Blocking the NOTCH Pathway Inhibits Vascular Inflammation in Large-Vessel Vasculitis

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Background—Giant cell arteritis is a granulomatous vasculitis of the aorta and its branches that causes blindness, stroke, and aortic aneurysm. CD4 T cells are key pathogenic regulators, instructed by vessel wall dendritic cells to differentiate into vasculitic T cells. The unique pathways driving this dendritic cell–T-cell interaction are incompletely understood, but may provide novel therapeutic targets for a disease in which the only established therapy is long-term treatment with high doses of corticosteroids.

Methods and Results—Immunohistochemical and gene expression analyses of giant cell arteritis-affected temporal arteries revealed abundant expression of the NOTCH receptor and its ligands, Jagged1 and Delta1. Cleavage of the NOTCH intracellular domain in wall-infiltrating T cells indicated ongoing NOTCH pathway activation in large-vessel vasculitis. NOTCH activation did not occur in small-vessel vasculitis affecting branches of the vasa vasorum tree. We devised 2 strategies to block NOTCH pathway activation: γ-secretase inhibitor treatment, preventing nuclear translocation of the NOTCH intracellular domain, and competing for receptor-ligand interactions through excess soluble ligand, Jagged1-Fc. In a humanized mouse model, NOTCH pathway disruption had strong immunosuppressive effects, inhibiting T-cell activation in the early and established phases of vascular inflammation. NOTCH inhibition was particularly effective in downregulating Th17 responses, but also markedly suppressed Th1 responses.

Conclusions—Blocking NOTCH signaling depleted T cells from the vascular infiltrates, implicating NOTCH-NOTCH ligand interactions in regulating T-cell retention and survival in vessel wall inflammation. Modulating the NOTCH signaling cascade emerges as a promising new strategy for immunosuppressive therapy of large-vessel vasculitis. (Circulation. 2011;123:309-318.)

Key Words: arteries ■ costimulation ■ inflammation ■ interferon-γ ■ interleukin-17 ■ NOTCH ■ T cell

Giant cell arteritis (GCA) is characterized by intramural and perivascular granulomatous lesions that destroy the vascular wall structure and induce luminal occlusion through fast and concentric neointimal outgrowth.¹ Clinical manifestations include blindness, stroke, and aortic aneurysm, and arterial inflammation is almost always combined with a syndrome of severe systemic inflammation.²

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Vascular lesion formation is mediated by a maladaptive immune response, characterized by in situ activation of CD4 T cells.³ CD4 T cells receive activating signals from tissue-resident vascular dendritic cells (DCs).⁴⁵ Apart from the strength of the antigen:T-cell receptor (TCR) signal, the microenvironment and accessory signals derived from the antigen-presenting cell are critical in T-cell activation. Antigen-presenting cell surface receptors costimulate or coinhibit TCR-mediated signals, and ultimately shape the outcome of the T-cell activation cascade.

The present project examined how NOTCH-NOTCH ligand interactions affect T-cell activation in vasculitis, with the goal of targeting such interactions therapeutically. NOTCH is critically involved in lymphocyte development; its overexpression in T-cell acute lymphoblastic leukemia points to a potential central position for the pathway in regulating T-cell growth. Experimental data suggest crosstalk between the TCR signaling cascade and the canonical NOTCH signaling pathway.⁷ Signal transduction in the NOTCH pathway⁸ is initiated by ligand binding, which leads to two proteolytic cleavage processes catalyzed by ADAM metalloproteases and γ-secretase, respectively.⁹ The latter cleavage liberates the NOTCH intracellular domain (NICD)
facilitating its nuclear translocation and induction of target genes such as Hairy enhancer of split (Hes).10

Because the instigator of T-cell activation in GCA is unknown, current therapies are restricted to long-term high doses of corticosteroids. Interfering with in situ T-cell activation in the vascular microenvironment emerges as an attractive alternative. Here, we report that in humanized mice carrying human arteries and human T cells, NOTCH pathway blockade has profound implications, suppressing vessel wall inflammation, cytokine production, and T-cell accumulation. Blockade of the NOTCH pathway with the γ-secretase inhibitor (GSI) N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-buty 1 ester (DAPT) and the soluble NOTCH ligand Jagged1-Fc effectively inhibits vascular inflammation. NOTCH-targeted immunosuppression is effective during the early and established phases of the disease process, opening the possibility for novel therapeutic interventions in treating GCA.

**Methods**

**Tissues and Cells**

GCA-affected temporal arteries were derived from diagnostic biopsies. Normal human temporal and axillary arteries were collected from early postmortem tissues. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and patients newly diagnosed with GCA. CD4+ T cells were positively selected with CD4 microbeads (Miltenyi Biotec, Auburn, CA). Monocyte-derived DCs were generated from CD14+ monocytes by 6-day culture in 1000 U/mL granulocyte macrophage colony-stimulation factor and 800 U/mL interleukin (IL)-4.

All protocols were approved by the Institutional Review Board. Informed consent was obtained.

**Antibodies and Reagents**

Antibodies to human CD4 and CD25 were purchased from BD PharMingen (San Diego, CA); antihuman NOTCH1-PE (clone 527425) was obtained from R&D Systems (Minneapolis, MN). Anti-NOTCH1 (ab27526; Abcam, Cambridge, MA) was used for flow cytometric detection of NOTCH1. Antibodies against NICD (ab8925; Abcam) were used for flow cytometry and immunohistochemistry. Goat anti-rabbit Alexa 488 was purchased from Stem Cell Technologies (Vancouver, Canada). The recombinant Jagged1-Fc fusion protein was manufactured by R&D Systems. DAPT and lipopolysaccharide (Escherichia coli, 0127:B8) were obtained from Sigma-Aldrich (St Louis, MO).

**Proliferation Assay**

Carboxyfluorescein succinimidyl ester (CFSE)–loaded T cells preincubated with 10 μmol/L DAPT or carrier dimethyl sulfoxide were stimulated with plate-bound α-CD3/CD28 or with monocyte-derived DCs (1:10) activated with lipopolysaccharide. Proliferation was measured by flow cytometry on day 3. Alternatively, T cells were cultured (2×10^5 cells per well) with plate-bound α-CD3/CD28 (1 μg/mL) antibodies and Jagged1-Fc (0.25 to 10 μg/mL) or isotype control IgG. After 48 hours, cells were pulsed with 1 μCi of ^3H thymidine; 24 hours later, proliferation was determined by ^3H thymidine incorporation.

**Flow Cytometric Analysis**

PBMCs were stained with the relevant primary antibodies or appropriate isotype controls (30 minutes at 4° C). For detection of intracellular molecules, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA). Permeabilized cells were incubated with anti-NOTCH1 antibody or isotype control followed by FITC-conjugated secondary antibodies (30 minutes at 4° C). Data were acquired with the LSRII instrument (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

**Immunohistochemistry**

Immunohistochemical analysis of optimal cutting temperature compound or paraffin-embedded arterial sections followed previously published procedures.4,11 Isotype-matched primary antibodies were used as controls.

**Human Artery–Severe Combined Immunodeficiency Mouse Chimeras**

Chimeras were generated as previously described.12 Three mice implanted with arteries from the same donor were assigned to 2 treatment arms and a control arm. On postimplantation day 7, mice received lipopolysaccharide (3 μg per mouse intraperitoneally) or PBS. Twenty-four-hour later, PBMCs (4×10^7 cells per mouse) were preincubated with 10 μmol/L DAPT for 30 minutes and adoptively transferred into the chimeras. On the same day, the chimeras received 1 mg DAPT or vehicle (dimethyl sulfoxide) intraperitoneally. PBMCs were derived from normal donors who had previously been tested for their ability to cause intrawall infiltrates in human artery–severe combined immunodeficiency (SCID) chimeras. Alternatively, PBMCs were isolated from patients with biopsy-proven GCA. Mice treated with soluble ligand received T cells preincubated with 100 μg Jagged1-Fc or control IgG intraperitoneally on day 8, followed by 100 μg Jagged1-Fc or control IgG on days 9 and 10. Gifts were harvested on day 15 and shock-frozen for RNA isolation or optimal cutting temperature compound embedded for immunostaining. For DAPT therapy of established disease, chimeras engrafted with human arteries were injected with lipopolysaccharide (day 7), received an adoptive cell transfer on day 8, and were treated with 1 mg DAPT by intraperitoneal injection on days 12 through 15. Tissues were harvested on day 20. All protocols were approved by the Animal Care and Use Committee.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated from human tissues, reverse transcribed, and analyzed by real-time polymerase chain reaction (PCR), as described.4 Relative RNA was normalized to 2×10^5 copies of β-actin. Specific PCR primers are listed in Table I of the online-only Data Supplement.

**Statistical Analysis**

The statistical methods used rely on an assumption of normality and homoskedasticity across groups. Because data were skewed with large heteroskedasticity, data were first log-base 2-transformed before analyses. The data were then averaged over 3 replicated measurements when appropriate. ANOVA techniques were used to assess differences between treatment conditions when independence across groups could be assumed (comparison of data obtained from control and GCA samples). Paired t tests were used to evaluate differences between treatment conditions when responses were expected to be correlated, as in experiments in which chimeras were engrafted with arteries from the same donor. In experiments in which engrafted human arteries were sham treated, treated with vehicle, or treated with NOTCH pathway blockers, paired t tests were used to first compare sham and vehicle treatment to assess for induction of vascular inflammation. Once this was established, a paired t test was conducted to compare expression between vehicle and active treatment. A mixed-effects model that assessed a synergistic effect between dose level and treatment condition on expression and that allowed a nonlinear relation between dose level and expression was fitted to assess whether the effect of treatment conditions (control vs. Jagged 1-Fc) varied by dose. Such a model accounted for correlation of responses across dose levels.

All tests were 2 sided and conducted with a 0.05 significance level. All analyses were performed with SAS version 9.2 (SAS Institute, Inc, Cary, NC).

**Results**

**NOTCH Expression in the Vascular Lesions of GCA**

In GCA, granulomatous vessel wall infiltrates are composed of highly activated macrophages and T cells.13 Intramural T
cells produce IL-2 and interferon-γ (IFN-γ) but essentially no IL-4. The role of costimulatory receptor-ligand pairs for in situ T-cell activation is unexplored. To examine whether NOTCH1 and its major ligands are expressed in GCA lesions, temporal artery specimens from patients with typical histology were analyzed. NOTCH1 transcripts were 6-fold higher in tissue extracts from GCA arteries than in normal arteries (Figure 1A). In addition, the NOTCH ligand Jagged1 was highly abundant, detected at >10-fold higher transcript levels in diseased vessels (Figure 1B). Similar to Jagged1, Delta1 transcripts were highly increased in vasculitic arteries (Figure 1C), whereas levels of Delta4-specific sequences were indistinguishable between normal and inflamed arteries (Figure 1D). Cellular analysis of tissue sections revealed dense infiltrates penetrating deep into the media, with almost all mononuclear cells positive for the T-cell marker CD3 (Figure 1F and I). The majority of intramural lymphocytes expressed NOTCH1 receptor (Figure 1G). Staining for the cleaved NICD (Figure 1J) demonstrated in situ activation of the NOTCH pathway in the tissue. NOTCH activation in T-cell infiltrates was confirmed at the single-cell level by costaining for NICD and CD3 (Figure 1K). Widespread

Figure 1. Activated NOTCH1 is abundant in GCA arteries. RNA was isolated from temporal artery biopsies that either had no evidence of inflammation (control) or showed granulomatous infiltrates typical of GCA (n=4). Expression levels of NOTCH1 (A), Jagged1 (B), Delta1 (C), and Delta4 (D) transcripts were quantified by real-time PCR. Data shown are mean±SD. Paraffin-embedded temporal artery specimens affected by GCA were stained with anti–human CD3 (F and I), anti–human NOTCH1 (G), or antibodies to the cleaved intracellular domain of the NOTCH1 receptor (J). Isotype antibodies served as control (E and H). Activated NOTCH1 and CD3 were visualized by immunofluorescence using Alexa 488–labeled (green) and Alexa 546–labeled (red) antibodies, respectively (K). Colocalization of NICD and CD3 resulted in a yellow cellular stain (merge). Original magnification: E, ×100, scale bar=500 μm; F, G, H, and K, ×200, scale bar=200 μm; I and J, ×400, scale bar=100 μm.
NICD detection provided confirmatory evidence that cells encountered NOTCH ligands in the tissue environment.

To explore whether NOTCH pathway activation is a feature of all T cells involved in vascular inflammation, a distinct, yet related, type of vasculitis was examined. In a subset of GCA patients, intramural infiltrates are localized in the small arteries of the vasa vasorum and not in the main temporal artery.14 Although T cells were crowded in the walls of the vasa vasorum branches (Figure 2A and 2B), there was no detectable expression of the NOTCH1 receptor (Figure 2C).

NOTCH Expression on Circulating T Cells in GCA

The intense systemic inflammation in GCA patients suggests that antigen encounter also occurs outside the artery.15,16 We therefore explored whether NOTCH expression is limited to the vascular lesions or also present in circulating immune cells. PBMCs were collected from freshly diagnosed patients and examined for NOTCH1 and Jagged1 transcript expression. Induction of the NOTCH target gene Hes-1 was assessed (Figure 3) to provide evidence for ongoing stimulation of the NOTCH pathway. Compared with PBMCs from age-matched controls, GCA PBMCs contained 20-fold higher NOTCH1 transcript levels (Figure 3A). By flow cytometry, essentially the entire population of peripheral CD4 T cells in GCA patients was strongly positive for NOTCH1 (Figure 3B).

Similarly, Jagged1 sequences were abundantly expressed in GCA samples, and barely detectable in control subjects (Figure 3C). Ongoing NOTCH signaling was confirmed by the upregulation of Hes-1 (Figure 3D). Thus, both NOTCH
receptors and Jagged1 ligands are highly induced in GCA PBMCs, and NOTCH-derived signals are constantly reaching the nucleus.

To test whether NOTCH1 expression in circulating GCA T cells was associated with functional changes, expression patterns of 2 major T-cell cytokines recently implicated in GCA were assessed. PBMCs from untreated GCA patients contained high levels of both IFN-γ and IL-17 sequences, demonstrating activation of the Th1 and the Th17 pathway (Figure 3E and 3F). The concentrations of IFN-γ-specific sequences were more than doubled in patients. The differences were even more pronounced for IL-17. A 4- to 5-fold increase in IL-17 transcript levels distinguished GCA PBMCs from those of healthy control subjects.

NOTCH Blockade Inhibits CD4 T-Cell Function
To explore functional implications of NOTCH activation, we inhibited NOTCH activity using the GSI DAPT. Cross-linking of the CD3 and CD28 receptors resulted in vigorous T-cell proliferation; by day 3, >50% of cells had entered the cell cycle. Cell cycle kinetics were unaffected in dimethyl sulfoxide vehicle controls (53.2%; Figure 4A). Cells stimulated in the presence of DAPT showed a 25% decrease in proliferation, with only 39% of cells progressing through the cell cycle (Figure 4A). To confirm that DAPT was efficient in disrupting NOTCH signaling, we assessed the accumulation of NICD, a process directly dependent on enzymatic activity of the γ-secretase complex. DAPT treatment abrogated NICD accumulation, with intracellular NICD levels diminished after short-term and prolonged T-cell activation (Figure 4B). In DAPT-treated cells, the level of the NOTCH target gene Hes-1 was reduced by 80% (Figure 4C), providing confirmation that γ-secretase inhibition was actively targeting the NOTCH pathway.

To more closely approximate in vivo T-cell–DC interactions, we explored the impact of NOTCH inhibition in T-cell–DC coculture assays. In control cultures, 63% of the T cells upregulated the activation marker CD25, and a similar proportion of cells underwent activation in the presence of dimethyl sulfoxide (Figure 4D). Inhibiting intracellular
NOTCH cleavage reduced T-cell activation, with only 47% of the T cells acquiring CD25 expression. In essence, NOTCH-NOTCH ligand interactions provide costimulatory signals, amplifying the process of T-cell activation and regulating T-cell expansion.

Kinetic studies of NOTCH1 expression on T cells pointed toward interesting kinetics in the upregulation of this co-stimulatory receptor (Figure I in the online-only Data Supplement). Anti-CD3/CD28 stimulation increased both the level of NOTCH1 transcripts and cell surface expression. By 24 hours after stimulation, levels were 4-fold higher, and by 48 hours, even 8-fold higher (Figure I in the online-only Data Supplement). Thus, changes in NOTCH1 receptor expression are an integral component of the T-cell activation program, and NOTCH-NOTCH ligand interactions may not be relevant early after antigen recognition but days after TCR ligation.

**NOTCH Signaling Inhibition Restricts Early CD4 T-Cell Infiltration Into the Arterial Wall**

To study the contribution of the NOTCH pathway in vascular inflammation, a global NOTCH inhibitor was applied in a humanized mouse model of vasculitis. In this disease model, human arteries are implanted into SCID mice, and human allogeneic T cells from selected healthy donors or from GCA patients are adoptively transferred into the chimeras. Tissue tolerance is broken through injection of Toll-like receptor ligands; eg, lipopolysaccharide and cellular infiltrates. Tissue tolerance is broken through injection of Toll-like receptor ligands; eg, lipopolysaccharide and cellular infiltrates.

To study the role of NOTCH during the early phase of vascular inflammation, human T cells were coadministered with DAPT or vehicle (Figure 5). Explanted arteries were analyzed for TCR and Hes-1 transcript levels to monitor composition and activity of the infiltrates. NOTCH inhibition had a profound effect on T-cell invasion into the wall layers. The density of infiltrating T cells was measured by immunohistochemical quantification of CD3+ T cells in tissue sections (Figure 5A through 5C). Chimeras without vessel wall inflammation (did not receive lipopolysaccharide injection) served as controls. Arteries with fully developed inflammation harbored ∼50 cells per high-powered field (Figure 5D). Coadministration of DAPT inhibited T-cell recruitment/retention, and only 10 cells per high-powered field were detected in the human grafts. T-cell depletion correlated with an almost 2-fold reduction of TCR transcript levels (Figure 5E).

To test the effectiveness of NOTCH pathway blockade, we determined the expression levels of the NOTCH target gene Hes-1. Mice infused with DAPT-treated human T cells produced 4-fold less Hes-1 in the arterial grafts than mice with fully developed vascular infiltrates (Figure 5F). NOTCH pathway blockade resulted in a marked suppression of tissue IFN-γ (Figure 5G) and IL-17 (Figure 5H).

Similar results were obtained if the adoptively transferred PBMCs were derived from patients with biopsy-proven GCA. Infiltration of GCA PBMCs into the human vessels elicited strong Th1 and Th17 responses (Figure 5I and 5J). Both arms of T-cell immunity were susceptible to DAPT-dependent suppression. However, blocking NOTCH activation was more effective in downregulating IL-17 than IFN-γ (Figure 5I and 5J).

Monitoring of TCR transcript levels and histochemical analysis of the explants from treated and untreated chimeras strongly suggested that the NOTCH pathway is involved in the recruitment/retention of proinflammatory T cells. Alternative mechanisms of action include the recruitment/activation of immunosuppressive regulatory T cells or swaying of immune responses from the Th1 to the Th2 lineage. To address these possibilities, IL-4 as a marker of Th2 cells and transforming growth factor-β as a marker for regulatory T cells were evaluated in the explanted blood vessels (Figure 5K and 5L). IL-4 transcripts were low in vehicle- and DAPT-treated grafts, consistent with the absence of IL-4 in temporal artery biopsies from GCA patients (Figure III in the online-only Data Supplement). Transforming growth factor-β transcripts were detectable in vehicle-exposed arteries but declined with blocking NOTCH activation (Figure 5L). In essence, interfering with NOTCH cleavage and translocation had profound implications for the inflammatory infiltrates, essentially disrupting T-cell recruitment/retention.

**Jagged1-Fc Treatment Is Protective Against Vessel Wall Inflammation**

In a complementary approach, we used another strategy to prevent NOTCH pathway activation by occupying NOTCH1 with the exogenous decoy ligand Jagged1-Fc. Immunosuppressive effects of Jagged1-Fc were first explored in vitro. Triggering of CD4 T cells by TCR cross-linking induced the activation marker CD25 on essentially all T cells within 24 hours. In the presence of exogenous soluble Jagged1-Fc protein, T-cell activation was curbed with a minor population of T cells acquiring CD25. Stimulation in the presence of 5 µg/mL Jagged1-Fc over a 3-day course effectively blocked NOTCH pathway activation by occupying NOTCH1 inhibitory Fc receptors. Jagged1-Fc protein impaired T-cell proliferation in a dose-dependent manner (Figure 6B). At doses of 5 to 10 µg/mL Jagged1-Fc, T-cell proliferation reached only 50% of that in untreated T cells.

In vivo therapeutic effects of Jagged1-Fc were probed in the humanized mouse model. To explore immunosuppressive effects of Jagged1-Fc, 100 µg of the soluble ligand was administered concurrently with the adoptively transferred human T cells. The chimeras received 2 additional doses 24 and 48 hours later. Control animals were injected with human IgG. Treatment with Jagged1-Fc over a 3-day course effectively inhibited NOTCH-dependent signaling as indicated by the marked reduction of Hes-1 sequences (Figure 6C). The loss of NOTCH target gene induction was accompanied by a loss of TCR sequences, indicating reduced T-cell accumulation (Figure 6D). Administration of Jagged1-Fc suppressed in situ production of both IFN-γ (∼4-fold reduction; Figure 6E).
and IL-17 (≈10-fold reduction; Figure 6F), essentially eliminating the major inflammatory drivers of the vasculitic process. As seen in the DAPT experiments, IL-4 expression was low in the controls and Jagged1-Fc had no effect on IL-4 transcript levels (Figure 6G).

NOTCH Blockade Ameliorates Established Vascular Inflammation

Treatment with either DAPT or Jagged1-Fc protein markedly decreased tissue-infiltrating T cells and suppressed T-cell cytokine production. To assess whether blockade of the
NOTCH pathway could be successfully applied after T cells had already established intramural infiltrates, we inhibited NOTCH-derived signals in established vessel wall inflammation. Prior studies have shown that the subset of CCR6^+^CD4 T cells plays an important role in forming panarteritic infiltrates.\(^1\) As vascular smooth muscle cells transition from a contractile to a noncontractile phenotype, they lose production of smoothelin as an indication of damage to the vessel wall.\(^1\)

Preventing NICD cleavage by administering the GSI DAPT 96 hours after induction of vascular inflammation caused a marked reduction in the tissue production of both TCR (Figure 7A) and the T-cell subset marker CCR6 (Figure 7B). Thus, NOTCH inhibitor treatment at the peak of vascular disease evolution was highly effective in thinning the T-cell infiltrate. DAPT therapy essentially eliminated CCR6 transcripts from the lesions (Figure 7B), depleting a T-cell subset implicated in the formation of transmural infiltrates. The suppression of T-cell function was associated with a decline in both IFN-\(\gamma\) (Figure 7C) and IL-17 (Figure 7D).

Again, as seen in earlier experiments, DAPT appeared to be more effective in abrogating tissue IL-17 than tissue IFN-\(\gamma\). Blockade of NOTCH-dependent activation was confirmed by the suppression of the NOTCH target gene Hes-1 after DAPT injection (Figure 7E). Vascular damage quantified by the levels of smoothelin production was intense in the arteries invaded by the T cells; vascular smooth muscle cells literally ceased transcribing this gene, diminishing smoothelin levels by almost 10-fold of that in normal tissue (Figure 7F). DAPT was able to partially reverse the smoothelin loss, restricting the reduction of smoothelin transcripts to 2-fold of the undamaged tissue grafts, indicative of protection of vascular smooth muscle cells from the inflammatory injury.

**Discussion**

The NOTCH signaling pathway is mostly recognized for its role in determining cell fate, especially during development and tissue homeostasis.\(^1\)\(^8\) The pathway transduces signals between neighboring cells, emphasizing its participation in the 3-dimensional structuring of tissues. Here, we report that the NOTCH pathway is of disease relevance in large-vessel vasculitis, regulating the activity of vessel wall–infiltrating T cells that orchestrate tissue damage in blood vessels. Blocking NOTCH signaling has profound effects on T-cell–dependent immune responses and suppresses the production of proinflammatory cytokines, in particular IL-17. Both early and later stages of vasculitis are sensitive to disrupting NOTCH-NOTCH ligand interactions, suggesting that this pathway has potential as a therapeutic target in inflammatory vasculopathies.

In GCA, CD4 T-cell invasiveness, cytotoxicity, and in situ cytokine production lie at the core of the pathogenic process.\(^1\)\(^9\) Initiation of vascular inflammation requires DC activation; DC depletion abrogates chronic vasculitis.\(^4\) Vasculitic T cells depend on continuous instructions provided by DCs, which sustain T-cell activation\(^2\) and shape overall disease architecture.\(^1\)\(^1\) DCs express NOTCH ligands, including Jagged and Delta ligands, and differences in DC activation have been associated with the induction of distinct T-cell differentiation trajectories.\(^1\)\(^2\)\(^2\) In the present study, NOTCH signaling blockade markedly reduced T-cell infiltration into the vessel wall during both early and late stages of vasculitis. NOTCH blockade particularly affected the density of wall-invading cell populations, essentially depleting T cells from the infiltrates. This antivasculitic effect may result from either impairing T-cell recruitment or survival in the tissue niche. It is unlikely that the NOTCH pathway affects only T-cell
migration because T-cell cytokine production was also profoundly reduced and the kinetics of NOTCH receptor induction suggested a delayed role of NOTCH in T-cell activation. The NOTCH pathway has been associated with the regulation of lymphocyte activation and proliferation.22,23 This may be the mechanism underlying the loss of T cells from the infiltrates. Alternatively, NOTCH may interfere with survival signals delivered to wall-infiltrating cells. Thus, it would be important to know where the NOTCH-triggering signals derive from and whether this is unique to the arterial wall.

The contribution of cytokines in GCA is well appreciated both in the systemic inflammatory response and in the blood vessels.25 Tissue cytokine profiles in temporal artery biopsies suggest that local IFN-γ production drives disease progression.3 The recent implication of Th17 cells in the pathogenesis of autoimmune disease26 led us to investigate the expression of both inflammatory cytokines. Upregulation of inflammatory T-cell cytokines correlated with persistent activation of the NOTCH pathway in both tissue lesions and blood. Differential effects of NOTCH blockade on IFN-γ versus IL-17 production suggested a formidable role of NOTCH-NOTCH ligand interactions in T-cell differentiation. Regardless of the approach taken to suppress canonical NOTCH signaling, IL-17 production appeared more sensitive and IFN-γ more resistant. A selective role of NOTCH-NOTCH ligand interaction in regulating the function of certain T-cell subsets is supported by the finding that regulatory T cells appear to be unaffected by NOTCH blockade. The outcome of NOTCH signaling is cell-type specific, and T-cell cytokine production obviously displays a differential dependence on the NOTCH pathway.

Gaps in the understanding of GCA pathogenesis restrict therapeutic options. Therefore, dissecting the intricacies of the unique signals that regulate and sustain a self-propagating inflammation is warranted. Overall, our in vivo studies assign a role of NOTCH triggering in the initiation of vascular wall inflammation. Disrupting NOTCH activation via GSI inhibition or with decoy ligands is profoundly immunosuppressive. Therapeutic targeting of the NOTCH signaling pathway, however, may be limited by intestinal toxicity; NOTCH1 and NOTCH2 receptors expressed on intestinal epithelium have been connected to secretory goblet cell accumulation and impaired cell proliferation.27 Whether parental GSI formulations could reduce this side effect remains to be explored. The current standard of care in GCA, high doses of glucocorticoids given chronically, is associated with serious side effects. A recent study examining the antileukemic effects of GSI in T-cell acute lymphoblastic leukemia, a malignancy characterized by activating mutations in the NOTCH receptor gene, demonstrated synergistic beneficial effects of glucocorticoids and GSI.28 Moreover, the combination therapy protected mice from gut toxicity. Thus, combining NOTCH signaling inhibition with steroids may allow improved anti-inflammatory control in GCA and enable steroid sparing, an important need in the optimization of the management of large-vessel vasculitis.
REFERENCES


CLINICAL PERSPECTIVE

Giant cell arteritis (formerly called temporal arteritis) is an inflammatory vasculopathy that causes aortic wall damage and lumen-stenosing lesions in medium-sized arteries. Giant cell arteritis preferentially targets extracranial branches of the aorta, resulting in ischemic optic neuropathy, stroke, pulselessness, and aortic aneurysm/dissection. If diagnosed and treated promptly, ischemic organ damage such as blindness can be prevented. The standard of care involves high doses of corticosteroids, often given over prolonged periods. Alternative treatment approaches are urgently needed. A critical step in the disease process is the activation of proinflammatory T cells in the vessel wall. Here, we searched for receptor-ligand pairs relevant in T-cell activation, with the ultimate goal of developing novel antiinflammatory interventions. We found that vascular lesions of giant cell arteritis patients abundantly expressed NOTCH and NOTCH ligands and accumulated NOTCH+ T cells. The NOTCH signaling pathway has mostly been recognized for its role in determining cell fate, especially during development and tissue homeostasis. NOTCH pathway signaling depends on 2 proteolytic cleavage processes, including the action of a γ-secretase, which can be inhibited by an enzyme blocker. Treatment with the γ-secretase inhibitor or alternatively a soluble NOTCH ligand effectively suppressed vascular inflammation in a humanized mouse model. NOTCH signaling blockade suppressed activity of interferon-γ-producing Th1 cells and almost depleted interleukin-17–producing Th17 cells. Targeting the NOTCH-NOTCH ligand pathway may provide a novel strategy to suppress the activity of vasculitic T cells in large and medium vessel vasculitis.
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Supplemental Figure 1

A. Notch1 mRNA expression

B. Flow cytometry analysis of Notch1 expression over time:
- Unstimulated (0hr)
- CD3/CD28 stimulation (6hr, 24hr, 48hr)

C. Notch1 MFI over time:
- 0hr
- 6hr
- 24hr
- 48hr
Supplemental Figure 1. T cell activation enhances Notch1 surface expression. A. CD4 T cells purified from PBMC of healthy donors were stimulated with immobilized anti-CD3/CD28 mAbs. The cells were harvested at 24 h and Notch1 expression was analyzed by real time PCR. B. Alternatively, cell surface expression of the Notch1 receptor was quantified by flow cytometry on CD4 T cells stimulated with anti-CD3/CD28 (1µg/ml) for 0 (light grey line), 24 (dark grey line) or 48 h (black line). Shaded histogram represents isotype control. Representative data from one of six independent experiments are shown. C. Cell surface expression of Notch 1 on CD4 T cells was measured as mean fluorescence intensity 0, 6, 24, or 48 h post stimulation. Data shown are the means ± SEM from 6 independent experiments.
Supplemental Figure 2

Adjusted copy number

0 24 72 120 168 240

0 120 240 600

IL-6
IFN-γ
IL-17

Hours post adoptive transfer of PBMC
Supplemental Figure 2. Time course of vascular inflammation in the humanized mouse model. Axillary arteries were implanted into SCID mice. On day 7, chimeras were injected with LPS (3µg/mouse). Twenty-four hours later, PBMC (3×10^7/mouse) from healthy donors were adoptively transferred into chimeras by intravenous injection. Arterial tissues were explanted at different time points (0, 24, 72, 120, 168 hours after adaptive transfer).

Transcripts for the inflammatory cytokine IL-6, the Th1 T cell cytokine IFN-γ and the Th17 T cell cytokine IL-17 in the tissue extracts were quantified by RT-PCR. Results are shown as mean ± SEM from three independent experiments.
**Supplemental Figure 3. Th2 responses in GCA.** RNA was isolated from temporal artery biopsies, which either had no evidence for inflammation (control) or showed granulomatous infiltrates typical of GCA (GCA) (n=4). Transcript expression levels of the Th2 cytokine IL-4 were quantified by RT-PCR. Data shown are the mean ± SEM.
Supplemental Table 1. Human primer pairs for qRT-PCR

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<th>Gene</th>
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<td>5'-CCTTCAACAACACAGATTATTATTCCAG-3</td>
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<td>5'-CTCCGAGACAGTCTCGTGAC-3</td>
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<td>Smoothelin</td>
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<td>5'-CGCTGCTCTCTCTTCTTGT-3</td>
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<td>Jagged-1</td>
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<td>5'-TGG GTG CCA AGC ACT GC-3</td>
<td>5'-TCG TGA CCA CCT TGT TTT TCT G-3</td>
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<td>5'-TTCTGGAAGAAGTCGACAGCTCTTT-3</td>
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<td>5'-TGCAACAAGGTTACCGCCACATTC-3</td>
<td>5'-TGAAATGCCCACCTCAGGACTCAT-3</td>
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