC-1 tumor sections. (D and E) TC-1 tumor growth in *c5ar1*$^{+/+}$ (blue) and *c5ar1*$^{-/-}$ (red) mice in the absence of treatment (CTRL) or after transfer of 5 × 10^6 E7-primed CD3$^+$ T-cells. (F–I) Chimeras were generated by transferring wild-type bone marrow from B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice to *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ littermatemice. Mice were inoculated in the back with TC-1 tumors and then given an effective dose of E7 vaccine-primed adoptive CD45.2 T cells (5 × 10^6 CD3$^+$ cells/mouse). Flow cytometry analysis showing the number of CD8$^+$ (F) and E7-specific CD8$^+$ (G) engrafting TC-1 tumors in *c5ar1*$^{+/+}$ and *c5ar1*$^{-/-}$ mice reconstituted with bone marrow cells from WT mice. (H and I) TC-1 tumor growth in BM-transplanted *c5ar1*$^{+/+}$ (blue) and *c5ar1*$^{-/-}$ (red) mice in the absence of treatment (CTRL) or after the transfer of 5 × 10^6 E7-primed CD3$^+$ T-cells.

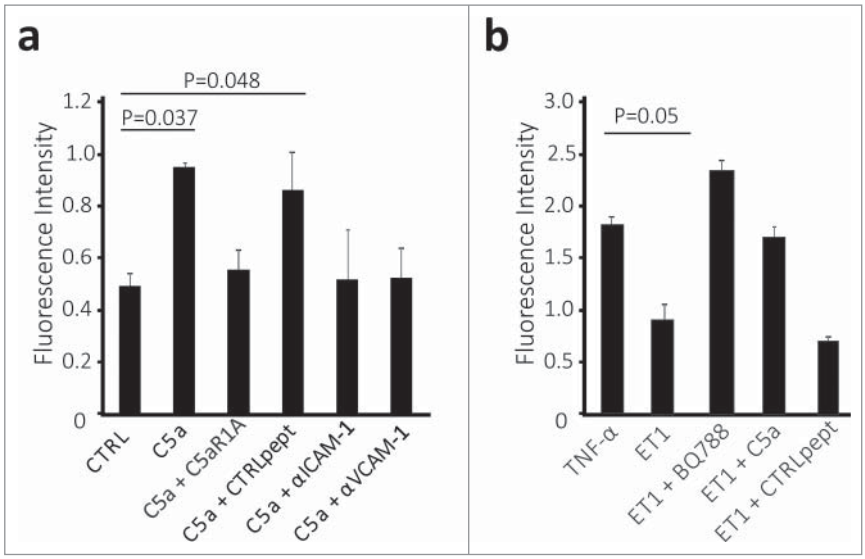

Figure 4. C5a-C5aR1 axis activation reverses endothelial cell quiescence. (A) HUVECs were treated with C5a in the presence of the C5aR1 antagonist (C5aR1A), control peptide (CTRLPept), anti-ICAM-1, or anti-VCAM-1 and incubated with CFSE-labeled T cells. T-cell adhesion was measured by detection of total fluorescence using a fluorocounter microplate reader. (B) HUVECs were activated with TNF-α, and endothelial quiescence was induced by treatment with endothelin-1 (ET1) in the presence of the ET_BR antagonist (BQ-788), C5a, or control peptide. After the addition of CFSE-labeled T cells, cell adhesion was measured by detection of total fluorescence using a fluorocounter microplate reader.

in vitro to ET-1, which inhibits T-cell adhesion through downregulation of CAMs, even in the presence of TNF-α (Fig. 4B). Like the ET_BR antagonist BQ-788, C5a (but not control peptide) reversed the inhibiting effect of ET-1, restoring T-cell adhesion (Fig. 4B) and suggesting that local complement activation is sufficient to activate endothelium and can counter ET_BR-induced signaling, maintaining the expression of CAMs in the endothelium and preventing endothelial dormancy

Th1 cytokines activate the endothelium through endothelial complement activation

Complement activation on the surface of human endothelial cells and the extracellular matrix has previously been observed after cell stimulation with IFNγ or TNF-α, either alone or in combination.[34] We found that supernatant from CD3/CD28-activated T cells strongly induced the production of C3 by HUVECs *in vitro*, and the activation of HUVECs with IFNγ or TNF-α, alone or in combination, also promoted the production of C3 (Fig. 5A). More interestingly, complement activation and deposition could be detected on the surface of HUVECs activated with TNF-α or supernatant from CD3/CD28-activated T cells, as evidenced by deposition of the C3 activation fragments C3b/iC3b/C3c (Fig. 5B). Further, *de novo* release of C5a was detected in the supernatant of activated HUVECs (Fig. 5C), supporting the concept of local complement activation on the surface of endothelial cells activated by pro-inflammatory cytokines. Notably, the addition of C5aR1 antagonist, which abrogates C5a signaling, attenuated the ability of HUVECs stimulated with supernatants from CD3/CD28-activated human T cells to support adhesion of T cells (Fig. 5D). A similar reduction in T-cell adhesion was observed after treatment of the HUVECs with blocking antibodies against ICAM-1 or VCAM-1 (Fig. 5D), indicating that C5a is required for optimal cytokine-induced expression of CAMs on endothelial cells. Indeed, the upregulation of VCAM-1 by IFNγ was abrogated in the presence of the C5aR1 antagonist or the C3 inhibitor Compstatin (Fig. 5E). This finding was corroborated using purified primary mouse lung microvascular endothelial cells from wild-type, *c5ar1*$^{-/-}$ and *c3*$^{-/-}$ mice treated with IFNγ or TNF-α, either alone or in combination. Although complement-sufficient endothelial cells mounted a robust VCAM-1 and ICAM-1 response to Th1 cytokines, this response was severely attenuated in endothelial cells that lacked C3 or C5aR1 (Fig. 5F and Fig. S5). Thus, cytokines released by activated effector type 1 T cells upregulate the expression of C3 in the endothelium, allowing local complement activation and the release of C5a, which in turn modulates the expression of endothelial CAMs and the attachment of T cells to the endothelium. We propose that such a mechanism plays a key role in tumors, where type 1 cytokines released by tumor-infiltrating $CD4^+$ and $CD8^+$ T cells activate the cascade just described, promoting the attachment, and extravasation of effector T cells.

Complement hyperactivation enhances T-cell engraftment of tumors and promotes tumor rejection by suboptimal numbers of tumor-specific T cells

The notion that complement activation allows for improved extravasation of T cells into the tumor bed suggests that the activation of the tumor vasculature by C5a could reduce the requirement for infusing a large dose of tumor-reactive T cells to abate endothelial quiescence. Decay accelerating factor (DAF/CD55) is a regulatory protein that limits complement activation on cell surfaces[18]; a deficiency of *daf1* allows unopposed complement activation in areas of inflammation and has been shown to exacerbate autoimmunity in mouse models.[35] Therefore, we asked whether *daf1* deficiency allowed for efficient tumor suppression when a low (suboptimal) dose (2.5×10^6, ineffective in wild-type mice) of E7-primed T cells was injected. Indeed, an increased number of E7-specific $CD8^+$ T cells homed to the tumors in *daf1*-deficient mice when

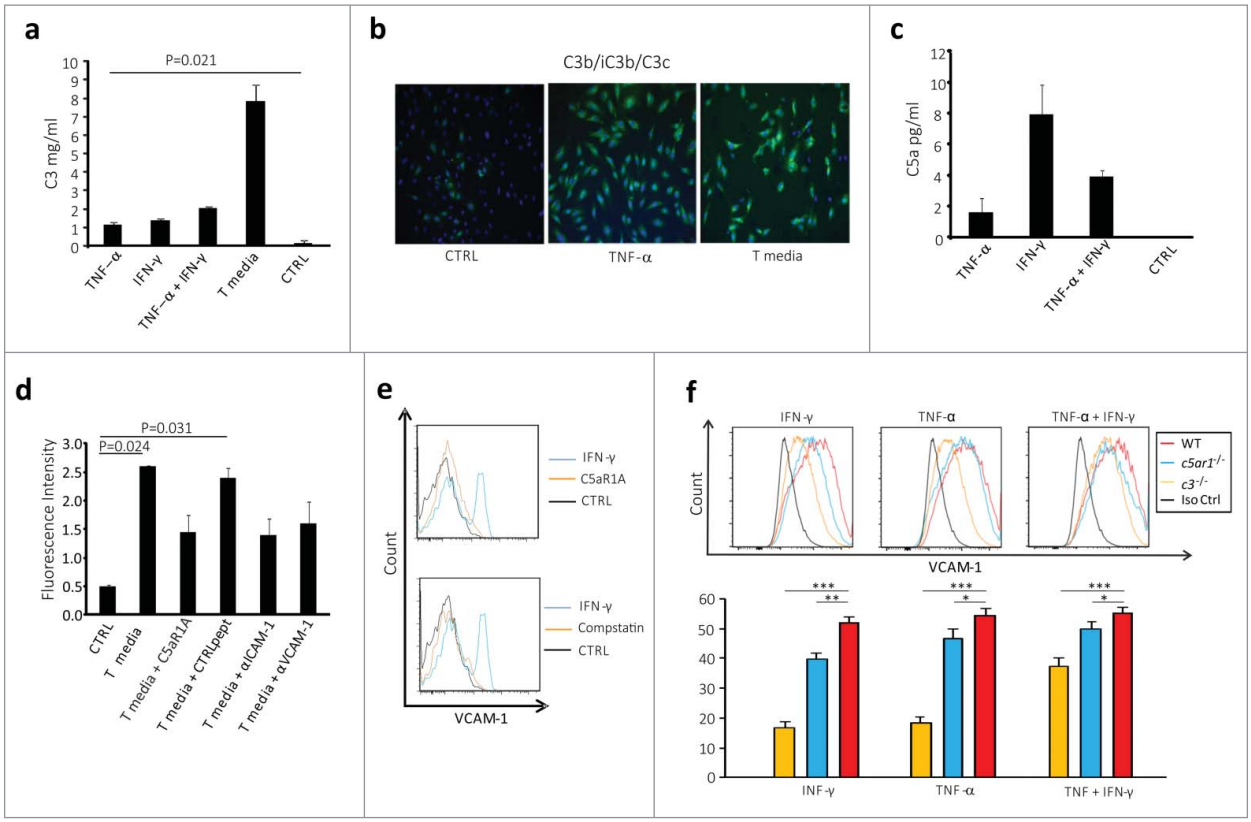

Figure 5. Th1 cytokines activate the endothelium through endothelial complement activation. (A) HUVEC cells were treated with TNF-α or IFNγ alone or in combination, or with the supernatant of T cells activated with anti-CD3/CD28, and the levels of C3 were measured in the culture supernatants by ELISA. (B) HUVECs activated by TNF-α or medium from anti-CD3/CD28-co-stimulated human T cells (T medium) were stained for deposition of the C3 activation fragments C3b, iC3b, and C3c. (C) HUVEC cells were treated with TNF-α or IFNγ alone or in combination, and the levels of C5a were measured in the culture supernatants by ELISA. (D) Adhesion of activated T cells to HUVECs was determined after treatment of HUVECs with supernatants of activated T cells (T medium) in the presence of C5a receptor 1 antagonist (C5aR1A), control peptide (CTRLPept), or antibody neutralizing ICAM-1 or VCAM-1. After addition of CFSE-labeled T cells, cell adhesion was measured by detecting total fluorescence using a fluorocounter microplate reader. CTRL indicates the adhesion of activated T cells on HUVECs in the absence of T-cell medium or any of the above factors. (E) Expression levels of VCAM-1 in response to the treatment with IFNγ in the presence of C5aR1 antagonist (C5aR1A) or the C3 inhibitor Compstatin were measured by flow cytometry. (F) Primary mouse lung micro vascular endothelial cells from wild-type, *c5ar1*$^{-/-}$,or *c3*$^{-/-}$ mice were treated with IFNγ or TNF-α alone or in combination, and the expression levels of VCAM-1 were measured by flow cytometry. Right panels depict quantification of VCAM-1 expression on the different mouse endothelial cells. $^{*}p < 0.05$; $^{**}p < 0.02$; $^{***}p < 0.0002$.

compared those in their wild-type counterparts (Fig. 6A). More importantly, whereas 2.5×10^6 T cells were not sufficient to suppress tumor growth in wild-type mice, the same number of cells significantly inhibited tumorigenesis in *daf1*-deficient mice (Fig. 6B and C). Co-administration of the C5aR1 antagonist abrogated the advantage seen in the *daf1*-deficient mice (Fig. 6D and E), confirming the role of C5a in T-cell homing and tumor suppression.

Pioneering CD4$^+$ and CD8$^+$ cells are both required for local complement activation, T-cell homing and tumor suppression

Based on our data, we hypothesized that pro-inflammatory cytokines such as IFNγ and TNF-α promote the production of C3 by the tumor endothelium and mediate local complement activation, with generation of C5a. The activation fragment C5a, in turn, upregulates the expression of CAMs in the tumor vasculature, allowing for successful engraftment of effective T cells. Of note, this process would require a pioneering population of tumor-reactive T cells to release sufficient cytokines and activate endothelial cells, with consequent complement deposition. To investigate this assumption, we used FACS to determine the number of adoptively transferred T cells over time in the tumors of *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ mice. Whereas expansion of total and E7-specific CD8$^+$ T cells in the spleen was similar in both recipient groups, reduced levels of CD8$^+$ T cells were observed in the tumors of *c5ar1*$^{-/-}$ mice when compared with their wild-type counterparts (Fig. 7A). Notably, at day 5, a similar low-level T-cell engraftment was present in the tumors of *c5ar1*$^{-/-}$ and *c5ar1*$^{+/+}$ mice. Remarkably, up to 25% of these TILs were CD4$^+$ (Fig. 7B). A marked increase in T-cell engraftment in *c5ar1*$^{+/+}$ mice occurred by day 12, with 95% CD8$^+$ cells (Fig. 7B), consistent with prior evidence that CD8$^+$ cells mediate TC-1 tumor suppression.[17] Overall, these data suggest that an early, complement-independent phase of T-cell tumor infiltration allows few pioneering T cells (among which are many CD4$^+$ cells) to migrate to the tumor, whereas complement activation is critical for the second phase of T-cell homing and infiltration, allowing marked escalation of CD8$^+$ T-cell engraftment.

To evaluate the relative contribution of CD4$^+$ and CD8$^+$ cells to the priming of the tumor endothelium and consequent complement activation, E7-primed CD3$^+$ (1×10^7,

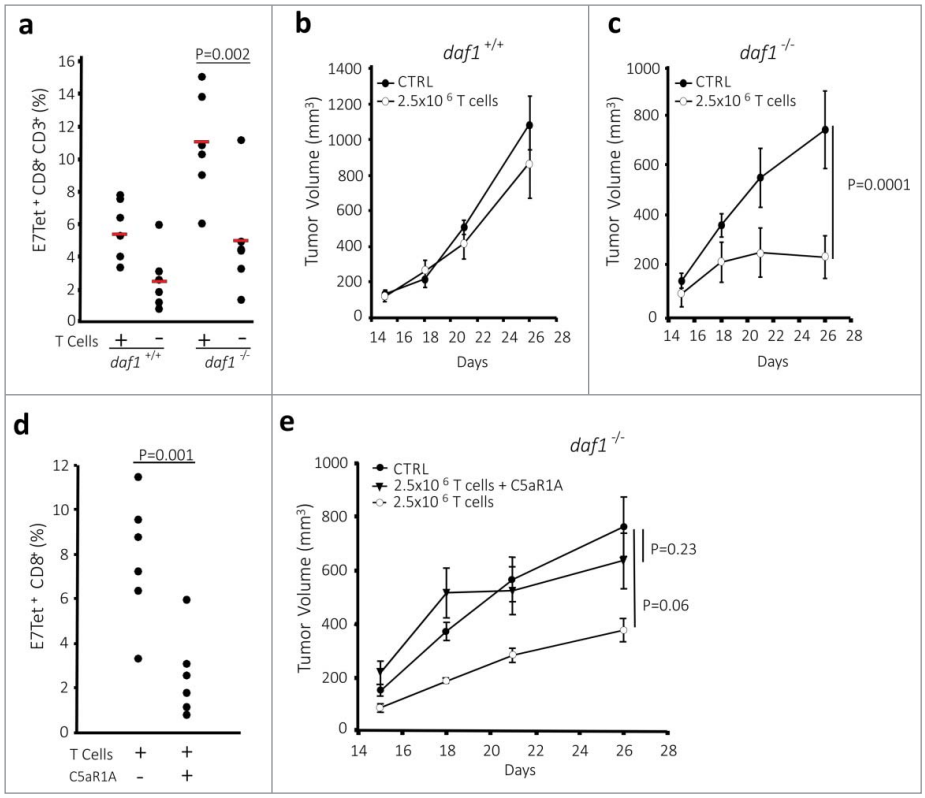

Figure 6. Complement hyperactivation enhances T-cell engraftment in tumors and facilitates tumor rejection by suboptimal numbers of tumor-specific (T)cells. (A) Mice were given an ineffective dose of T cells (2.5 × 10^6 $CD3^+$ T cells/mouse) by adoptive transfer, and the frequency of E7-specific $CD8^+$ cells engrafted in the tumors of *daf1*$^{+/+}$ and *daf1*$^{-/-}$ mice was determined by flow cytometry. (B and C) TC-1 tumor growth curve in *daf1*$^{+/+}$ and *daf1*$^{-/-}$ mice in the absence of treatment (CTRL) or after the transfer of 2.5 × 10^6 E7 primed $CD3^+$ T cells. (D) Some *daf1*$^{-/-}$ mice also received C5aR1 antagonist (C5aR1A), and the number of E7-specific $CD8^+$ cells engrafted in the tumors was determined by flow cytometry. **(E)** TC-1 tumor growth curve in *daf1*$^{-/-}$ mice treated with C5aR1 antagonist (C5aR1A) after the transfer of 2.5 × 10^6 $CD3^+$ T cells/mouse harvested from spleens of donor mice vaccinated with pConE6-E7 DNA.

approximately composed by 4 × 10^6 $CD8^+$ and 6 × 10^6 $CD4^+$ T cells), $CD8^+$ (4 × 10^6), or $CD4^+$ (6 × 10^6) T cells were transferred to wild-type mice bearing TC-1 tumors. The $CD8^+$ and $CD4^+$ cell populations, when transferred alone, failed to establish the second (complement-dependent) phase of escalated tumor engraftment beyond day 5 (Fig. 7C and D) and, unlike $CD3^+$ cells, they failed to suppress tumor growth (Fig. 7E). Although we cannot exclude the possibility that higher doses of $CD8^+$ cells could bypass the tumor's endothelial barrier, at the dose used in our experiment, both $CD4^+$ and $CD8^+$ cells were required in the early (complement-independent) phase in order for the second (complement-dependent) phase of escalated tumor engraftment and the consequent therapeutic effect to occur successfully.

Tumors were examined on day 12 for complement deposition; importantly, enhanced deposition of C3b/iC3b/C3c fragments on the tumor endothelium was seen only after the transfer of whole T cells, and not after the transfer of $CD4^+$ or $CD8^+$ cells alone (Fig. 7F). Thus, enhanced activation of endothelial complement requires cooperation between $CD4^+$ and $CD8^+$ cells. Confirming the role of complement activation in T-cell homing and tumor suppression, we found that the C5aR1 antagonist abrogated both the ability of whole T cells to home to tumors and their therapeutic benefit (Fig. 7D and G), but it had no effect when $CD4^+$ or $CD8^+$ cells were infused alone (Fig. 7D). These results support the previously observed dependency of $CD8^+$ adoptive tranfer on the co-administration of $CD4^+$ cells.[15]

Discussion

Cancer vaccines represent the possibility of awakening the host's immune system to efficiently recognize and kill tumor cells. Thus far, many approaches have been positively tested in preclinical settings,[36-39] and recent improvements, such as the identification of tumor neo-antigens and the exploitation of immune checkpoints, are increasing the suitability of these vaccines for cancer treatment in the clinic.[40,41] T-cell homing and infiltration into the tumor are essential for the success of cancer immunotherapy. Emerging evidence suggests that the tumor endothelium plays a critical function in regulating T-cell traffic: Roles for endothelial adhesion molecules and Th1 cytokines have already been described.[42,43] Tumors shut down endothelial adhesion molecules and upregulate inhibitory and death ligands to prevent T-cell homing.[8] Overcoming the blood-tumor endothelial barrier is thus essential for successful cancer immunotherapy.

Here, we show that tumors are not endowed with an irreversible immune-privileged status, since endothelial quiescence could be reversed by cytokine-mediated activation of the tumor vasculature followed by complement production, local generation of C5a, and consequent upregulation of endothelial adhesion molecules, allowing for efficient T-cell extravasation and infiltration into the tumor. We have shown that effective doses of T cells can thus reverse the endothelial barrier, provided that they can induce endothelial complement activation, which requires the generation of

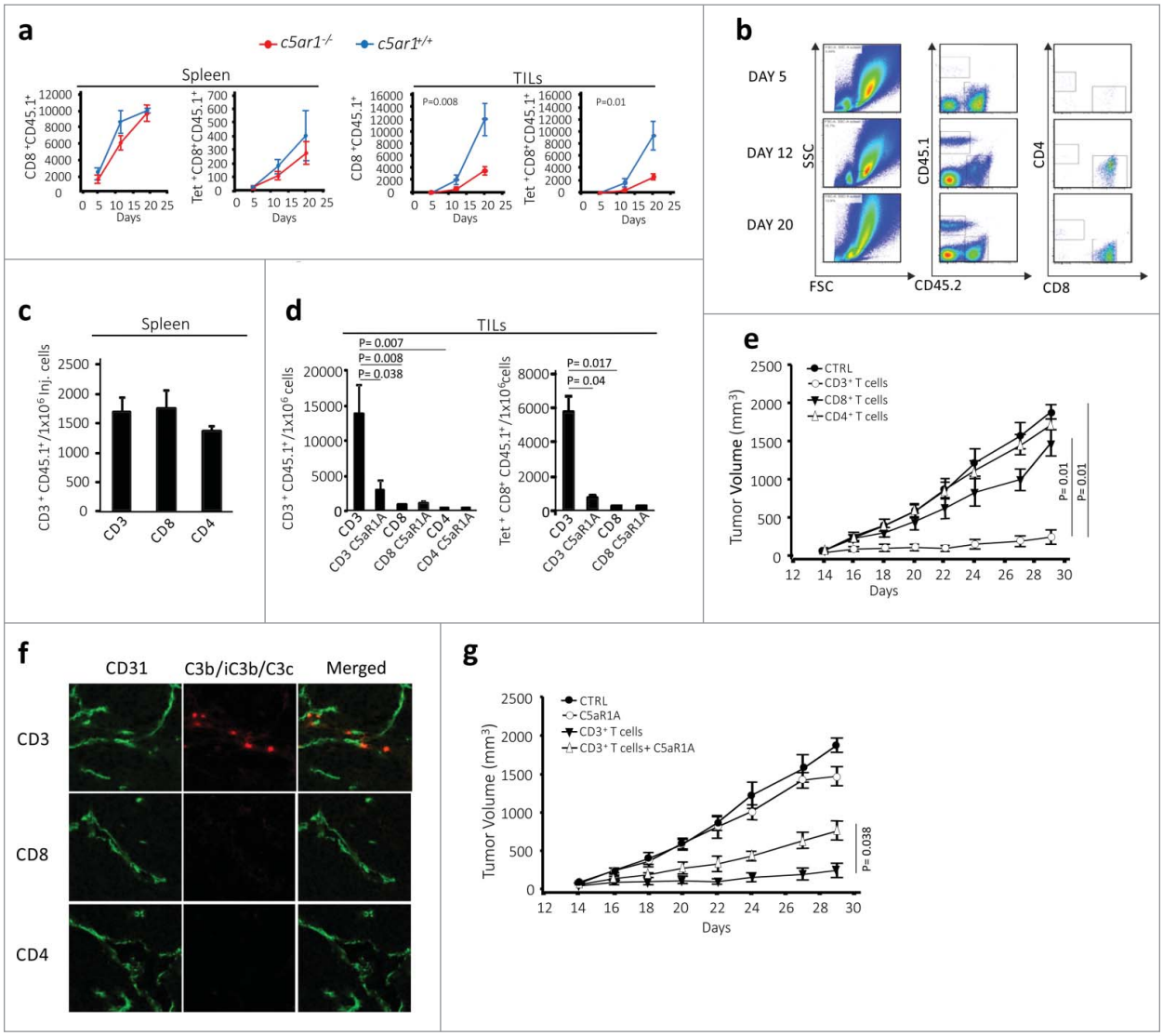

Figure 7. Pioneering CD4$^+$ and CD8$^+$ cells are both required for local complement activation, T-cell homing, and tumor suppression. (A and B) *c5ar1*$^{+/+}$ or *c5ar1*$^{-/-}$ C57BL/6 tumor-bearing mice were treated with 5 × 10^6 CD3$^+$ T cells isolated from the spleen of pConE6E7-vaccinated B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice, and the expansion of the T cells was determined over time in the spleen and tumor. (C–G) C57BL/6 mice were injected s.c. with the TC-1 tumor, and after 1 week they were given total CD3$^+$ (1 × 10^7/mouse), CD8$^+$ (4 × 10^6/mouse) or CD4$^+$ (6 × 10^6/mouse) T cells isolated from pConE6E7 vaccinated B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice by adoptive transfer. (C) Frequency of total CD3$^+$ CD45.1 cells in the spleens of mice receiving a transfer of CD3$^+$, CD8$^+$, or CD4$^+$ T cells. Numbers are normalized for millions of analyzed cells. (D) Frequency of CD3$^+$CD45.1 cells (left) and E7-specific CD8$^+$CD45.1 cells (right) in tumors from mice that received CD3$^+$ or CD8$^+$ cells, followed by treatment with C5aR1 antagonist (C5aR1A). (E) Tumor growth curves of C57BL/6 mice that received CD3$^+$, CD4$^+$, or CD8$^+$ cells. (F) Immunohistochemical staining for the endothelial cell marker CD31 and C3 activation fragments in TC-1 tumor sections from mice given the CD3$^+$, CD4$^+$, or CD8$^+$ cell populations by adoptive transfer. (G) Tumor growth curves of C57BL/6 mice that received CD3$^+$ T cells and treated with C5aR1 antagonist (C5aR1A). CTRL- control mice did not receive any T- cell therapy.

local C3 and C5a in the tumor stroma and vasculature. In turn, C5a can directly act on the endothelium to upregulate CAMs, allowing unopposed extravasation of tumor-reactive T cells. Consistent with this mechanism, effective numbers of E7-primed T cells transferred into mice bearing TC-1 tumors failed to penetrate to tumor sites in *c3*$^{-/-}$ or *c5ar1*$^{-/-}$ or C5aR1A treated WT mice. Furthermore, genetic or pharmacologic blockade of C3 or the C5aR1 in human or mouse endothelial cells impaired cytokine-induced upregulation of adhesion molecules. Collectively, our results identify the C5a activation fragment as a key effector in tumor endothelial activation and T-cell extravasation, consistent with previous evidence that C5a activates the nuclear translocation of NF-κB and upregulates VCAM-1 expression in endothelial cells.[44]

We have previously identified a pivotal role for ET_BR in maintaining the endothelial barrier via the binding of its ligand ET-1, which is overexpressed by nearby tumor cells. This interaction establishes a local, tumor-restricted paracrine axis that suppresses endothelial T-cell adhesion.[7,45] Interestingly, we found that C5a-mediated activation of the C5aR1 on the endothelium was able to override this inhibitory axis, rescuing endothelial adhesion and optimal infiltration of effective T cells into the tumors. ET_BR suppresses the expression of endothelial adhesion molecules via nitric oxide, which upregulates guanylyl cyclase and intracellular cyclic (cGMP) in the endothelium.[7,46,47] Conversely, C5a activates endothelial NF-κB, leading to a reduction in endothelial cGMP and neutralizing the ability of nitric oxide to suppress VCAM-1 expression.[44] Our results indicate that this effect is mediated by C5a rather than

by the deposition of C5b-9 complexes, as previously considered in hypoxia/reoxygenation.[44] This effect likely occurs when enough T cells encounter cognate antigen to release sufficient Th1 cytokines locally, but it can also happen when local complement activation is unopposed, as in *daf1*-deficient mice.

Given that complement activation can override the suppressive effect of ET-1/ET_BR, preservation of the tumor's endothelial barrier would require continuous local complement inhibition in the tumor microenvironment. In the tumor context, VEGF is not only required for angiogenesis and tumor development, but it also promotes endothelial quiescence by upregulating the expression of the complement inhibitor DAF in the endothelium via protein kinase-C, thereby significantly hindering complement deposition in the tumor vasculature.[34,48] Similarly, VEGF overexpression by the tumor is associated with the upregulation of ET_BR expression by the endothelium.[49] As such, these findings are in line with the inverse association between TILs and VEGF expression in ovarian cancers, the increment in the number of TILs when VEGF is inhibited, and the efficacy of combining anti-angiogenic therapy and immunotherapy in cancer.[50-54] It should further be noted that Th1 cytokines also upregulate endothelial DAF.[55,56] Evidently, tumor endothelial barrier mechanisms are finely balanced, and the processes controlling T-cell engraftment and function in tumors are intricate. Our findings cannot exclude the possibility that complement activation also has additional effects on the endothelium or other cell types as well as blood flow.[57]

The proposed model requires that certain tumor-reactive T cells home to and infiltrate the tumors in the presence of low-level or no complement activation, and then after antigen encounter, release cytokines that are able to upregulate the local production of C3 by the endothelium. It is noteworthy that complement activation on the surface of human endothelial cells and in the extracellular matrix has previously been observed after cell stimulation with IFNγ or TNF-α, alone or in combination.[34] Indeed, we found a low-level extravasation of scouting T cells in tumors a few days after adoptive transfer; this process did not require complement activation, since it also occurred in $c3^{-/-}$ mice. However, tumor suppression required a significant amplification of the $CD8^+$ T-cell infiltrate that was found to be complement-dependent, since it failed to occur in $c3^{-/-}$ mice. Furthermore, we observed that the presence of $CD4^+$ T cells was fundamental among the early scouting T cells, and in agreement with prior evidence,[58] we found that it was required for the subsequent amplification of the $CD8^+$ T-cell infiltration that was responsible for TC-1 tumor rejection.[15,58] Since $CD8^+$ and $CD4^+$ T cells differ in integrin expression and their degree of adhesion to the endothelium,[59] it is likely that T-cell subsets also differ in their requirements for complement-dependent endothelial activation for tumor infiltration. Indeed, cytotoxic $CD8^+$ T cells express high levels of the ICAM-1/2 and VCAM-1 receptors CD11a (LFA-1) and CD49d,[59] respectively, implying that $CD8^+$ T cells are particularly dependent on endothelial activation for extravasation. Moreover, the role of $CD4^+$ T cells in the recruitment of $CD8^+$ has been already established in other disease settings. $CD4^+$ cells have been observed to predominate in early multiple sclerosis lesions, whereas $CD8^+$ cells predominate in late lesions,[60] and in a model of experimental autoimmune encephalomyelitis, complement deficiency impairs infiltration of $CD8^+$ but not TNF-α/IFNγ-secreting $CD4^+$ cells in the brain.[61]

In apparent contrast to our findings, a significant body of evidence suggests that complement activation in the tumor microenvironment leads to tumor growth and metastasis. C5aR1-mediated signaling has been implicated in establishing a microenvironment that promotes tumor growth via the recruitment of myeloid-derived suppressor cells (MDSCs) and inhibition of antitumor immune responses mediated by $CD8^+$ and $CD4^+$ T cells.[22,23,25,62] Complement activation has been associated with increased CCL2-mediated recruitment of immunosuppressive macrophages in tumors via CCL2 production.[63] C5a-induced local inflammation has also been shown to support angiogenesis and tumor progression, as demonstrated by the development of small and poorly vascularized tumors in the absence of C3- or C5aR1-mediated signals.[27] In addition to determining a pro-angiogenic and pro-tumorigenic microenvironment, C3a and C5a derived from tumor cells have an autocrine effect on tumor proliferation via the activation of the PI3K/AKT signaling pathway in cancer cells.[24] We have also previously highlighted the role of C3 and complement activation in tumor growth in animals exhibiting low or no spontaneous antitumor immunity. In the absence of antitumor T cells, complement activation promotes tumor growth by recruiting and activating myeloid-derived suppressor cells[22] or proangiogenic B cells.[27]

We now demonstrate an opposite effect for C3 and complement activation in mice that possess a robust antitumor immune response. These superficially contradictory observations illustrate the critical role of complement in permitting tumors to orchestrate leukocyte homing and infiltration. Our study reflects the double-edged role of complement in regulating the trafficking of immune cells peripherally in many diseases, especially cancer. The fate of the tumor depends on whether the infiltrating immune cells are friends or foes.[64] If effective T cells are present, complement facilitates tumor rejection, whereas it would seem to promote inflammation and tumorigenesis when other immune cell types predominate over antitumor T cells.

These results have important therapeutic implications. We have shown that a threshold dose of tumor-reactive T cells is required *in vivo* to circumvent the tumor's endothelial barrier. Although tumor rejection is mediated by $CD8^+$ cells, $CD4^+$ cells have to ensure adequate T-cell engraftment in tumors, and we found that this effect was C5a-dependent. These findings may guide the development of more effective immunotherapy protocols and are in agreement with previous evidence.[15] Tumor vaccines typically induce low numbers of low-avidity T cells; thus, activation of endothelial complement specifically in the tumor endothelium could represent a powerful immune adjuvant strategy to improve the efficacy of both active and passive immunotherapy approaches. Our results might be model or strain dependent and additional work might be required to ultimately prove the relevance of this mechanism in mice and human.

Material and methods

Mice

The $c3^{-/-}$, $daf1^{-/-}$, and $c5ar1^{-/-}$ mice used in our studies have been described previously.[22,35,65,66] Mice were backcrossed for at least nine generations onto the C57BL/6 background, and their homozygous wild-type littermates were used as controls. Wild-type 6- to 8-week-old female C57BL/6 and B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ congenic mice were purchased from the Jackson Laboratory. Mice were housed in a barrier animal facility, with a 12-h light/dark cycle. Water and standard rodent diet were provided *ad libitum*. To generate chimeras, mice were lethally irradiated and injected i.v. with 1×10^7 CD45.1 BM cells isolated from B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ congenic mice.[20] Standard antibiotic was added in the drinking water for 2 weeks. BM engraftment was checked on a regular basis by surface staining of peripheral blood for the CD45.1/CD45.2 markers. All experiments were repeated three times with similar results and included 8–10 mice per group unless stated otherwise. All mouse studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee according to the National Institutes of Health (NIH) guidelines.

In vivo immunotherapy

Mice were inoculated with 1×10^5 TC-1 tumor cells (ATCC, Manassas) s.c. in the back. After 7 d, the mice were vaccinated weekly with a total of three i.m. injections of the pConE6E7 plasmid DNA vaccine (100 μg in PBS) expressing the HPV16 E6 and E7 genes, kindly provided by Dr David B. Weiner[16]; or with Lm-LLO-E7, encoding the HPV16 E7 gene fused with *Listeria monocytogenes* LLO, kindly provided by Dr Yvonne Patterson.[67] In adoptive transfer experiments, $CD3^+$ splenocytes were sorted using a Miltenyi CD3 pan-T cell purification kit and inoculated i.v. into recipient tumor-bearing mice 1 week after the last immunization. CD4 and CD8 positive selection (Myltenyi Biotec) was performed to sort $CD4^+$ and $CD8^+$ fractions out of the $CD3^+$ cell population. Where indicated, mice were treated i.p. with a dose of 300 μg of the ET_BR antagonist BQ-788 (American Peptide) or with the C5aR1 antagonist[68,69] AcF[OPdChaWR] or control inactive peptide AcF[OPdChaA(d)R][27] at 1 mg/kg, every other day for 14 d, beginning 2 weeks after tumor inoculation. Tumor progression was monitored every other day by caliper measurements.

Evaluation of the immune response

ELISPOT was performed as described previously.[70] Briefly, splenocytes (5×10^5/well) were plated in duplicate in 96-well MAIP plates (Millipore) pre-coated with anti-mouse IFNγ antibody (BD PharMingen). Cells were incubated for 20 h at 37°C with 1 μg/mL H2-Db E7 (RAHYNIVTF) or control OVA (SIINFEKL) peptide. Concanavalin A (Sigma) or anti-CD3 (eBioscience) was used at 5 μg/mL as a positive control. Plates were sequentially incubated for 12 h at 4 °C with biotin-conjugated rat anti-mouse IFNγ (BD PharMingen) and for 3 h at room temperature with streptavidin-AKP (BD PharMingen), followed by the addition of NBT/BCIP (Pierce) for color development. Spots were counted using an automated ELISPOT reader (AID, Germany).

For flow cytometry analysis of TILs, tumors were minced and incubated for 30 min at 37 °C under continuous rotation in a RPMI solution containing 1 mg/mL collagenase IV, 0.1 mg/mL hyaluronidase, and 30 U/mL DNAse. The resulting material was passed through a 70-μm cell strainer, followed by washing and pelleting. Cells (5×10^6) were then incubated with anti-mouse CD16/32 (Biolegend) and subsequently stained as described below. E7 tetramer labeling (Beckman Coulter) was performed according to the manufacturer's instructions. Staining for human VCAM-1 (clone: 51–10C9, BD Bioscience) or mouse ICAM-1 (clone: YN1/1.7.4, Biolegend) and mouse VCAM-1 (clone: 429, Biolegend) was performed according to the manufacturer's recommendations.

Intracellular cytokine staining was performed as follows: 5×10^6 splenocytes were incubated with H2-Db E7 or OVA peptide (5 μg/mL) or a combination of anti-CD3 and anti-CD28 antibody (10 μg/mL) in 1 mL RPMI 10% FCS plus 1 μg/mL brefeldin A (BD Biosciences) at 37 °C overnight. Cells were washed, stained for the CD45.1, CD45.2, CD3, CD4, and $CD8^+$ markers (all antibodies from Biolegend), fixed and permeabilized with Perm/Wash Buffer (BD Biosciences), then further stained with anti-IFNγ (eBioscience). Cells were analyzed on a FACS-Canto flow cytometer (Becton Dickinson) with FlowJo software, version 10.

T-cell cytotoxicity was measured by ^{51}Cr release assay. T cells isolated from the spleen of vaccinated mice were re-stimulated for 5 d with E7 peptide (MLPC), counted, and co-cultured with ^{51}Cr-labeled MBL-2 cells that had been previously pulsed with the E7 or OVA peptide. T cells were mixed at different ratios with MBL-2 target cells, and after 5 h of co-culture, the supernatants were harvested to measure ^{51}Cr release.

In vitro cell assays

For lymphocyte–endothelial adhesion assays, HUVECs were grown to 40% confluence in 96-well plates (Biocoat laboratory) in EGM2 medium (Cambrex) and treated with recombinant human endothelin 1 (ET1; 10 nM, Anaspec), BQ-788 (200 nM-American Peptide Company), C5aR1A (20 μM), Compstatin-analog CP30[71] (100 μM), or control peptide for 24 h, followed by the addition of TNF-α (20 ng/mL, Sigma) for 4 h. Where indicated, HUVECs cells were stimulated with 50 ng/mL of recombinant C5a (Sigma) according to literature.[72,73] Control HUVECs were kept in EGM2 medium (Cambrex) or treated with TNF-α alone. Cells were rinsed twice, supplemented with fresh EGM2 medium, and 2×10^5 T lymphocytes were added to the plates. Human peripheral blood lymphocytes were obtained through elutriation of blood from normal donors. Before use every batch of recombinant C5a was tested for LPS contamination using the Pyrosate LPS detection kit (sensitivity of 0.03 EU/mL). When required, T cells were stimulated with anti-CD3/CD28 (2 μg/mL plate-bound anti-CD3, clone OKT3, Biolegend; and 1 μg/mL soluble anti-CD28, clone 15E8, Sigma) in RPMI-1640 for 48 h. T cells (>97% pure) were labeled with Carboxyfluorescein succinimidyl

ester (CFSE) (Sigma), washed, allowed to adhere to pre-treated HUVECs for 1 h, and washed five times with PBS. Fluorescence was detected in 96-well plates using a Packard FluoroCounter microplate reader. Where indicated, ICAM-1- (clone LB-2, 20 μg/mL, R&D) or VCAM-1-neutralizing antibodies (clone m/k-2.7, 20 μg/mL, R&D) were added to the HUVEC culture before the addition of T cells. To measure the expression levels of adhesion molecules, HUVECs were plated in 6-well plates pre-coated with 1% gelatin and treated with IFNγ (5 ng/mL, Sigma) for 1 h before the addition of T cells. The presence of complement components in the supernatant of HUVECs was measured using a C3 (Genway) or C5a (R&D Duoset) ELISA kit. Primary mouse lung microvascular endothelial cells were isolated from the lungs of *c3*$^{-/-}$, *c5ar1*$^{-/-}$, and wild-type mice. Lungs were minced with scissors and digested with Collagenase/Dispase (Roche) according to the manufacturer's instructions. Primary microvascular endothelial cells were obtained through sequential magnetic depletion of CD45^{+} cells and enrichment of CD31^{+} cells (Miltenyi Biotec). To measure the expression levels of adhesion molecules, primary lung endothelial cells were stimulated for 6 h with TNF-α (5ng/mL, Peprotech) or IFNγ (125U/mL, Peprotech), alone or in combination. All *in vitro* experiments were repeated in duplicate for three times with similar results.

Immunohistochemistry

Tumors were flash-frozen in optimal cutting temperature medium (Tissue-Tek, Sakura), and 5-μm sections of frozen tissue were cut on a cryotome. Sections were incubated with anti-mouse CD3 (clone sp7, Sigma, 1:100 dilution) and anti-rabbit IgG (Sigma, 1:1000 dilution), or biotin-conjugated anti-mouse-CD45.1 (clone A20, Sigma, 1:250 dilution) and HRP-conjugated streptavidin (Sigma, 1:1000 dilution). CD3 or CD45.1 infiltration was quantified by manual counts in 10 representative fields (30X) of five independent samples per group. For immunofluorescent assessment of complement activation, sections were stained sequentially for mouse complement proteolytic fragments C3b/iC3b/C3c (clone 2/11, Hycult Biotech, 1:200 dilution) and Alexa Fluor 647-conjugated anti-rat IgG (Invitrogen, 1:1000 dilution), and Alexa Fluor 488-CD31 (BD PharMingen) for the vascular endothelial marker CD31 (DAPI nuclear counter stain was applied at the time of coverslipping). A total of 12 $\times$ 200 high-power fields were imaged for each of three tumor samples per mouse from three mice in each group, and the spectral components were deconvoluted using the Nuance FX multispectral imaging system (Cambridge Research and Instrumentation). Regions of interest were designated manually containing approximately 20 nuclei each. These were arbitrarily designated without regard to C3b/iC3b/C3c staining. The C3b/iC3b/C3c fluorescence intensities were quantified automatically by the Nuance FX multispectral imaging system. A similar method was used to quantify human C3b/iC3b/C3c (clone 1H8/C3b, Biolegend, 1:50 dilution) deposition on HUVECs *in vitro*. Approximately 8 $\times$ 10^{3} HUVECs were plated in a Lab-Tek II chamber slide system pre-coated with 1% gelatin, and treated for 16 h with IFNγ (5 ng/mL, Sigma), TNF-α (20 ng/mL,Sigma), or supernatants of human T-cell cultures co-stimulated with anti-CD3/CD28 (2 μg/mL plate-bound anti-CD3 and 1 μg/mL soluble anti-CD28, Biolegend).

Isolation of human tumor endothelial cells and real-time PCR

TECs were sorted from tumors with a MoFlo Cell Sorter (Cytomation) as the CD146^{+}VE^{-}cadherin^{+}CD45^{-} population, as described previously.[74] RNA from TECs (>95% pure), HUVECs, or whole tumor specimens was isolated using TRIzol. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) as detailed elsewhere[75] using the following primers: 5′–ACACTACCTCATGTGG GGTCTC–3′ (forward); 5′–GGCATTGTTTCTGGTTCTCT TC–3′ (reverse). All transcripts were confirmed using 3% agarose gel electrophoresis. In all cases, expression was normalized to β-actin: 5′–ACACAGGGGAGGTGATAGCATT–3′ (forward); 5′–ATACATCTCAAGTTGGGGGACAA–3′ (reverse). Relative gene expression was calculated using the comparative Ct method ($2^{\text{delta (Ct-treated- Ct-untreated)}}$).

Statistical analyses

Descriptive statistics were performed with SPSS®. *In vitro* experiments and *in vivo* tumor volumes were analyzed using ANOVA with Tukey post-tests to compare all conditions, and with Student's *t*-test. All experiments were repeated three times; in the case of *in vivo* studies, each group consisted of 8–10 animals, unless otherwise noted. All figures portray one representative experiment.

Disclosure of potential conflicts of interest

J.D. Lambris is inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes. J.D. Lambris is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors. Other authors declare no financial interest or conflict.

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