Inhibition of endothelial NOTCH1 signaling attenuates inflammation by reducing cytokine-mediated histone acetylation at inflammatory enhancers

Lars la Cour Poulsen[1,6], Reidunn Jetne Edelmann[1,6], Stig Krüger[1], Rodrigo Diéguez-Hurtado[2], Akshay Shah[3], Tor Espen Stav-Noraas[1], Anastasia Renzi[1], Monika Szymanska[1], Junbai Wang[1], Manuel Ehling[2], Rui Benedito[2], Monika Kasprzycka[1], Espen Bækkevold[1], Olav Sundnes[1], Kim Midwood[4], Helge Scott[1], Philippe Collas[3], Christian W. Siebel[5], Ralf H. Adams[2], Guttorm Haraldsen[1,*], Eirik Sundlisæter[1,7], and Johanna Hol[1,7]

[1]Department of Pathology, Oslo University Hospital Rikshospitalet and University of Oslo, 0424 Oslo, Norway
[2]Max Planck Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, University of Münster, 48149 Münster, Germany
[3]Department of Molecular Medicine, Institute for Basal Medical Sciences, University of Oslo, 0317 Oslo, Norway
[4]The Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford OX3 7FY, UK
[5]Department of Discovery Oncology, Genentech, Inc., South San Francisco, California 94080, USA
[6]Co-first author
[7]Contributed equally to this work

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*Correspondence: Guttorm Haraldsen, Department of Pathology, Oslo University Hospital Rikshospitalet, PO Box 4950 Nydalen, NO-0424 Oslo, Norway. Phone: (+47) 23 07 14 92 Fax: (+47) 23 07 15 11. E-mail: gharalds@rr-research.no

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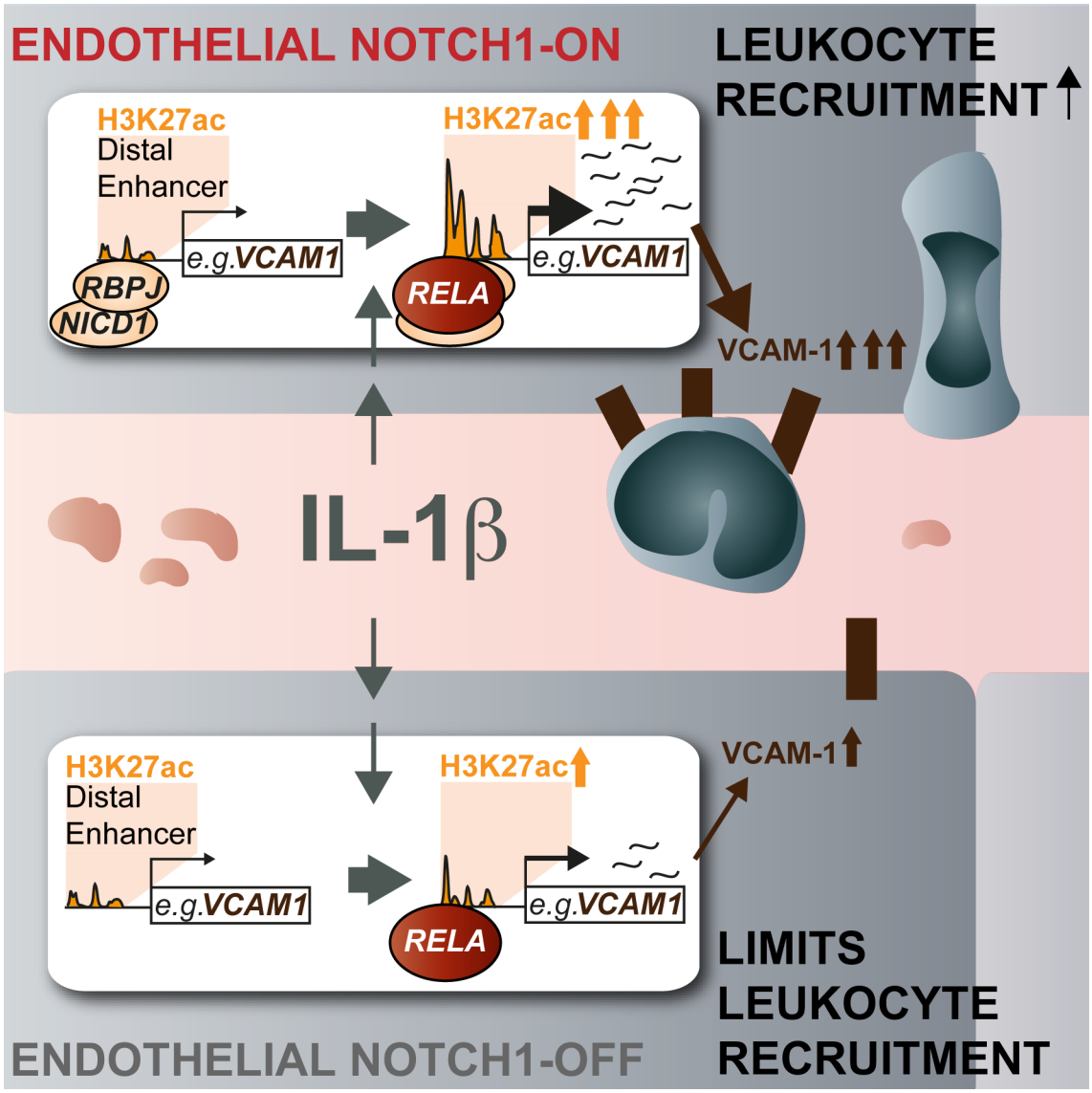
Summary

Objective: Endothelial upregulation of adhesion molecules serves to recruit leukocytes to inflammatory sites and appears to be promoted by NOTCH1; however, current models based on interactions between active NOTCH1 and NF-κB components cannot explain the transcriptional selectivity exerted by NOTCH1 in this context.

Approach and Results: Observing that Cre/Lox-induced conditional mutations of endothelial Notch modulated inflammation in murine contact hypersensitivity, we found that IL-1β stimulation induced rapid recruitment of RELA to genomic sites occupied by NOTCH1-RBPJ, and that NOTCH1 knockdown reduced H3K27 acetylation at a subset of NF-κB-directed inflammatory enhancers.

Conclusions: Our findings reveal that NOTCH1 signaling supports the expression of a subset of inflammatory genes at the enhancer level and demonstrate how key signaling pathways converge on chromatin to coordinate the transition to an inflammatory endothelial phenotype.

Graphical abstract



Abbreviations

NICD/NICD1 - NOTCH/NOTCH1 intracellular domain
HUVECs - Human umbilical vein endothelial cells

Introduction

The Notch signaling system coordinates cell differentiation during embryogenesis and postnatal development by linking the fate of one cell with that of its neighbors. To achieve this coordination, mammals have five ligands (Delta-like (DLL)-1, -3, and -4, and Jagged (JAG)-1 and -2) that signal via four Notch receptors (NOTCH 1-4). Such signaling leads to γ-secretase-dependent release of the NOTCH intracellular domain (NICD) and translocation of NICD to the nucleus, where it recruits coactivators and forms an active transcription complex with the transcription factor RBPJ (also known as CBF1, Su(H), Lag1 or CSL)[1,2].

Recent evidence reveals that the Notch pathway is involved in regulation of inflammation. This is not entirely surprising, because NOTCH1 has been found to interact with the NF-κB pathway in cancer[3,4] and therefore has the potential to modulate the actions of proinflammatory cytokines such as TNF (tumor necrosis factor)-α and IL-1 (interleukin-1)-β as well as Toll-like receptor agonists. Indeed, inhibition of Notch signaling ameliorates experimental arthritis[5], acute colitis[6], acute lung injury[7], and graft-versus-host disease[8].

A requirement for effective inflammation is the activation of endothelial cells in postcapillary venules. Vascular upregulation and presentation of adhesion molecules and chemokines direct the sequential recruitment of specific leukocyte subsets, and, while the molecular events involved in leukocyte transmigration have been thoroughly described[9], our understanding of the mechanisms that control the endothelial response to inflammatory activation is still incomplete.

Notably, the role of endothelial Notch signaling in inflammatory activation appears to be highly context-dependent: While hemizygous deletion of endothelial *Notch1* predisposed for atherosclerosis development and monocyte recruitment in the L-sIDOL model[10], endothelial-specific deletion of the canonical Notch transcription factor *Rbpj* in ApoE$^{-/-}$ mice reduced atherosclerotic plaque inflammation and leukocyte recruitment[11]. Notably, NOTCH1 augmented TNF-driven upregulation of the adhesion molecules VCAM1 and ICAM1 in human endothelial cells[12], and endothelial-specific overexpression of active Notch1 in mouse melanoma promoted a pro-inflammatory, dysfunctional phenotype that included enhanced VCAM1 expression[13]. In contrast, inhibition of NOTCH4 boosted the upregulation of VCAM1 in TNF-stimulated human endothelial cells, suggesting that NOTCH1 and NOTCH4 play opposing roles in the regulation of VCAM1[14]. The effect of NOTCH1 on other classes of inflammatory mediators is, however, less clear-cut, exemplified by the neutrophil-recruiting chemokines CXCL1 and CXCL8 that appear to be repressed by NOTCH1[10,13]. Inflammatory events in endothelial cells are to a large extent driven by NF-κB signaling[15], and it has been suggested that the activated NOTCH1 receptor fragment drives adhesion molecule expression by directly interacting with NF-κB subunits to facilitate their nuclear translocation and retention[11]. However, this mechanism does not account for the apparent selectivity with which NOTCH1 modulates the expression of inflammatory genes in endothelial cells.

A recently identified molecular event of endothelial cell activation is the NF-κB-directed activation of inflammatory enhancer regions, including BRD4-dependent super enhancers essential to the transcriptional inflammatory response[16]. Enhancers have also been identified as the main regions containing functional Notch binding sites that exert transcriptional control over long distances[17,18]. However, it is not known whether Notch associates with inflammatory enhancers or affects inflammation at the genomic level.

We here show that endothelial NOTCH1 signaling promotes leukocyte recruitment by facilitating the activation of inflammatory enhancers supporting a distinct vascular transcription profile. Our findings demonstrate that NOTCH1 signaling is fundamentally involved in regulating the inflammatory response in endothelial cells, supporting the assumption that it may serve as a novel therapeutic target in chronic inflammatory disease.

Materials and methods

Materials and Methods are available in the online-only Data Supplement.

Results

Proinflammatory cytokines selectively drive JAG1 expression in endothelial cells
Endothelial *JAG1* is upregulated by TNF-α via activation of NF-κB and AP-1[19]. We therefore assessed the proinflammatory responsiveness of all endothelial Notch ligands in human umbilical vein endothelial cells (HUVECs), observing that the response was restricted to *JAG1,* and that IL-1β, compared to TNF-α, induced an even stronger upregulation of *JAG1* mRNA (Figure 1A). In addition, IFN-γ also gave a moderate response (Figure 1A). At the protein level, cell surface expression of JAG1 was upregulated by IL-1β in a dose- and time-dependent manner, peaking at 5 ng/ml and 12 hours, respectively (Figures IA-B in the online-only supplement).

JAG1 promotes inflammatory gene expression in endothelial cells
To address whether JAG1 plays a role in inflammatory activation of endothelial cells, we next performed genome-wide transcriptome analysis of IL-1β-stimulated HUVECs treated with siRNA targeting *JAG1* or scrambled siRNA, identifying 135 differentially expressed genes (Fold change>1.5, Benjamini-Hochberg´s adj.p<0.05; Figure IC in the online-only supplement, genes listed in Table I in the online-only supplement). Gene ontology analysis revealed that genes supported by *JAG1* (Table II in the online-only supplement) were enriched for biological terms associated with leukocyte recruitment (chemokine-mediated signaling pathway, positive regulation of leukocyte chemotaxis), response to proinflammatory stimuli (IFN-gamma-mediated signaling pathway, cellular response to IL-1, cellular response to TNF, response to lipopolysaccharide, response to virus), and inflammatory activation (immune effector process, inflammatory response) (Figure 1B). In contrast, genes repressed by JAG1 (Table III in the online-only supplement) were enriched for biological terms associated with hypoxia and apoptosis (Figure 1B). Interestingly, while knockdown of *JAG1* markedly inhibited the expression of selectins (*SELE*, *SELP*) and adhesion molecules (*VCAM1* and *ICAM1*) in IL-1β-stimulated HUVECs, the effect on chemokines and associated molecules was much more variable, with some being markedly reduced (*DARC*, *CCL8, CX3CL1*) while others remained unaffected (*CXCL8*, *CCL2*) (Figure 1C).

Results were validated by means of RT-qPCR (Figure ID in the online-only supplement). The modulation of VCAM1*,* CX3CL1, and DARC after *JAG1* knockdown was confirmed at the protein level by flow cytometry (Figure 1D and Figure IE in the online-only supplement). To corroborate the specificity of the knockdown, we also inhibited the function of JAG1 in IL-1β-stimulated cells by means of a neutralizing antibody, observing modulation of target transcripts similar to that observed in response to siRNA treatment (Figure 1E). Furthermore, we inverted the experiment by seeding cells on immobilized recombinant JAG1 in the absence of proinflammatory cytokines, observing enhanced expression of target genes (Figure 1F).

Altogether, these results show that inhibition of endothelial JAG1 attenuates the upregulation of a subset of inflammatory mediators with established roles in leukocyte recruitment.

JAG1 maintains expression of NOTCH1-supported genes in activated endothelial cells
Considering the dynamic and competitive balance between JAG1 and DLL4 during angiogenic sprouting[20], and the suggested role of JAG1 in priming endothelial cells for angiogenesis after inflammatory activation[21], we next asked if JAG1 reinforced or inhibited the expression of NOTCH1-supported genes in IL-1β-stimulated HUVECs. Comparing transcriptome data from confluent HUVECs treated with siRNA targeting NOTCH1 to two published data sets investigating the effect of inhibiting of overexpressing NOTCH1 in cultured human endothelial cells[10,22], we compiled lists of genes supported or repressed by NOTCH1 in at least two of the three data sets (Figure 2A). Gene set enrichment analysis

showed that *JAG1* knockdown mediated negative enrichment of NOTCH1-supported genes (Figure 2B) and positive enrichment of NOTCH1-repressed genes (Figure 2C) in IL-1β-stimulated HUVECs.

JAG1 inhibition is mirrored by targeting DLL4 or NOTCH1

HUVECs seeded on immobilized recombinant DLL4 substantially increased their expression of *VCAM1* and *DARC* (Figure 2D), and inhibition of DLL4 by a neutralizing antibody reduced the IL-1β-induced transcription of *VCAM1*, *DARC,* and *CX3CL1* in a similar manner to JAG1 inhibition (Figure 2E). Moreover, administration of a neutralizing antibody against NOTCH1 reduced the IL-1β-induced expression of *VCAM1*, *DARC,* and *CX3CL1* (Figure 2F). Finally, siRNA-mediated knockdown of *NOTCH1, JAG1,* or *DLL4* inhibited VCAM1 protein expression as detected by a cell-based ELISA assay (Figure 2G). Notably, and despite the marked increase in JAG1 expression during inflammatory activation, *JAG1*, *DLL4* and *NOTCH1* knockdown all attenuated VCAM1 induction with similar kinetics. It should also be noted that the effect of IL-1β on NOTCH1-supported genes was diverse (Figure II in the online-only supplement), with some genes (e.g. *FABP4*, *FOXC1*) being induced and others (e.g. *HES1*, *HES4*, *IL33*) being repressed by inflammatory activation.

Taken together, these data show that JAG1, DLL4, and NOTCH1 all support the upregulation of a similar subset of inflammatory mediators in cultured human endothelial cells and confirm previous observations that the cytokine-induced upregulation of JAG1 serves to maintain NOTCH1 signaling under inflammatory conditions.

Endothelial NOTCH1 is activated in endothelial cells of inflamed human appendix

Considering that NOTCH1 signaling supported the upregulation of leukocyte-recruiting factors *in vitro*, we next asked if active NOTCH1 could be observed in endothelial cells of inflamed human tissues. When immunostaining biopsies) with an antibody that recognizes the intracellular domain of human NOTCH1 only when released by cleavage before Val1754[23] (csNICD1), we observed strong csNICD1 signal in endothelial cells in a subset of vessels in inflamed appendix (Figure 3A). Endothelial identity was confirmed by co-staining with the endothelial-specific transcription factor ERG. We next quantified the nuclear csNICD1 signal in lamina propria endothelial cells (ERG+) and observed a significantly higher levels of csNICD1 in biopsies with moderate to severe inflammation (inflammation score ++ or +++) than in biopsies with no or little inflammation (inflammation score 0 or +).

Because leukocyte recruitment takes place at the level of post-capillary venules, we next mapped the endothelial csNICD1 signal in different branches of the vascular tree. To this end, we used morphological criteria and a panel of molecular markers detailed in Table IV (online-only supplement) to identify arterioles, venules, capillaries, and lymphatic vessels and found that the csNICD1 signal in venules (positive for the postcapillary venule-specific marker DARC[24], not shown) were negative or only weakly positive under matching conditions for staining and exposure (Figures 3J, 3K, labelled a, L and v, respectively). By contrast, postcapillary venules of inflamed specimens (Figures 3L-3N, labelled v) altogether showed an induction of nuclear endothelial signal for csNICD1 at the mucosal-submucosal border. Together, these findings suggest that endothelial NOTCH1 signaling in the gastrointestinal tract is subject to spatial and temporal regulation in the context of inflammation.

Notch signaling modulates the contact hypersensitivity response

To evaluate the functional importance of our observations, we used the DNFB-induced contact hypersensitivity model (Figure IVA in the online-only supplement). We observed that subcutaneous injection of the γ-secretase inhibitor DAPT 3 hours before DNFB challenge (blocking Notch signaling in all cell types) inhibited ear swelling at 12 hours and that the effect of DAPT became even more pronounced 24 hours after challenge (Figure 4A).

To understand the relative contribution of endothelial Notch signaling to inflammation, we next asked to what extent endothelial cell-targeted loss and gain of Notch function would affect the contact hypersensitivity response. We found that mice with deletion of *Rbpj* in endothelial cells ($Rbpj^{i\Delta EC}$) showed a significant reduction in ear swelling 24 hours after

challenge (Figure 4B). Complementary, mice that expressed constitutively active endothelial Notch1 (*NICD*$^{iEC\text{-}OE}$) responded by a significantly stronger ear swelling compared to controls (Figure 4C). Collectively, these results show that not only global but also endothelial Notch signaling modulates the contact hypersensitivity response.

Endothelial Notch signaling supports leukocyte recruitment

We next asked if Notch signaling also affected leukocyte recruitment to the inflamed lesion. By immunostaining and quantifying CD45+ cells in DNFB-treated ears 24 hours after challenge, we observed increased numbers of total leukocytes (Figures 4D and S4B), CD3+ dermal T cells (Figure 4E and S4C), and neutrophils (Ly6G+ cells, Figure 4F) in *NICD*$^{iEC\text{-}OE}$ mice when compared to control mice. *NICD*$^{iEC\text{-}OE}$ mice also showed increased recruitment of monocytes (CD11b+Ly6C+ cells, fraction of total CD45+ population, Figure 4G). These findings demonstrate that an amplification of endothelial Notch1 signaling promotes leukocyte recruitment to the inflamed dermis in murine contact hypersensitivity.

Notch regulates common inflammatory genes in human and mouse endothelial cells

To identify genes that were consistently altered by Notch signaling in activated endothelial cells, we next compared transcriptome data from mouse endothelial cells isolated from DNFB-treated ears of *NICD*$^{iEC\text{-}OE}$ mutants and HUVECs treated with siRNA targeting *JAG1* or *NOTCH1* and stimulated with IL-1β. This approach identified 11 genes that were differentially regulated by all conditions (Figure 4H), including *VCAM1* and *CX3CL1* as well as Notch target genes with established roles in vascular differentiation (*HEY1*, *EFNB2*).

Confirming our transcriptional data, immunostaining of sections from mouse ears with an antibody recognizing VCAM1 showed increased VCAM1 signal in *NICD*$^{iEC\text{-}OE}$ mice compared to controls (Figures 4I and S4D). VCAM1 is upregulated by DNFB exposure[25] and allows firm adhesion of T cells, monocytes, and eosinophils that express the integrin heterodimer α4β1/VLA-4. A neutralizing antibody to VLA-4 is furthermore reported to partially block DNFB-induced contact dermatitis[26], suggesting that the observed increase in VCAM1 is likely to be physiologically significant.

In conclusion, the NOTCH1 effect observed in cultured human endothelial cells translates at least in part to the *in vivo* situation and contributes to explain the phenotype of *NICD*$^{iEC\text{-}OE}$ mice in DNFB-induced contact hypersensitivity.

Inhibition of NOTCH1 does not affect NF-κB and AP-1 signal transduction in endothelial cells

To dissect the mechanism by which NOTCH1 supported endothelial inflammatory activation, we next assessed if NOTCH1 inhibition altered IL-1β-induced signaling in endothelial cells. The IL-1β-induced signaling cascade involves IKK- and MAPK-dependent activation of the master inflammatory transcription factors NF-κB (RELA and p50) and AP-1 (cJUN and cFOS)[27] and NOTCH1 has been reported to directly[3,4,11] or indirectly[28] promote the activity of the IKK signalosome complex. We found, however, no evidence for such crosstalk in IL-1β-stimulated HUVECs since *NOTCH1* knockdown by siRNA altered neither the phosphorylation, degradation or resynthesis of IκBα (Figure VA in the online-only supplement), nor the levels of DNA-binding NF-κB subunits RELA and p50 in nuclear lysates (Figures VB and VC in the online-only supplement). Furthermore, assessing AP-1 activation in IL-1β-stimulated HUVECs, we detected no change in the DNA-binding potential of active cJUN (cJUN pSer73) upon *NOTCH1* knockdown (Figure VD in the online-only supplement), and levels of cJUN phosphorylation remained unaltered (Figure VE in the online-only supplement). Levels of DNA-binding cFOS in nuclear lysates were undetectable (data not shown). Taken together, these data show that inhibition of NOTCH1 does not markedly alter the signal transduction cascades of NF-κB and AP-1 in HUVECs.

Genomic sites occupied by the NOTCH1 transcription complex are also binding sites for RELA

Considering that NOTCH1 inhibition markedly reduced the upregulation of selected inflammatory genes but had little impact on inflammatory signaling cascades, we asked if the

NOTCH1 transcription complex might in itself promote favorable conditions for transcriptional activation of these genes. We therefore profiled the genome-wide binding of canonical components of the NOTCH1 transcription complex (NOTCH1-RBPJ) and the NF-κB subunit RELA, as well as the deposition of histone H3 acetylated at lysine 27 (H3K27ac), a measure of enhancer activity[29] that can be promoted by the genomic impact of both NOTCH1[17,18] and NF-κB[16,30]. Chromatin immunoprecipitation combined with deep sequencing (ChIP-seq) was first performed in confluent, resting HUVECs to generate genome-wide binding profiles of NOTCH1 and RBPJ, identifying a total of 4,328 and 11,345 peaks (Figure VIA in the online-only supplement). Strikingly, when profiling the binding of RELA by ChIP-seq in IL-1β-stimulated HUVECs, we discovered that the vast majority of DNA binding sites for NOTCH1 and RBPJ in unstimulated cells overlapped with sites bound by RELA in IL-1β-stimulated cells (97% and 83% basepair overlap; Figure 5A). Moreover, global RELA binding was not reduced by knockdown of *NOTCH1* (Figures VIA and VIB in the online-only supplement), supporting the notion that NOTCH1 is dispensable for RELA signal transduction and its genomic binding in endothelial cells.

The NOTCH1 transcription complex promotes activation of inflammatory enhancers
We next manually explored the binding of the NOTCH1 transcription complex in the proximity to JAG1/NOTCH1-supported genes. RBPJ peaks were identified in or close to transcriptional start sites of canonical NOTCH1 target genes including *HES1* (Fig 5B) and *IL33* (Figure VIIA in the online-only supplement), as well as some of the JAG1/NOTCH1-supported genes involved in leukocyte recruitment, including *ICAM1* (Fig 5C) and *SELP* (Figure VIIB in the online-only supplement). In contrast, RBPJ did not bind in or near transcriptional start sites of the JAG1/NOTCH1-supported genes *VCAM1* (Fig 5D), *CCL7*, or *CCL8* (Fig 5E), but instead occupied nearby (+/- 100kB) regions recently described as "inflammatory super-enhancers", based on strong, TNF-α-induced BRD4 enrichment[16]. Interestingly, the RBPJ-peak close to the *ICAM1* transcriptional start site was also associated with one of these inflammatory enhancers (Fig 5C). In all examined regions, NOTCH1 binding patterns mirrored those of RBPJ, but the signal was generally weaker, resulting in fewer called peaks.

Notably, the identified NOTCH1/RBPJ peaks in the *VCAM1*-assigned enhancer overlapped with binding of RELA in IL-1β-stimulated cells (Figure 6A), and NOTCH1 knockdown almost completely abrogated the IL-1β-induced activity around this site as judged by the deposition of H3K27ac (Figure 6A). The same pattern was observed in the inflammatory enhancer region associated with the *CCL2-CCL7-CCL11-CCL8* locus (Figure 6B).

Our findings prompted us to ask whether the NOTCH1 transcription complex exerted a net effect on the activation of inflammatory enhancers. Taking advantage of the previously defined set of inflammatory enhancers[16], we detected RELA binding within all of these regions and found that a large subset was also bound by NOTCH1 (41%) and RBPJ (77%) (Figure 6C). Importantly, when focusing on those regions containing NOTCH1 peaks (all of which also contained RBPJ peaks, Figure 6C), we discovered that the increased mean peak intensity of H3K27ac elicited by IL-1β stimulation was strongly attenuated upon *NOTCH1* knockdown (Figure 6D). Furthermore, the extent of H3K27ac peak overlap within the same regions was reduced by 31% following *NOTCH1* knockdown (Figure 6E). Taken together, these results suggest that the transcription complex of NOTCH1-RBPJ facilitates activation of a subset of strong NF-κB-directed enhancers central to the endothelial inflammatory response that mediates leukocyte recruitment.

Discussion

Endothelial cell activation is a crucial component of the inflammatory response. Here, we provide the first evidence that NOTCH1 supports the inflammatory endothelial phenotype that mediates leukocyte recruitment by facilitating activation of inflammation-driven enhancers. Our conclusions build on the following observations: First, we demonstrate that JAG1/NOTCH1 globally modulates the transcriptional response to inflammatory cytokines. Second, we observe that NOTCH1 signaling contributes to the inflammatory response *in*

vivo, and, third, we show that the transcription complex acting downstream of NOTCH1 signaling occupies inflammatory loci targeted by NF-κB and facilitates activation of inflammatory enhancers central to the endothelial inflammatory program[16].

Previous data demonstrate that NOTCH1 signaling may promote inflammatory activation by crosstalk with inflammatory signaling cascades, perhaps best illustrated in the NF-kB pathway. For example, NOTCH1 can induce NF-κB activation and cellular survival in cancer cells by direct interactions between NICD1 and the IKK signalosome that facilitates cytoplasmic IκBα degradation[3,4]. NICD1 has also been reported to directly interact with NF-κB subunits, affecting their nuclear import and retention[11,31,32]. It nevertheless appears, based on our analyses, that these mechanisms are of less importance in human endothelial cells. Our combined findings demonstrate that acute NF-κB signaling to RELA-p50 remains unaffected by *NOTCH1* knockdown, and that the recruitment of RELA to chromatin, as assessed by genome-wide ChIP-sequencing, is not reduced. This agrees with observations made by ourselves and others[10] that some NF-κB targets, like CXCL8 and CXCL1, remain unaffected or are even induced by NOTCH1 inhibition. Moreover, the current model can not explain how NOTCH1 supports the expression of P-selectin, another leukocyte-recruiting molecule that is not driven by NF-κB activation.

By contrast, our data indicate that NOTCH1 supports inflammatory activation of endothelial cells at the chromatin level by globally promoting inflammatory enhancer activity, and that its transcription complex also binds directly to the transcriptional start sites of some inflammatory genes (*SELP, ICAM1*). Indeed, we discovered that most sites occupied by the NOTCH1 transcription complex in endothelial cells are inflammatory in the sense that they are bound by RELA after IL-1β-stimulation. Although our data are the first to reveal an effect of NOTCH1 on inflammatory enhancers, NOTCH1 has been reported to promote recruitment of IKKα to IKKα-stimulated promoters in cervical cancer cells [4], indicating that it may also support the ability of IKKα to mediate derepression of NF-κB target genes [33]. Altogether, our data takes the understanding of NOTCH1 function in inflammation to a new level by demonstrating a profound involvement in inflammatory enhancer activation.

Our data support a model in which the NOTCH1 transcription complex assists the NF-κB-driven machinery in orchestrating activation of inflammatory enhancers central to controlling the endothelial inflammatory phenotype. Notably, this mode of regulation was apparent at the *VCAM1* locus (Figures 5D and 6A), which stood out as one of the most robustly NOTCH1-supported genes in our transcriptional analyses. It should be noted that while the depicted enhancer was recently assigned to *VCAM1*[16], its functional impact on *VCAM1* expression remains to be confirmed, *e.g.* by genomic deletion or epigenetic inactivation of the enhancer. While the enhancer is directly associated with a long intergenic nonprotein-coding RNA (LINCO1349), NOTCH1 has also previously been shown to coregulate coding transcripts without an obvious NOTCH1 binding site near the transcriptional start site and noncoding transcripts associated with distal NOTCH1-bound enhancers[34]. Another interesting enhancer region is associated with the *CCL2-CCL7-CCL11-CCL8* locus (Figures 5E and 6B). We observed that while basal *CCL2* expression was consistently supported by NOTCH1 in unstimulated HUVECs, neither JAG1 nor NOTCH1 inhibition had any effect on its upregulation after IL-1β-stimulation. In contrast, JAG1 and NOTCH1 supported the IL-1β-stimulated transcriptional upregulation of the adjacent genes *CCL7* and *CCL8*, perhaps by facilitating the activation of the enhancer region associated with the *CCL2* transcriptional start site (Figures 5E and 6B). Because many enhancers regulate cognate genes over considerable distances[35], target gene(s) of a given enhancer can not be accurately predicted simply by genomic distance. So far, no studies have reported chromosome conformation data from inflammatory activated HUVECs, making it difficult to make valid predictions of chromatin folding. To promote a better understanding of how NOTCH1 controls the expression of inflammatory genes in HUVECs, it is therefore crucial that future experimental approaches are designed to map the three-dimensional chromatin structure in relation to RBPJ-NOTCH1 binding sites in the context of inflammatory activation.

Emerging evidence adds support to the notion that cell fate-regulating transcription factors other than the NOTCH1 transcription complex also modulate chromatin at inflammation-inducible enhancers. Illustrating this, lineage-specific expression of stress-inducible target genes of NF-κB, AP-1, and IRF1 in monocytes/macrophages are programmed by the cell fate-regulating transcription factor PU.1 in synergy with C/EBPα[36,37]. In a complementary manner, our findings demonstrate that the Notch signaling system dynamically modulates the responsiveness of endothelial cells to proinflammatory cytokine activation.

A fascinating aspect of Notch signaling is the relative contribution of individual Notch ligands to receptor activation. Ligand-specific biological outcomes appears to depend on differences in signal strength and have been demonstrated in inner ear development[38], T cell differentiation[39], and during angiogenesis where endothelial JAG1 acts as a competitive antagonist to DLL4[20]. The selective responsiveness of JAG1 to proinflammatory endothelial activation motivated our initial transcriptional screen of the response to *JAG1* knockdown, showing a profound effect of JAG1 on endothelial activation. However, subsequent, unpublished experiments in mice with endothelial cell-specific deletion of *Jag1* failed to show significant effects on inflammation in the DNFB-induced contact hypersensitivity model (Edelmann *et al.*, unpublished). In contrast, endothelial loss of the canonical Notch transcription factor *Rbpj* markedly reduced inflammation, suggesting that signaling by other endothelial Notch ligands or ligands expressed by cell types other than endothelial cells promoted inflammatory activation in this model. Supporting the concept that several Notch ligands can drive the proinflammatory effect of NOTCH1, we found that knockdown of either *JAG1* or *DLL4* in confluent endothelial cell cultures inhibited VCAM1 expression in a similar manner, despite the dramatic increase in JAG1 levels during the time period investigated. Notably, we did not observe any evidence of increased NOTCH1 activity in IL-1β-stimulated cells, despite the upregulation of JAG1 expression. It is therefore tempting to speculate that the upregulation of JAG1 by pro-inflammatory cytokines at least partly serves to compensate for the downregulation of other more potent Notch ligands, like DLL4. Moreover, the ability of individual Notch ligands to induce or inhibit signaling will vary according to the presence of other Notch ligands[38], the *cis*- and *trans*-expression of Notch receptors[40], and the glycosylation status of these receptors[20]. The functional impact of JAG1 induction in a physiological setting therefore remains unsettled, but invites further investigation.

Although this study was focused on understanding the role of NOTCH1 signaling in endothelial cell activation, assessment of global Notch inhibition as a treatment modality in inflammatory disease may be of even greater therapeutic interest. To this end, the currently most impressive demonstration of Notch involvement in an inflammatory context is perhaps the observation that inhibition of selected Notch ligands control murine graft-versus-host-disease with no overt side effects[8]. We therefore propose that the relative contribution of individual Notch components to different inflammatory processes is highly deserving of future exploration. To this end, it will be interesting to systematically assess how Notch ligands and receptors contribute to chronic inflammatory diseases and to what extent components of the Notch pathway are suitable for therapeutic targeting.

Accession numbers

Microarray and ChIP-seq data are accessible through GEO accession number GSE85987.

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Disclosures

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References

1. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *Journal of Cell Science*. 2013;126(Pt 10):2135–2140. doi:10.1242/jcs.127308.

2. Schwanbeck R, Martini S, Bernoth K, Just U. The Notch signaling pathway: molecular basis of cell context dependency. *European Journal of Cell Biology*. 2011;90(6-7):572–581. doi:10.1016/j.ejcb.2010.10.004.

3. Vilimas T, Mascarenhas J, Palomero T, Mandal M, Buonamici S, Meng F, Thompson B, Spaulding C, Macaroun S, Alegre M-L, Kee BL, Ferrando A, Miele L, Aifantis I. Targeting the NF-κB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med*. 2006;13(1):70–77. doi:10.1038/nm1524.

4. Song LL, Peng Y, Yun J, Rizzo P, Chaturvedi V, Weijzen S, Kast WM, Stone PJB, Santos L, Loredo A, Lendahl U, Sonenshein G, Osborne B, Qin J-Z, Pannuti A, Nickoloff BJ, Miele L. Notch-1 associates with IKKα and regulates IKK activity in cervical cancer cells. *Oncogene*. 2008;27(44):5833–5844. doi:10.1038/onc.2008.190.

5. Park J-S, Kim S-H, Kim K, Jin C-H, Choi KY, Jang J, Choi Y, Gwon A-R, Baik S-H, Yun UJ, Chae SY, Lee S, Kang YM, Lee KC, Arumugam TV, Mattson MP, Park JH, Jo D-G. Inhibition of Notch signalling ameliorates experimental inflammatory arthritis. *Ann Rheum Dis*. November 2013. doi:10.1136/annrheumdis-2013-203467.

6. Aziz M, Ishihara S, Ansary MU, Sonoyama H, Tada Y, Oka A, Kusunoki R, Tamagawa Y, Fukuba N, Mishima Y, Mishiro T, Oshima N, Moriyama I, Ishimura N, Sato S, Yuki T, Kawashima K, Kinoshita Y. Crosstalk between TLR5 and Notch1 signaling in epithelial cells during intestinal inflammation. *Int J Mol Med*. 2013;32(5):1051–1062. doi:10.3892/ijmm.2013.1501.

7. Han H, Gong G, Bai X, Lin Y-C, Sun J, Wang W, Zhao Y, Yang L, Wang X, Zhang Z, Dong H, Hou L, Xiong L. Inhibition of notch signaling protects mouse lung against zymosan-induced injury. *Shock*. 2013;40(4):312–319. doi:10.1097/SHK.0b013e3182a102e5.

8. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, Radojcic V, Friedman A, Gridley T, Shelton A, Reddy P, Samuelson LC, Yan M, Siebel CW, Maillard I. Blockade of individual Notch ligands and receptors controls graft-versus-host disease. *J Clin Invest*. 2013;123(4):1590–1604. doi:10.1172/JCI65477DS1.

9. Vestweber D. How leukocytes cross the vascular endothelium. *Nat Rev Immunol*. 2015;15(11):692–704. doi:doi:10.1038/nri3908.

10. Briot A, Civelek M, Seki A, Hoi K, Mack JJ, Lee SD, Kim J, Hong C, Yu J, Fishbein GA, Vakili L, Fogelman AM, Fishbein MC, Lusis AJ, Tontonoz P, Navab M, Berliner JA, Iruela-Arispe ML. Endothelial NOTCH1 is suppressed by circulating lipids and antagonizes inflammation during atherosclerosis. *J Exp Med*. 2015;212(12):2147–2163. doi:10.1084/jem.20150603.

11. Nus M, Martínez-Poveda B, Macgrogan D, Chevre R, D'Amato G, Sbroggio M, Rodríguez C, Martínez-González J, Andrés V, Hidalgo A, la Pompa de JL. Endothelial Jag1-RBPJ signalling promotes inflammatory leukocyte recruitment and atherosclerosis. *Cardiovasc Res*. August 2016:cvw193. doi:10.1093/cvr/cvw193.

12. Verginelli F, Adesso L, Limon I, Alisi A, Gueguen M, Panera N, Giorda E, Raimondi L, Ciarapica R, Campese AF, Screpanti I, Stifani S, Kitajewski J, Miele L, Rota R, Locatelli F. Activation of an endothelial Notch1-Jagged1 circuit induces VCAM1 expression, an effect amplified by interleukin-1β. *Oncotarget*. 2015;6(41):43216–43229. doi:10.18632/oncotarget.6456.

13. Wieland E, Rodriguez-Vita J, Liebler SS, Mogler C, Moll I, Herberich SE, Espinet E, Herpel E, Menuchin A, Chang-Claude J, Hoffmeister M, Gebhardt C, Brenner H, Trumpp A, Siebel CW, Hecker M, Utikal J, Sprinzak D, Fischer A. Endothelial Notch1 Activity Facilitates Metastasis. *Cancer Cell*. February 2017. doi:10.1016/j.ccell.2017.01.007.

14. Quillard T, Coupel S, Coulon F, Fitau J, Chatelais M, Cuturi MC, Chiffoleau E, Charreau B. Impaired Notch4 Activity Elicits Endothelial Cell Activation and Apoptosis: Implication for Transplant Arteriosclerosis. *Arterioscler Thromb Vasc Biol*. 2008;28(12):2258–2265. doi:10.1161/ATVBAHA.108.174995.

15. Kempe S. NF- B controls the global pro-inflammatory response in endothelial cells: evidence for the regulation of a pro-atherogenic program. *Nucleic Acids Res*. 2005;33(16):5308–5319. doi:10.1093/nar/gki836.

16. Brown JD, Lin CY, Duan Q, Griffin G, Federation AJ, Paranal RM, Bair S, Newton G, Lichtman AH, Kung AL, Yang T, Wang H, Luscinskas FW, Croce KJ, Bradner JE, Plutzky J. NF-kB Directs Dynamic Super Enhancer Formation in Inflammation and Atherogenesis. *Mol Cell*. 2014;56(2):219–231. doi:10.1016/j.molcel.2014.08.024.

17. Wang H, Zang C, Taing L, Arnett KL, Wong YJ, Pear WS, Blacklow SC, Liu XS, Aster JC. NOTCH1-RBPJ complexes drive target gene expression through dynamic interactions with superenhancers. *Proc Natl Acad Sci U S A*. 2014;111(2):705–710. doi:10.1073/pnas.1315023111.

18. Castel D, Mourikis P, Bartels SJJ, Brinkman AB, Tajbakhsh S, Stunnenberg HG. Dynamic binding of RBPJ is determined by Notch signaling status. *Genes Dev*. 2013;27(9):1059–1071. doi:10.1101/gad.211912.112.

19. Johnston DA, Dong B, Hughes CCW. TNF induction of jagged-1 in endothelial cells is NFκB-dependent. *Gene*. 2009;435(1-2):36–44. doi:10.1016/j.gene.2009.01.003.

20. Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, Adams RH. The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis. *Cell*. 2009;137(6):1124–1135. doi:10.1016/j.cell.2009.03.025.

21. Sainson RCA, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, Hughes CCW. TNF primes endothelial cells for angiogenic sprouting

by inducing a tip cell phenotype. *Blood*. 2008;111(10):4997–5007. doi:10.1182/blood-2007-08-108597.

22. Brütsch R, Liebler SS, Wüstehube J, Bartol A, Herberich SE, Adam MG, Telzerow A, Augustin HG, Fischer A. Integrin cytoplasmic domain-associated protein-1 attenuates sprouting angiogenesis. *Circulation Research*. 2010;107(5):592–601. doi:10.1161/CIRCRESAHA.110.217257.

23. Kluk MJ, Ashworth T, Wang H, Knoechel B, Mason EF, Morgan EA, Dorfman D, Pinkus G, Weigert O, Hornick JL, Chirieac LR, Hirsch M, Oh DJ, South AP, Leigh IM, Pourreyron C, Cassidy AJ, DeAngelo DJ, Weinstock DM, Krop IE, Dillon D, Brock JE, Lazar AJF, Peto M, Cho RJ, Stoeck A, Haines BB, Sathayanrayanan S, Rodig S, Aster JC. Gauging NOTCH1 Activation in Cancer Using Immunohistochemistry. Martinez Climent JA, ed. *PLoS ONE*. 2013;8(6):e67306. doi:10.1371/journal.pone.0067306.g009.

24. Middleton J, Americh L, Gayon R, Julien D, Mansat M, Mansat P, Anract P, Cantagrel A, Cattan P, Reimund J-M, Aguilar L, Amalric F, Girard J-P. A comparative study of endothelial cell markers expressed in chronically inflamed human tissues: MECA-79, Duffy antigen receptor for chemokines, von Willebrand factor, CD31, CD34, CD105 and CD146. *J Pathol*. 2005;206(3):260–268. doi:10.1002/path.1788.

25. Henseleit U, Steinbrink K, Sunderkötter C, Goebeler M, Roth J, Sorg C. Expression of murine VCAM-1 in vitro and in different models of inflammation in vivo: correlation with immigration of monocytes. *Exp Dermatol*. 1995;4(5):249–256.

26. Takeshita K, Yamasaki T, Akira S, Gantner F, Bacon KB. Essential role of MHC II-independent CD4+ T cells, IL-4 and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse. *International Immunology*. 2004;16(5):685–695. doi:10.1093/intimm/dxh073.

27. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol*. 2007;7(10):803–815. doi:10.1038/nri2171.

28. Espinosa L, Cathelin S, D'Altri T, Trimarchi T, Statnikov A, Guiu J, Rodilla V, Inglés-Esteve J, Nomdedeu J, Bellosillo B, Besses C, Abdel-Wahab O, Kucine N, Sun S-C, Song G, Mullighan CC, Levine RL, Rajewsky K, Aifantis I, Bigas A. The Notch/Hes1 Pathway Sustains NF-κB Activation through CYLD Repression in T Cell Leukemia. *Cancer Cell*. 2010;18(3):268–281. doi:10.1016/j.ccr.2010.08.006.

29. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, Hanna J, Lodato MA, Frampton GM, Sharp PA, Boyer LA, Young RA, Jaenisch R. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci USA*. 2010;107(50):21931–21936. doi:10.1073/pnas.1016071107.

30. Zhong H, May MJ, Jimi E, Ghosh S. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol Cell*. 2002;9(3):625–636.

31. Wang J, Shelly L, Miele L, Boykins R, Norcross MA, Guan E. Human Notch-1 inhibits NF-kappa B activity in the nucleus through a direct interaction involving a novel domain. *J Immunol*. 2001;167(1):289–295.

32. Shin HM, Minter LM, Cho OH, Gottipati S, Fauq AH, Golde TE, Sonenshein GE, Osborne BA. Notch1 augments NF-κB activity by facilitating its nuclear retention. *EMBO J*. 2005;25(1):129–138. doi:10.1038/sj.emboj.7600902.

33. Hoberg JE, Yeung F, Mayo MW. SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival. *Mol Cell*. 2004;16(2):245–255. doi:10.1016/j.molcel.2004.10.010.

34. Severson E, Arnett KL, Wang H, Zang C, Taing L, Liu H, Pear WS, Liu XS, Blacklow SC, Aster JC. Genome-wide identification and characterization of Notch transcription complex-binding sequence-paired sites in leukemia cells. *Sci Signal*. 2017;10(477). doi:10.1126/scisignal.aag1598.

35. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, Shiroishi T. Chromosomal Dynamics at theShhLocus: Limb Bud-Specific Differential Regulation of Competence and Active Transcription. *Dev Cell*. 2009;16(1):47–57. doi:10.1016/j.devcel.2008.11.011.

36. Ghisletti S, Barozzi I, Mietton F, Polletti S, De Santa F, Venturini E, Gregory L, Lonie L, Chew A, Wei C-L, Ragoussis J, Natoli G. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*. 2010;32(3):317–328. doi:10.1016/j.immuni.2010.02.008.

37. Jin F, Li Y, Ren B, Natarajan R. PU.1 and C/EBP synergistically program distinct response to NF- B activation through establishing monocyte specific enhancers. *Proc Natl Acad Sci U S A*. 2011;108(13):5290–5295. doi:10.1073/pnas.1017214108.

38. Petrovic J, Formosa-Jordan P, Luna-Escalante JC, Abelló G, Ibañes M, Neves J, Giraldez F. Ligand-dependent Notch signaling strength orchestrates lateral induction and lateral inhibition in the developing inner ear. *Development*. 2014;141(11):2313–2324. doi:10.1242/dev.108100.

39. Van de Walle I, Waegemans E, De Medts J, De Smet G, De Smedt M, Snauwaert S, Vandekerckhove B, Kerre T, Leclercq G, Plum J, Gridley T, Wang T, Koch U, Radtke F, Taghon T. Specific Notch receptor-ligand interactions control human TCR-αβ/γδ development by inducing differential Notch signal strength. *J Exp Med*. 2013;210(4):683–697. doi:10.1084/jem.20121798.

40. Sprinzak D, Lakhanpal A, LeBon L, Santat LA, Fontes ME, Anderson GA, Garcia-Ojalvo J, Elowitz MB. Cis-interactions between Notch and Delta generate mutually exclusive signalling states. *Nature*. 2010;465(7294):86–90. doi:10.1038/nature08959.

Highlights

- Conditional genetic targeting of endothelial Notch modulates inflammation in murine contact hypersensitivity.
- RBPJ-NOTCH1 premarks sites of RELA genomic recruitment in endothelial cells.
- Endothelial NOTCH1 facilitates the activation of inflammatory enhancers.