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The microvascular niche instructs T cells in large vessel vasculitis via the VEGF-Jagged1-Notch pathway

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Abstract

Microvascular networks in the adventitia of large arteries control access of inflammatory cells to the inner wall layers (media and intima) and thus protect the immune privilege of the aorta and its major branches. In the autoimmune vasculitis giant cell arteritis (GCA), CD4 T helper 1 (T_H1) and T_H17 cells invade into the wall of the aorta and large elastic arteries to form tissue-destructive granulomas. Whether the disease microenvironment provides instructive cues for vasculitogenic T cells is unknown. We report that adventitial microvascular endothelial cells (mvECs) perform immunoregulatory functions by up-regulating expression of the Notch ligand Jagged1. Vascular endothelial growth factor (VEGF), abundantly present in GCA patients' blood, induced Jagged1 expression, allowing mvECs to regulate effector T cell induction via the Notch-mTORC1 (mammalian target of rapamycin complex 1) pathway. We found that circulating CD4 T cells in GCA patients have left the quiescent state, actively signal through the Notch pathway and differentiate into T_H1 and T_H17 effector cells. In an in vivo model of large vessel vasculitis, exogenous VEGF functioned as an effective amplifier to recruit and activate vasculitogenic T cells. Thus, systemic VEGF co-opts endothelial Jagged1 to trigger aberrant Notch signaling, biases responsiveness of CD4 T cells, and induces pathogenic effector functions. Adventitial microvascular networks function as an instructive tissue niche, which can be exploited to target vasculitogenic immunity in large vessel vasculitis.

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Introduction

Giant cell arteritis (GCA) is an autoimmune inflammation of the blood vessels (vasculitis) that targets vital vascular territories, specifically the aorta and its branch vessels (1, 2). Prototypic granulomatous lesions within the walls of affected arteries consist of CD4 T cells, macrophages and histiocytes, often presenting as multinucleated giant cells, implicating both the adaptive and innate immune systems in key pathogenic events (3–5). Despite the pathognomonic granulomatous formations, no disease-inducing antigen has been identified, and therapeutic management currently depends on broad and nonspecific immunosuppression given for many years (2, 6).

Besides inflammatory cells, vessel wall indigenous cells are now recognized for their pathogenic contributions. Specifically, endothelial cells (ECs) interact with T cells in a tightly controlled and antigen-specific manner (7). Dendritic cells (DCs) located between the tunica media and adventitia act as antigen-presenting cells and shape the disease lesions through chemokine and cytokine production (5, 8). Vascular smooth muscle cells (VSMCs) participate in immunostromal interactions, functioning as signal-sending and signal-receiving partners (9). A critical disease pathway is the fast growth of myofibroblasts accumulating as concentric hyperplastic intima, which occlude the vascular lumen and induce ischemic organ damage. Lesional macrophages and VSMCs serve as a source of platelet-derived growth factor and fibroblast growth factor (FGF), promoting VSMC expansion and migration (10, 11).

Although pathologic events focus on the media and intima, the adventitia and its cellular contents hold a key regulatory role in vascular diseases (9, 12–15). In GCA, interferon- γ (IFN- γ)–producing T cells preferentially sit in the adventitia and adventitial macrophages are the major suppliers of interleukin-1 β (IL-1 β) and IL-6 (16). Adventitial vasa vasorum, the network of small blood vessels that supply large arteries with oxygen and nutrients, not only control trafficking of T cells and macrophages into the arterial wall but also participate in the process of neovascularization, populating the inflamed media and the rapidly expanding and space-occupying intimal layer with neocapillaries (17). Whether ECs per se have a pathogenic role in GCA is unexplored.

T cells in the vascular lesions of GCA have a strong bias toward T helper 1 (T_H1) and T_H17 effector functions, with T_H2 cells essentially absent (9, 18). Recent reports that GCA patients are deficient in a population of CD8 regulatory T cells have shifted attention toward systematic abnormalities in T cell biology (19). Here we have explored whether an altered threshold setting in CD4 T cell activation could render GCA patients susceptible to T cell hyperresponsiveness and promote formation of chronic inflammatory lesions in an otherwise inaccessible tissue site.

Efforts to understand transcriptional programs that regulate T cell differentiation and function have pinpointed the Notch signaling pathway as a core element shared by multiple T cell subsets and functional lineages (20–23). Initially identified as a key determinant of cell lineage commitment in developing lymphocytes. Notch is now recognized as a broad regulator for differentiating T cells, able to support differentiation into T_H1, T_H2, T_H9, and

T_H17 effectors (24). To capitalize on the versatility of Notch signaling, introducing the Notch1 receptor into T cells for the engineering of antitumor effector cells (25). Tissue-residing T cells in GCA express Notch1 (26), but whether Notch1 signaling participates in conditioning of CD4 T cells to acquire disease-relevant functions is unknown.

Notch is recognized as a master regulator of the development, the activation and the function of vascular ECs. Endothelial expression of the Notch ligands Jagged1 and Delta-like 4 controls developmental as well as adult physiologic angiogenesis by regulating EC expansion, survival and stability and thus, the density of capillary networks and vascular patency (27, 28). Although endothelial Jagged1 is well established as a regulator in vessel sprouting and tip cell formation, recent work has emphasized Jagged1's impact on defining vascular niches, which deploy regulatory control over the tissue microenvironment (29). Expressed on pulmonary capillaries, endothelial Jagged1 promotes lung alveolar repair and regulates tissue fibrosis (30). Endothelial Jagged1 is required in the adult bone marrow to protect the self-renewal and regenerative capacity of hematopoietic stem cells (31). In addition, endothelial Jagged1 defines tumor vascular niches to induce B cell lymphoma invasiveness and chemoresistance (29). In essence, endothelial Jagged1 provides an important platform for heterocellular cell-cell contact, enabling cross-talk between vascular structures and Notch-expressing cells in the tissue environment.

Here, we report that endothelial Jagged1 expressed on adventitial vasa vasorum networks defines a tissue niche that controls the activation status of the adaptive immune system regionally and systemically. In patients with GCA, excess circulating vascular endothelial growth factor (VEGF) induces expression of the Notch ligand Jagged1 in perivascular microvessels. This results in the activation of Notch1 receptor-expressing CD4 T cells, their differentiation into proinflammatory T_H1 and T_H17 effector cells and their invasion into the currently unprotected arterial wall layers. Targeting one of the elements in the VEGF-Jagged-Notch axis could disrupt the state of chronic immune stimulation in GCA patients and protect them from tissue-destructive immunity.

Results

Microvascular ECs in the vasa vasorum networks of GCA-affected arteries express the Notch ligand Jagged1

Vasa vasorum networks in large elastic arteries such as the aorta provide access to the arterial wall, an immunoprivileged region usually protected from inflammation. In vasculitic arteries, the immunoprivilege is breached, enabling the formation of transmural inflammatory lesions (Fig. 1A). T cells enter the vessel wall from the adventitia and penetrate into all tissue layers, often accumulating on the outside of the difficult-to-pass elastic membranes (Fig. 1A). In contrast, healthy (normal) medium and large human arteries are T cell-free (Fig. 1A) (32). Comparative gene expression profiling in arteries with granulomatous vasculitis (GCA-positive) and normal elastic arteries (GCA-negative) revealed high expression of the Notch ligand Jagged1 in the vasculitic vessels (Fig. 1B). Transcripts for the Notch ligands Delta-like 1 and Delta-like 4 were barely detectable in inflamed and normal arteries, with Delta-like 1 slightly increased in the GCA-positive vessels (Fig. 1B). Jagged1- and Delta-like 1-expressing cells were identified by dual color

immunostaining (Fig. 1, C and D). In line with gene expression studies, staining signals for Delta-like 1 protein were minimal (Fig. 1D), whereas Jagged1 protein was strongly expressed in GCA-affected temporal arteries and aortic wall (Fig. 1C). Most Jagged1⁺ cells were localized in the adventitial layer and, by dual-color staining, expressed the endothelial marker CD31. The adventitia contained Jagged1⁺CD31⁻ cells and not all intramural microvessels were positive for Jagged1 protein expression. ECs lining the macrolumen of the temporal artery rarely had Jagged1⁺ staining. In some GCA arteries, medial VSMCs had a weak signal for anti-Jagged1 antibody binding. In artery sections from non-GCA patients, neither macrovascular ECs nor microvascular ECs (mvECs) had detectable Jagged1 staining (Fig. 1C). Thus, a characteristic feature of mvECs in vasculitic arteries is the ample expression of Jagged1 protein.

Circulating VEGF in GCA patients induces Jagged1 expression on ECs

Abundant Jagged1 expression on mvECs may be the result of the inflammatory environment in GCA (33). To identify possible Jagged1 inducers, we probed the effect of patient-derived plasma on endothelial Jagged1 expression. Human cardiac mvECs (HMVECs), as well as human umbilical vein endothelial cells (HUVECs), responded to GCA patient-derived plasma with up-regulation of Jagged1 transcripts and protein (Fig. 2, A and B). Plasma collected from age-matched healthy controls and from patients with the inflammatory syndrome rheumatoid arthritis (RA) served as controls, and both controls failed to induce Jagged1 expression in ECs (Fig. 2, A and B). Transcripts for Delta-like 1 were distinctly low in HMVECs and HUVECs and increased to slightly higher amounts in response to plasma from RA and GCA patients (Fig. 2A). In subsequent experiments, we identified IL-6 as a potential inducer for Delta-like 1 but not Jagged1 (fig. S1). None of the plasma sources were able to induce Delta-like 4 transcripts (Fig. 2A). RA and GCA plasma treatments up-regulated EC activation markers *ICAM1*, *VCAM1* and *CDH5* (vascular endothelial cadherin) (fig. S2), but only GCA patient plasma increased Jagged1 mRNA and protein expression (Fig. 2, A and B).

Having observed that GCA plasma converted ECs into Jagged1⁺ ECs, we wondered which constituent(s) in the GCA plasma could drive this process. GCA plasma contains high amounts of VEGF, which correlates with the incidence of ischemic events (34). Quantification of GCA plasma samples confirmed a fourfold increase in circulating VEGF concentrations (Fig. 2C). Samples collected from patients with RA revealed VEGF concentrations indistinguishable from healthy controls, providing an explanation for the selectivity of GCA plasma in up-regulating Jagged1. We tested whether human recombinant VEGF (hVEGF) protein could mimic the Jagged1-inducing capability of GCA plasma and saw robust induction of Jagged1 expression on HMVECs and HUVECs (Fig. 2D). We used the tyrosine kinase inhibitor axitinib, which blocks VEGF receptor signaling (35), and bevacizumab, an anti-VEGF neutralizing antibody (36), to mechanistically link circulating VEGF and Jagged1 induction. Both axitinib and bevacizumab inhibited Jagged1 induction in ECs (Fig. 2, E and F).

To test whether hVEGF and GCA plasma had similar effects on tissue-embedded ECs, we cultured normal medium-sized arteries in culture medium containing hVEGF or GCA

plasma for 5 days. Adventitial mvECs up-regulated Jagged1 protein when exposed to either hVEGF or GCA plasma (Fig. 2, G and H). Conversely, normal plasma failed to modulate Jagged1 on the surface of adventitial microvessels. Notably, blocking VEGF activity by anti-VEGF efficiently reduced GCA plasma-dependent Jagged1 induction on adventitial mvECs (Fig. 2H). These data implicated circulating VEGF in regulating EC function of adventitial vasa vasorum networks in GCA.

The Notch pathway is constitutively activated in GCA CD4 T cells

T cells and activated macrophages are key pathogenic drivers in the granulomatous vasculitis GCA. The vast majority of blood vessel wall-infiltrating T cells in GCA are CD4⁺CD45RO⁺ memory T cells (37). The pathogenic potential of such lesional CD4 T cells depends on their longevity and persistent activation. Memory CD4 T cell survival is controlled by Notch signaling (38). By considering the abundance of the Notch ligand Jagged1 on adventitial microvessels, we evaluated the status of Notch signaling in CD4 T cells from patients with active GCA. *NOTCH1* was up-regulated >4-fold in vasculitic tissues from GCA-positive arteries versus GCA-negative arteries (Fig. 3A), whereas *NOTCH4* transcripts were essentially undetectable in noninflamed and inflamed arteries. We explored whether GCA T cells express Notch1 receptor before infiltrating into the vessel wall. Compared to age-matched healthy donors, circulating CD4 T cells in GCA patients expressed increased surface Notch1 receptor as well as the Notch target protein HES1 (Fig. 3, B to D). One to 2% of healthy CD4 T cells were Notch1⁺, whereas 8 to 20% of CD4 T cells in GCA patients expressed the receptor (Fig. 3B). The extent of Notch1 and HES1 expression in CD4 T cells of individual patients was closely correlated (Fig. 3E). Gating on CD3⁺CD4^{neg} T cells revealed that Notch1 expression was unique for CD4 T cells and was not shared with CD8 T cells (fig. S3). In addition, patient-derived and healthy control CD4 T cells were essentially negative for Notch4 (fig. S4).

Frequencies of Notch1⁺ cells were expanded among CD45RA⁺ naïve and CD45RA⁺ memory CD4 T cells, but Notch pathway activation was insufficient to convert naïve into memory T cells; frequencies of CD45RA⁺ memory CD4 T cells were indistinguishable in healthy controls and GCA patients (Fig. 3F and fig. S5). To understand the functional impact of Notch pathway activation, we quantified the frequencies of CD4 T cells that had made a commitment to effector cell differentiation. CD4 T cells expressing the lineage-determining transcription factors T-bet and RORγt were significantly expanded ($P = 0.04$ and $P < 0.001$, respectively) in GCA patients (Fig. 3G). Multiparametric flow cytometry assigned T-bet and RORγt expression almost exclusively to Notch1⁺ T cells (Fig. 3H). In essence, circulating CD4 T cells in GCA patients have up-regulated Notch1 receptor and are actively signaling through the Notch pathway. Such CD4⁺Notch1⁺ T cells are partially activated and have entered effector differentiation.

mvECs conditioned with GCA plasma induce effector T cell function

To understand whether microvascular endothelial Jagged1 expression has functional relevance for Notch1⁺ CD4 T cells, we investigated whether plasma-conditioned ECs are able to induce T effector cells. To standardize T cell receptor signaling and mimic antigen recognition, we loaded plasma-pretreated ECs with anti-CD3 antibody and

cocultured with purified CD4 T cells from healthy individuals for 4 days (Fig. 4A). The plasma-induced increase in Jagged1 expression was stable over the entire culture period (fig. S6). To test for effector functions, we analyzed T cells for lineage-determining transcription factors or intracellular cytokines. Together with an anti-CD3-mediated signal, HMVECs pretreated with GCA plasma up-regulated both T-bet and ROR γ t expression in CD4 T cells, whereas the frequency of GATA3⁺CD4⁺ T cells remained unaffected (Fig. 4, B to F). Neither healthy control plasma nor RA plasma could endow T cell-activating capabilities upon ECs. To examine which T cell subsets responded to the conditioned ECs, we purified CD4⁺CD45RO⁻ naïve and CD4⁺CD45RO⁺ memory T cells, before overlaying them on the plasma-treated endothelial monolayers. A small proportion of naïve CD4 T cells acquired T-bet expression when exposed to GCA-plasma conditioned ECs (Fig. 4G). Most of the responding CD4 T cells had a memory phenotype. About one-third of the memory CD4 compartment turned positive for T-bet expression and about 8% of memory CD4 T cells made a commitment to ROR γ t expression. Less than 1% of naïve CD4 T cells and 2–10% of memory CD4 T cells reached a T-bet⁺ or ROR γ t⁺ effector status when interacting with control-plasma conditioned ECs (Fig. 4G). Protein expression of the lineage-determining transcription factors was closely associated with the ability to secrete IFN- γ or IL-17 (Fig. 4, H to K). mvECs pretreated with GCA plasma induced IFN- γ production in 16% of interacting CD4 T cells (Fig. 4, H to I) and IL-17 production in 7.5% of CD4 T cells (Fig. 4, J and K). This process depended on the presence of anti-CD3 antibodies and was the most efficient when the ECs were preconditioned with GCA plasma. In all experiments, RA plasma was indistinguishable from the effects imposed by plasma from healthy controls. For both controls, frequencies of CD4 T cells with intracellular IFN- γ stayed at 10% (Fig. 4H), and only 2% had a positive signal for intracellular IL-17 (Fig. 4J). GCA plasma-conditioned HMVECs were even more potent in biasing CD4 T cells toward proinflammatory effector functions when the T cells originated from GCA patients (Fig. 4L). More than one-third of GCA CD4 T cells became positive for detectable T-bet and 20% committed to ROR γ t expression (Fig. 4L). Similar to HMVEC-induced T cell differentiation, HUVECs were able to function as antigen-presenting cells and convert CD4 T cells into IFN- γ and IL-17 producers, once the HUVEC were pretreated with GCA plasma (fig. S7). In contrast, IL-6-conditioned ECs, which express increased Delta-like 1 but not Jagged1 (fig. S1), failed to instruct T effector cells (fig. S8). We examined whether the excess VEGF in GCA patients directly affected T cell function. Exogenous VEGF added to EC–T cell cocultures had no effect on T cell activation (fig. S9). These data implicate Jagged1⁺ ECs in promoting proinflammatory T cells, specifically fostering activation and differentiation of T_H1 and T_H17 cells.

EC-induced T effector cell activity depends on Jagged-Notch signaling

By considering the impact that GCA plasma-exposed ECs had on T cell functional behavior, we explored whether interaction between Jagged1⁺ ECs and Notch1⁺ T cells participated in inducing effector T cells. To seek evidence for Notch pathway activation, we first analyzed induction of the Notch target protein HES1. HMVECs pretreated with control or RA plasma were indistinguishable, but exposure to GCA plasma, and thus induction of Jagged1, enabled interacting CD4 T cells to up-regulate HES1 protein expression (Fig. 5, A and B). We subsequently interfered with Jagged-Notch pathway activation through four strategies:

blocking access to Jagged1 with a monoclonal antibody (Fig. 5, C and D); hindering Notch-dependent signaling through the γ -secretase inhibitor DAPT (Fig. 5E–F) (26), blocking Notch trafficking and processing via FLI-06 (Fig. 5G) (39), and knocking down Notch1 expression by small interfering RNA (siRNA) technology (Fig. 5H and fig. S10). All four interventions effectively blocked the induction of T-bet⁺ and ROR γ ⁺ CD4 T cells in the EC–T cell cocultures. Inhibiting access to the Jagged1 ligand prevented the up-regulation of lineage-determining transcription factors and, thus, the functional differentiation of the interacting T cells (Fig. 5, C and D, and fig. S11), implicating Jagged1 as an essential facilitator of EC–T cell interaction. Notch1 knockdown in GCA CD4 T cells was even more effective in preventing EC-induced activation than in healthy CD4 T cells (Fig. 5H), in line with the constitutive up-regulation of Notch1 in patient-derived cells. Including axitinib or anti-VEGF in the pretreatment of ECs with GCA plasma brought the frequencies of T-bet⁺ and ROR γ ⁺ T cells down to those found with control-treated ECs (Fig. 5, I and J). Notch pathway activation did not occur if the ECs were pretreated with RA plasma, excluding systemic inflammatory disease as the sole mechanism of EC conditioning (fig. S12).

Notably, Notch1 receptor expression on CD4 T cells was itself subject to EC-dependent regulation. The density of Notch1 receptor protein on the surface of CD4 T cells doubled when they interacted with GCA plasma-pretreated EC (fig. S13), suggesting a feed-forward amplification loop, which enhances Notch1 expression when Jagged1 is available for engagement. Together, the data implicate endothelial Jagged1 in triggering Notch signaling activity in communicating CD4 T cells, shaping the functional behavior of such CD4 T cells.

EC-driven T effector cell induction relies on Notch-dependent mammalian target of rapamycin complex 1 activation

CD4 T cells encountering GCA plasma-conditioned mvECs were biased to differentiate into proinflammatory effector cells, choosing either the T_H1 or the T_H17 functional lineage while avoiding up-regulation of GATA3. Mammalian target of rapamycin complex 1 (mTORC1) activation acts as a critical checkpoint to guide T cells toward proliferative and inflammatory activity (40). mTORC1 activation results in phosphorylation of ribosome protein S6 (pS6) by S6 kinase (40, 41). We therefore tested whether preconditioning of HMVECs with GCA plasma affects the phosphorylation status of S6 in CD4 T cells. T cells in contact with GCA plasma-pretreated HMVECs responded with prompt up-regulation of pS6, indicating activation of mTORC1 (Fig. 6, A and B). This response was dependent on Notch pathway signaling, and was abrogated when Notch processing was inhibited by DAPT (Fig. 6, C and D). Further evidence for the contribution of the AKT-mTORC1 pathway came from experiments in which the CD4 T cells were incubated with either the AKT inhibitor VIII or the mTORC1 inhibitor rapamycin. Both series of experiments confirmed that induction of T-bet⁺CD4⁺ and ROR γ ⁺CD4⁺ T cells required the AKT-mTORC1 signaling pathway (Fig. 6, E to H). Rapamycin consistently minimized T_H1 and T_H17-committed effector T cells (Fig. 6, E and F). Rapamycin's suppressive effect was maintained if HUVECs were used as ECs (fig. S14). To overcome nonspecific effects associated with the small-molecule inhibitors, we used siRNA targeting *RAPTOR* to show that T-bet and ROR γ induction in T cells depended on mTORC1 (Fig. 6, I and J).

To explore the relevance of these findings for GCA patients, we recruited a cohort of 23 individuals with active GCA and age-matched healthy controls and quantified protein expression of pS6 in freshly isolated CD4 T cells. In GCA patients, spontaneous pS6 mean fluorescence intensities were at least 1 SD higher than in the control cohort (Fig. 6, K and L). We correlated the intensity of pS6 in circulating CD4 T cells in individual patients with Notch1 receptor expression and the frequencies of T-bet⁺ and ROR γ ⁺ CD4 populations. High expression of pS6 in CD4 T cells predicted high expression of Notch1 and expansion of T-bet and ROR γ -expressing effector T cells (Fig. 6, M to O). Overall, T cells from GCA patients have constitutive activation of the Notch-AKT-mTORC1 signaling axis, sustained by aberrant Jagged1 expression on mvECs.

VEGF exacerbates vascular inflammation in human artery-severe combined immunodeficient mouse chimeras

To directly examine the disease-amplifying effects of VEGF in vivo, we induced vasculitis in NOD-nonobese diabetic-*scid*IL-2R γ ^{null} (NSG) mice engrafted with human axillary arteries and reconstituted with PBMCs from patients with active GCA (26, 42). This model system recapitulates T cell-dependent vasculitis in human arteries. Healthy T cells fail to invade the human artery, but patient-derived T cells induce robust vasculitis with tissue gene expression profiles resembling those in temporal artery biopsies from GCA patients. Factors defining the vasculitogenic potential of patient-derived T cells are only partially understood (42). Immunohistochemical evaluation of tissue sections and comparison of gene expression profiles confirmed that patient-derived T cells (identified through *TRB* mRNA and CD3) and macrophages (identified through *CD68* mRNA) infiltrated into the engrafted human artery (Fig. 7). Chimeric mice carrying vasculitic arteries were treated with recombinant hVEGF to mimic the oversupply of the growth factor in GCA patients, which was given either alone or combined with axitinib to inhibit VEGF receptor signaling. Infusion of VEGF resulted in marked up-regulation of tissue Jagged1 expression and increased vessel wall-infiltrating T cells (Fig. 7, A to E). With increasing density of wall-infiltrating T cells, human *NOTCH1* transcripts were elevated as well (Fig. 7A). Immunohistochemical studies confirmed that VEGF increased T cell accumulation in the vascular wall layers, an effect abrogated by the co-administration of the VEGF receptor blocker axitinib (Fig. 7C). As measured by in situ dual-color immunohistochemistry, about 25% of tissue-infiltrating T cells had activated Notch1; VEGF administration resulted in almost 90% of tissue-resident T cells activating the Notch signaling pathway (Fig. 7D). Cotreatment with axitinib effectively counteracted the Notch-activating signal (Fig. 7D). The exacerbation of vasculitis and Notch activation in the tissue-resident T cells went hand-in-hand with the appearance of Jagged1 protein on CD31⁺ ECs. VEGF administration induced Jagged1 on most of the CD31⁺ ECs, whereas axitinib prevented endothelial Jagged1 expression (Fig. 7E). VEGF-induced intensification of the vasculitic response was associated with increased tissue expression of T-bet and IFN- γ as well as IL-17 and ROR γ transcripts (Fig. 7, F and G). Evidence for augmented innate immune responses came from the up-regulation of tumor necrosis factor- α (TNF- α) and IL-6 mRNA in the inflamed arteries (Fig. 7H). Counteracting the effect of VEGF by injecting the VEGF receptor blocker axitinib was effective in suppressing inflammatory activity: Arteries from axitinib-treated animals had few tissue-infiltrating T cells, and expressions of T cell effector molecules (IFN- γ and IL-17) as well as innate

cytokines (TNF- α and IL-6) were markedly reduced (Fig. 7, B to H). Quantification of GATA3, IL-4 and forkhead box P3 demonstrated that T_H2-committed cells and regulatory T cells were distinctly low in the inflamed vessel wall (Fig. 7, I and J). Spontaneously, 40% of tissue-resident T cells produced IFN- γ and more than 80% of lesional T cells differentiated into IFN- γ producers if the chimeras were treated with VEGF (Fig. 7K). This shift in the differentiation of tissue-resident T cells was almost completely offset by cotreatment with axitinib (Fig. 7K). Aberrant VEGF increased T_H1 and T_H17 immunity but did not broaden the inflammatory responses to additional T cell lineages. In summary, the angiogenic growth factor VEGF can mediate immunoregulatory functions by enabling mxECs to activate and instruct CD4 T cells.

Discussion

Here, we define a vascular niche in the adventitial layer of human arteries which has immunoregulatory functions. By up-regulating Jagged1, adventitial mvECs educate circulating CD4 T cells and induce a state of incomplete T cell activation. Notch1 engagement on CD4 T cells induces the lineage-defining transcription factors T-bet and ROR γ , biasing the CD4 T cell repertoire toward T_H1 and T_H17 and rendering the host susceptible to excessive inflammatory activity. In GCA, this manifests as a wall-destructive granulomatous inflammation in vital arteries and life-threatening clinical complications(9). Aberrant T cell stimulation through adventitial microvessels requires three components, which co-occur in GCA patients: (i) excess circulating VEGF; (ii) up-regulation of Jagged1 on adventitial ECs; and (iii) Notch1 expression on CD4 T cells. Implicating a vascular niche in sustaining inappropriate T cell activation through Notch signaling opens multiple new routes of immunomodulatory therapy.

VEGF is a master regulator of angiogenesis and vascular permeability, promoting EC proliferation and migration while reducing EC barrier function (43, 44). VEGF receptors are also expressed on nonvascular cells, specifically neuronal cells, and VEGF is considered to be neuroprotective. Beyond its beneficial effects on brain perfusion, it has been implicated in regulating neurogenesis, neuronal survival and neuronal differentiation (45). VEGF is regulated by hypoxia and glucose deficiency, and overproduction of VEGF has been reported in clinical conditions associated with insufficient tissue perfusion such as scleroderma, POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin changes) syndrome, and diabetic retinopathy (46–48). VEGF is a therapeutic target in patients with tumors and ophthalmic diseases (49–51). Current data add a new dimension to the functional spectrum of VEGF by assigning an immunomodulatory role to the growth factor, mediated by endothelial Jagged1 expressed on the vasa vasorum and the periarterial microvascular networks.

The source of excess VEGF in GCA patients remains to be clarified. In previous studies, VEGF but not FGF-2 tissue expression was associated with the degree of neovascularization within the vasculitic lesions (17) and macrophages, multinucleated giant cells and mast cells were identified as a cellular source (17, 52, 53). Notably, VEGF production and neovascularization were strongest in inflamed arteries rich in tissue IFN- γ , suggesting that the strength of aberrant T cell responses regulates VEGF production (17). Thus, besides

hypoxia and glucose deficiency, systemic VEGF concentrations may reflect the uncontrolled T cell stimulation occurring in a VEGF-rich host (34, 54, 55). Previous reports have raised the possibility that VEGF can directly inhibit T cell activation (56). We tested the effect of VEGF on patient-derived T cells undergoing EC-driven activation and found that T cell activation was unaffected by the added VEGF.

Localization of IFN- γ -producing T cells to the adventitia of GCA arteries provided the first evidence for a disease relevant role of this tissue layer (57). Subsequent work placing vascular DCs at the media-adventitia border spatially separated the immunological injury and the tissue destructive granuloma formation (58–60). In contrast to atherosclerotic disease, where the adventitia is attracting attention because it is the site of tertiary lymphoid organs (TLOs) (61–63), GCA-affected arteries do not form organized lymphoid architectures in the adventitia. In an elegant work, Hu et al. (62) have implicated localized TLOs in regulating the immune responses promoting atherosclerotic lesions in the murine aorta, affecting T cell recruitment, T cell priming, and induction of regulatory T cells. Such TLOs are occupied by antigen-presenting B cells and DCs and convert naïve CD4 T cells into memory cells, mimicking events in a lymph node germinal center. As in the development of lymph nodes, lymphotoxin- β s plays a critical role in the genesis of aortic TLO (64). TLOs do not occur in the perivascular tissue of GCA-affected arteries; the pathognomic granuloma formation is tightly restricted to the arterial wall layers, centering on the media. In this context it is important to consider that GCA has a stringent age restriction, exclusively affecting the elderly, and immune aging is associated with a profound reduction in germinal center reactions (65–67). Age-related loss of germinal centers is noticeable in human tonsil already during middle age and age-related deficiencies of follicular T_H cells correlate with reduced influenza vaccine responses (68, 69).

CD4 T cells are the major drivers of the vascular pathology in GCA, promoting granuloma formation in the arterial wall that eventually remodels the wall layers and causes luminal occlusion and tissue ischemia (5, 70). Current data show that CD4 T cell abnormalities extend beyond the tissue-residing pool and include circulating CD4 T cells. High surface-localized Notch1 and HES1 protein expression indicated constitutive Notch signaling. We mapped the signaling pathway elicited by Jagged1⁺ ECs and found that T effector cell induction was sensitive to γ -secretase inhibition as well as inhibitors of AKT and mTORC1. mTORC1 is a critical regulator in the generation of T_H1 and T_H17 cells, whereas mTORC2 signaling is necessary to induce T_H2 cells (71, 72). Whereas the Notch signaling pathway has been linked to direct regulation of GATA3, and thus T_H2 cell generation (73, 74), recent work has emphasized that activation of the mTOR-Rictor (rapamycin-insensitive companion of mTOR) complex lies downstream of noncanonical Notch signaling (75). In essence, aberrant expression of Notch1, present even on naïve CD4 T cells, exposes the CD4 T cell population to constant canonical Notch-AKT-mTORC1 signal activity, lifting such T cells into a partially activated state with reduced threshold for additional stimuli (76). Such partially activated CD4 T cells should easily escape from peripheral tolerance mechanisms. Notably, despite the constitutive activity of the Notch pathway, such CD4 T cells did not convert to the memory status, in line with the finding that GCA patients often lack classical features of immunosenescence. Defining the defect that causes deviant Notch1 expression should provide key insights into the immunopathogenesis of GCA.

Like all translational studies, the current study has limitations, imposed by the limited numbers of disease-relevant tissues that can be accessed. This is particularly important for the aorta, which cannot be biopsied for diagnostic purposes. In addition, it is impossible to test disease mechanisms directly in patients. To overcome that barrier, we relied on a human artery-severe combined immunodeficient (SCID) chimera model, which allowed us to explore in vivo, whether VEGF acted as a disease amplifier. In addition, the current study did not identify the cellular source of the VEGF circulating in patients with GCA; a question that needs to be addressed.

Data presented here define three intersection points in the pathogenic cascade leading to GCA and all three have the potential to lead to novel therapeutic interventions. Blocking excess VEGF should reverse the Jagged1-mediated immunostimulatory functions of adventitial microvessels. Anti-VEGF is available to block tumor angiogenesis and local delivery has become a routine intervention to treat angioproliferative retinal disease (77). Alternatively, blocking access to endothelial Jagged1 would provide the means to interrupt the chronic immunostimulation in the adventitial vascular niche. Finally, intercepting the Notch-AKT-mTORC1 pathway with appropriate small molecule inhibitors would disrupt the ongoing T cell activation and restore T cell quiescence.

Materials and Methods

Study design

This study investigated the role of microvascular cells in protecting the immunoprivilege of large arteries, such as the aorta and its major branches. This immunoprivilege is breached in GCA, where CD4 T cells invade into the vessel wall and cause aneurysm formation or vaso-occlusive lesions. Temporal arteries and aortas affected by GCA were collected from diagnostic biopsies or aortic repair surgeries. Molecules involved in the disease process were identified from tissue transcriptome studies. Microvessels in the adventitia of healthy and GCA-affected arteries were examined by immunohistochemistry. Eighty patients with biopsy-positive GCA and 58 age-matched healthy controls were enrolled into the study. Peripheral T cells were cultured on microvascular endothelial monolayers and analyzed for activation markers, transcription factor expression and intracellular cytokines by flow cytometry. For some experiments, patients with the inflammatory disease RA served as controls. To induce and manipulate vasculitis, immunodeficient mice were engrafted with noninflamed human arteries and reconstituted with PBMCs of patients with ongoing GCA. Characteristics of GCA and RA patients are summarized in Table 1. The numbers of independent experiments or individuals are given in each figure legend. All experiments were approved by the Stanford Institutional Review Board and appropriate informed consent was obtained from all participants.

Cell isolation

PBMCs were isolated by gradient centrifugation with Lymphocyte Separation Medium (Lonza) and cultured in RPMI 1640 supplemented with 10% fetal calf serum (HyClone) plus penicillin/streptomycin/gutamine. CD4 T cells were purified from PBMCs using an EasySep

Human CD4 T Cell Enrichment kit (STEMCELL Technologies). Purity of cell populations was consistently >90%.

Reagents

Human Jagged1 neutralizing antibody (clone 188323) was purchased from R&D Systems. The Notch signaling inhibitors DAPT and FLI-06, the mTORC1 inhibitor rapamycin, and the VEGF receptor blocker axitinib, were from Sigma-Aldrich. Human Notch1 siRNA and control siRNA originated from OriGene. Human Raptor siRNA and control were from Santa Cruz Biotechnology. AKT inhibitor VIII was from Merck Millipore's Calbiochem. hVEGF protein was obtained from SinoBiological. The hVEGF ELISA Kit (enzyme-linked immunosorbent assay kit) was a product bought from Thermo Fisher Scientific. The anti-VEGF blocking antibody Avastin (bevacizumab) was provided by Y. Levina from the Byers Eye Institute, Stanford University. Nucleofector kits from Lonza were used for the siRNA knockdown experiments. All reagents were used according to the manufacturer's instructions.

Tissues and ECs

GCA-affected temporal arteries and aortic specimens were collected from diagnostic biopsies. Normal human temporal and axillary arteries were harvested as early postmortem tissues. HMVECs and HUVECs from Lonza were cultured in EGM-2MV BulletKit medium (Lonza) and EGM BulletKit medium (Lonza), respectively. ECs within the third to eighth passage under active growing conditions were used for the experiments.

Flow cytometry

For intracellular staining, cells were fixed with Fix Buffer I (BD Biosciences) and permeabilized with Perm Buffer III (BD Biosciences). Multiparametric flow cytometry panels were assembled with antibodies to Notch1, CD45RA, CD25, HES1, T-bet, ROR γ t and pS6, combined with the following anti-CD3 and anti-CD4 antibodies: APC-Cy7 (allophycocyanin-Cy7) anti-human CD3 antibody (clone HIT3a, BioLegend), FITC (fluorescein isothiocyanate) or PE-Cy7 (phycoerythrin-Cy7) anti-human CD4 antibody (clone RPA-T4, BioLegend), APC or APC-Cy7 anti-human CD45RA antibody (clone HI100, BioLegend), PE anti-human CD25 antibody (clone M-A251, BioLegend), APC or PE anti-human Notch1 antibody (clone MHN1-519, BioLegend), purified anti-human HES1 antibody (Clone 4H1HES, eBioscience) plus PE-Cy7 anti-mouse IgG1 antibody (clone M1-14D12, eBioscience), PE or PE-Cy7 anti-human T-bet antibody (clone eBio4B10, eBioscience), APC anti-human ROR γ t antibody (clone AFKJS-9, eBioscience) and PE anti-human pS6 antibody (clone D57.2.2E, Cell Signaling Technology), respectively. For the quantification of IFN- γ and IL-17-expressing CD4 T cells, cells were treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Tocris) plus ionomycin (500 ng/ml, Tocris) in the presence of Brefeldin A (5 μ g/ml, BioLegend) for 5 hours, fixed, permeabilized and stained with FITC anti-human CD4 antibody (clone RPA-T4, BioLegend), and PerCP-Cy5.5 anti-human IFN- γ antibody (clone 4S.B3, BioLegend) plus APC anti-human IL-17 antibody (clone eBio64DEC17, eBioscience). Jagged1 expression on ECs was detected with PE anti-human Jagged1 antibody (clone MHJ1-152BD, Biosciences). Cells were stained for 45 min

at 4°C. Flow cytometry was performed on a LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.).

Immunofluorescence and immunohistochemistry

Protein expression of Jagged1, Delta-like 1, activated Notch1, IFN- γ and CD3 in tissue sections was visualized as previously described (78). Briefly, paraffin-embedded GCA-positive and GCA-negative artery sections, as well as frozen sections of axillary arteries and arterial explants, were stained with rabbit anti-human Jagged1 antibody (1:200; clone EPR4290, Abcam), rabbit anti-human Delta-like 1 antibody (1:100; PA5-23457, Thermo Fisher Scientific), rabbit anti-human activated Notch1 antibody (1:200; ab8925, Abcam), rabbit anti-human IFN- γ antibody (1:100; ab25101, Abcam) or mouse anti-human CD3 antibody (clone F7.2.381:50, DAKO). EC in artery sections were stained with mouse anti-human CD31 antibody (1:100; clone 89C2, Cell Signaling Technology). Antibody binding was visualized with Alexa Fluor 594 anti-mouse IgG (1:200; A-11032, Thermo Fisher Scientific) and Alexa Fluor 488 anti-rabbit IgG (1:200; A-11034, Thermo Fisher Scientific) as secondary antibodies, or developed with the VECTASTAIN ABC kit (Vector Laboratories) plus DAB (3,3'-diaminobenzidine) substrate (Vector Laboratories). All sections were analyzed using a confocal microscope system (Carl Zeiss).

Real-time PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen). Complementary DNA (cDNA) was synthesized with Maxima First Strand cDNA Synthesis Kits for real-time qPCR (Thermo Scientific). qPCR analyses were carried out using SYBR Green qPCR Master Mix (Biotool) following previously reported protocols (26, 42, 78). Gene expression was normalized to 18S ribosomal RNA.

EC-dependent T cell differentiation

EC monolayers were conditioned with control or patient-derived plasma [10% (v/v)] for 24 hours, loaded with anti-CD3 antibody (1 μ g/ml, BioLegend), and cocultured with purified CD4 T cells at an EC to T cell ratio of 1:5. CD4 T cells were analyzed for lineage-determining transcription factors after 4 days and intracellular cytokines after 6 days by flow cytometry.

Human artery-SCID mouse chimeras

Human artery-SCID mouse chimeras were generated as previously described (26, 42). NSG mice from the Jackson Laboratory were kept in pathogen-free facilities and used at the age of 8 to 12 weeks. Pieces of axillary arteries, which are a preferred target of GCA, were placed into a subcutaneous pocket on the midback of the mice. For postsurgical pain control, the mice received a single dose of buprenorphine (0.125 mg/kg body weight, subcutaneously). In this model, engraftment is reached after 1 week, with mouse microvessels connecting to human vasa vasorum microvessels. Mice implanted with pieces of arteries from the same donor were randomly assigned to two treatment groups and one control group. Mice received a single intraperitoneal injection of lipopolysaccharide (10 μ g/mouse, Sigma-Aldrich) on day 7 and were reconstituted with PBMCs (15×10^6 cells/mouse)

from patients with biopsy-proven GCA on day 8. Arterial grafts were harvested on day 15, shock frozen for RNA isolation and OCT (optimal cutting temperature)-embedded for immunostaining. hVEGF protein was injected intraperitoneally into the chimeras at 2 µg/mouse on day 7. Axitinib was given daily by intraperitoneal injection at a dose of 25 mg/kg body weight from days 7 to 15 (79). All protocols were approved by the Institutional Animal Care and Use Committee.

Statistical analysis

All data are means±SEM. Mann-Whitney test, Student's *t* test and nonparametric Spearman correlation analysis were applied for statistical analyses as appropriate using GraphPad Prism 6.0 software (GraphPad Software Inc.). *P* < 0.05 was considered significant. All data analyses were overseen by the Department of Health Research and Policy at Stanford University. Individual subject-level data are shown in table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Adventitial mvECs in GCA arteries express Jagged1

Tissue biopsies were collected from temporal arteries and from aortic wall specimens. Arteries affected by GCA had typical transmural granulomatous arteritis (GCA-positive and GCA aortitis). Temporal arteries with no inflammatory infiltrates (GCA-negative) served as controls. Nuclei were marked with 4',6-diamidino-2-phenylindole. Scale bars 50 μ m. **(A)** Tissue-infiltrating T cells were identified by staining sections from GCA-negative and GCA-positive temporal arteries and from GCA aortitis with mouse anti-human CD3 antibody. Antibody binding was visualized with Alexa Fluor 594-labeled anti-mouse immunoglobulin G (IgG) secondary antibody (red). Representative stains from eight samples each are shown here. **(B)** mRNA was extracted from GCA-positive and GCA-negative temporal arteries and analyzed by reverse transcription polymerase chain reaction (RT-PCR) for the expression of *JAG1*, *DLL1* and *DLL4* transcripts. Results (mean $\pm$ SEM) from six GCA arteries and six healthy arteries are shown. N.s., not significant. **(C and D)** Dual-color staining was applied to identify Jagged1 and Delta-like 1 expressed on endothelial cells in GCA-positive, GCA aortitis, and GCA-negative arteries. Tissue sections were double-stained with mouse anti-human CD31 antibody and rabbit anti-human Jagged1 antibody or rabbit anti-human Delta-like 1 antibody. Alexa Fluor 594 anti-mouse IgG (red) and Alexa Fluor 488 anti-rabbit IgG

(green) were used as secondary antibodies. Merged images demonstrate colocalization of both markers (yellow). Representative images from eight samples each.

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Figure 2. Circulating VEGF in GCA patients up-regulates microvascular endothelial Jagged1
 Plasma samples were collected from patients with GCA and age-matched healthy controls. Patients with RA served as disease controls. All data are mean $\pm$ SEM. **(A and B)** EC monolayers (HMVECs and HUVECs) were treated with 10% GCA plasma, RA plasma and control plasma. After 6 hours, mRNA transcripts for *JAG1*, *DLL1* and *DLL4* were quantified by RT-PCR (A). After 24 hours, Jagged1 protein was measured by flow cytometry (B). Representative histograms and mean fluorescence intensities (MFIs) corrected by background subtraction from four to six independent experiments. **(C)** Plasma VEGF concentrations in healthy controls, RA patients and GCA patients. Each dot represents one individual. **(D)** Jagged1 protein expression on HMVECs and HUVECs treated with hVEGF (10 ng/ml) for 24 hours. Results from three to four experiments. **(E)** HMVECs were treated with GCA plasma $\pm$ VEGF receptor inhibitor axitinib (1 μ M). Representative histogram of Jagged1 expression and MFIs are from four experiments. **(F)** HMVECs were treated with GCA plasma $\pm$ anti-VEGF blocking antibody (10 μ g/ml) for six hours. *JAG1* transcripts were measured by RT-PCR. Data from three experiments. **(G and H)** Slices of human medium-sized arteries were cultured for 5 days with hVEGF (100 ng/ml) or vehicle (G). Alternatively, the arteries were kept in medium containing 30% plasma from healthy donors or from GCA patients in the absence or presence of anti-VEGF antibody (100 μ g/ml) (H).

Jagged1 protein was visualized by immunohistochemical staining with anti-Jagged1 antibody. Isotype antibody was used as control for binding specificity. Representative images are from five experiments. Scale bars 20 μm .

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Figure 3. Constitutive Notch pathway activation in GCA CD4 T cells

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from patients with active GCA and age-matched controls, stained with antibodies specific for CD3, CD4, Notch1, activated Notch1, HES, CD45RA, CD25, T-bet and retinoic acid-related orphan receptor γ t (ROR γ t) and analyzed by multiparametric flow cytometry. All data are mean $\pm$ SEM. **(A)** Tissue sections from GCA-positive and GCA-negative arteries were processed as in Fig. 1 and gene expression for *NOTCH1* and *NOTCH4* was analyzed by RT-PCR. Results are from six GCA/control artery pairs. **(B)** Notch1 receptor expression was assessed on gated

CD3⁺CD4⁺ T cells. Expression of activated Notch1 and HES1 was determined on CD3⁺CD4⁺Notch1⁺ T cells. Representative images from five independent experiments. **(C, D)** MFI for Notch1 and HES expression on gated CD4 T cells from 35 GCA patients and 12 age-matched controls. Each dot represents data from an individual patient/control. **(E)** Correlation of Notch1 expression with HES1 expression in gated CD4 T cells. Each dot represents the data from an individual patient. **(F)** Phenotypic analysis of gated Notch1⁺ and Notch1⁻ CD4 T cells for the expression of CD45RA and the activation marker CD25. Representative contour plots from six different GCA patients are shown. **(G)** Frequencies of T-bet and ROR γ t-expressing CD4 T cells in GCA patients and age-matched healthy controls. Representative contour plots and quantitation from 35 patients and 12 controls are shown. **(H)** Comparison of T-bet and ROR γ t expression in gated Notch1⁻ and Notch1⁺ CD4 T cell populations. Representative contour plots are shown.

Figure 4. ECs treated with GCA plasma function as antigen-presenting cells and promote T cell effector functions

HMVEC monolayers were conditioned for 24 hours with control or patient-derived plasma, incubated with or without anti-CD3 antibody (1 μg/ml), and cocultured with purified CD4 T cells at an EC to T cell ratio of 1:5. All data are presented as mean ± SEM. **(A)** Scheme of experimental design. **(B to F)** Expression of the lineage-determining transcription factors T-bet, RORγt and GATA3 within CD4 T cells was analyzed by flow cytometry after 4 days. Representative histograms and quantitation from 5 to 10 independent experiments are shown. **(G)** Naïve CD4⁺CD45RO⁻ T cells and memory CD4⁺CD45RO⁺ T cells were purified from healthy individuals and cultured on plasma-pretreated HMVECs for 4 days. T-bet and RORγt within CD4 T cells were analyzed by flow cytometry as above. Data are from four to six experiments. **(H to K)** After 6 days of coculture, CD4 T cells were stained

for intracellular IFN- γ and IL-17. Representative images and collective data from 6 independent experiments. (L) CD4 T cells were isolated from healthy controls or GCA patients. Frequencies of T-bet⁺ and ROR γ t⁺ CD4 T cells were determined after EC-T cell coculture and pretreatment with GCA plasma as above. Results are from five to nine independent experiments.

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Figure 5. Plasma-conditioned mvECs trigger the Notch signaling pathway in CD4 T cells
 mvEC monolayers were treated with control or patient-derived plasma, loaded with anti-CD3 antibodies and overlaid with CD4 T cells as in Fig. 4A. After 4 days, CD4⁺T-bet⁺ and CD4⁺RORγt⁺ T cells were measured by flow cytometry. All data are mean ± SEM. (**A and B**) Frequencies of CD4 T cells expressing the Notch target protein HES1 were analyzed by flow cytometry after 24 hours. Representative images and collective MFIs from five experiments. (**C and D**) EC-T cell interaction was blocked with anti-Jagged1 antibody or isotype control and induction of T-bet and RORγt in T cells was assessed by flow cytometry.

Data from six experiments. **(E and F)** Induction of CD4⁺T-bet⁺ and CD4⁺RORγt⁺ T cells was quantified after pharmacologic inhibition of the Notch signaling pathway with DAPT (10 μM). Data from five experiments. **(G)** The Notch signaling pathway was inhibited with FLI-06 (10 μM) and the effect on T cell differentiation was assessed by flow cytometry for CD4⁺T-bet⁺ and CD4⁺RORγt⁺ T cells. Data from five experiments presented as a heat map. **(H)** The Notch1 receptor was knocked down by transfecting CD4 T cells from healthy subjects and GCA patients with Notch1 siRNA or control siRNA, respectively. T cells were cocultured with pretreated ECs as above. Frequencies of T cells acquiring expression of T-bet or RORγt were measured by flow cytometry. Data from six independent experiments. **(I and J)** The VEGF receptor inhibitor axitinib (1 μM) and anti-VEGF antibody (10 μg/ml) were included into the EC-T cell cultures. CD4⁺T-bet⁺ and CD4⁺RORγt⁺ T cells were quantified by flow cytometry. Data from five to six independent experiments.

CD4 T cells determined by multiparametric flow cytometry in freshly isolated PBMCs of GCA patients. Each dot represents an individual patient.

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Figure 7. VEGF amplifies vasculitogenic T cell responses

Pairs of NSG mice were engrafted with human axillary arteries, reconstituted with PBMCs from patients with GCA and treated with recombinant hVEGF (2 μ g per mouse) or vehicle. Alternatively, the chimeras were treated with axitinib by daily injection of the inhibitor (25 mg/kg) for eight consecutive days. Artery grafts were explanted on day 15 after transplantation and processed for immunohistochemistry or tissue transcriptome analysis by RT-PCR. All data are mean $\pm$ SEM. (**A and B**) Tissue gene expression of *JAG1*, *NOTCH1*, *TRB* and *CD68* mRNA in different treatment arms. *TRB* mRNA identified tissue-infiltrating T cells; *CD68* mRNA is derived from tissue-infiltrating macrophages. Data are from 8 to 11 different artery grafts. (**C**) Vessel wall-infiltrating CD3 T cells visualized by immunostaining. Representative images from eight grafts. Scale bars 20 μ m. (**D**) Activated Notch1 in vessel wall resident CD3 T cells visualized by immunofluorescence staining. Representative images and quantitation from six to seven grafts. Scale bars 50 μ m. DAPI, 4',6-diamidino-2-phenylindole. (**E**) Expression of Jagged1 protein in human CD31⁺ mvECs visualized by dual-color immunofluorescence staining. Representative images and quantitation are from six to seven grafts. Scale bars 50 μ m. (**F to J**) Tissue transcriptome analysis for lineage-determining transcription factors (*TBX21*, *RORC*, *GATA3* and *FOXP3*) and innate and adaptive cytokines (*TNF*, *IL6*, *IFNG*, *IL17A* and *IL4*) quantified by qPCR.

Data are from 8 to 12 grafts. **(K)** IFN- γ expression in vessel wall-infiltrated CD3⁺ T cells visualized by dual-color immunofluorescence staining. Representative images and quantitation are from six to seven grafts. Scale bars 50 μ m.

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Table 1

Clinical characteristics of patient cohorts. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Parameters	Patients with GCA	Patients with RA
Number of patients	80	54
Age (mean $\pm$ SD, years)	69.63 $\pm$ 7.95	53.16 $\pm$ 16.35
Female	77.5%	79.63%
Headaches	70%	
Eye involvement	31.25%	
Jaw claudication	26.25%	
Polymyalgia rheumatica	60%	
Aortic/large vessel involvement	43.75%	
Prednisone (mg/day, mean $\pm$ SD)	7.49 $\pm$ 11.29	
Disease duration (mean $\pm$ SD, months)	14.36 $\pm$ 12.40	14.88 $\pm$ 11.48
ESR (mean $\pm$ SD, mm/h)	51.43 $\pm$ 35.39	24.63 $\pm$ 19.53
CRP (mean $\pm$ SD, mg/dL)	6.25 $\pm$ 7.48	1.72 $\pm$ 4.47
Untreated patients	32.5%	16.67%