Regulation of Notch 1 signaling in THP-1 cells enhances M₂ macrophage differentiation

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Submitted 12 November 2013; accepted in final form 24 September 2014

Singla RD, Wang J, Singla DK. Regulation of Notch 1 signaling in THP-1 cells enhances M₂ macrophage differentiation. Am J Physiol Heart Circ Physiol 307: H1634–H1642, 2014. First published September 26, 2014; doi:10.1152/ajpheart.00896.2013.—Macrophage polarization is emerging as an important area of research for the development of novel therapeutics to treat inflammatory diseases. Within the current study, the role of Notch1R in macrophage differentiation was investigated as well as downstream effects in THP-1 monocytes cultured in “inflammation mimicry” media. Interference of Notch signaling was achieved using either the pharmaceutical inhibitor DAPT or Notch1R small interfering RNA (siRNA), and Notch1R expression, macrophage phenotypes, and anti- and proinflammatory cytokine expression were evaluated. Data presented show that Notch1R expression on M₁ macrophages as well as M₁ macrophage differentiation is significantly elevated during cellular stress (P < 0.05). However, under identical culture conditions, interference to Notch signaling via Notch1R inhibition mitigated these results as well as promoted M₂ macrophage differentiation. Moreover, when subjected to cellular stress, macrophage secretion of proinflammatory cytokines was significantly heightened (P < 0.05). Importantly, Notch1R inhibition not only diminished proinflammatory cytokine secretion but also enhanced anti-inflammatory protein release (P < 0.05). Our data suggest that Notch1R plays a pivotal role in M₁ macrophage differentiation and heightened inflammatory responses. Therefore, we conclude that inhibition of Notch1R and subsequent downstream signaling enhances monocyte to M₂ polarized macrophage outcomes and promotes anti-inflammatory mediation during cellular stress.

Regulation of Notch 1 signaling in THP-1 cells enhances M₂ macrophage differentiation.

THE STUDY OF MACROPHAGE POLARIZATION is emerging as a novel area of research that could yield new methods for the treatment of inflammatory diseases such as atherosclerosis. During an inflammatory response, monocytes migrate to the area of injury and differentiate into two types of macrophages known as M₁ and M₂ polarized macrophages based on diverse activation signals (2, 16). Each category of macrophages has distinct properties that can either negatively or positively affect the inflammatory response and progression of the disease. M₁ macrophages are activated by various macrophage-activating factors including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and bacterial lipopolysaccharide (LPS) (3, 19, 21). They are characteristically proinflammatory, secreting various cytokines and toxic reactive oxygen intermediates including inducible nitric oxide synthase (iNOS), IL-6, TNF-α, and MCP-1. Although M₁ macrophages are needed for normal host defense mechanisms, during diseased states they have the capability to enhance profligate inflammatory responses and exacerbate injury. On the other hand, M₂ macrophages, activated by unique cues including IL-4 and IL-13, are anti-inflammatory and promote wound repair and healing, in part, through the secretion of IL-10, IL-1RA, and arginase 1 (14, 19, 24). Based on the established properties of M₁ and M₂ macrophages, methods to direct differentiation and polarization of monocytes into M₂ macrophages offer new opportunities for the development of pharmaceutical therapies for the treatment of inflammatory diseases.

The Notch signaling pathway plays a fundamental role in cell-to-cell communication in a variety of tissues and organs throughout the body and is implicated in many genetic and acquired diseases and conditions (5, 13, 26, 34). Specifically, the Notch pathway is associated with cell fate determination and differentiation, proliferation, and apoptosis, to name a few. The Notch pathway is initiated by ligand binding to the transmembrane Notch signaling receptor, which results in proteolytic cleavage of the Notch receptor. The intracellular domain then translocates to the nucleus where gene regulation is altered. There are four types of Notch receptors: Notch1R, Notch2R, Notch3R, and Notch4R, all of which have been shown to be expressed on either primary monocytes or macrophages (5, 12, 18, 31). In fact, evidence provided suggests Notch signaling plays a critical role in monocytic cell fate determination and participates in macrophage modulation (11, 22). Recently, studies showed that increased expression of Notch1R results in the progression of lung and vascular inflammation and can induce M₁ macrophage differentiation and proinflammatory cytokine expression (23, 25, 26). Moreover, inhibition of the Notch 1 receptor by a γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyll ester (DAPT), which works by inhibiting the cleavage of transmembrane proteins including Notch receptors, has been shown to reduce atherosclerosis in apolipoprotein E-deficient mice (1). However, whether the mechanisms of inhibited atherosclerosis are mediated through monocyte-macrophage polarization regulation or another pathway altogether is completely unknown.

In the present study, we hypothesized that inhibition of Notch1R and subsequent Notch signaling would promote M₂ macrophage activation and diminish M₁ macrophage differentiation. Our data show that monocytes contain the Notch1R, and when these monocytes were treated with either DAPT or Notch1R small interfering RNA (siRNA), expression of Notch1R significantly decreased. Furthermore, our data demonstrate that following Notch1R inhibition, expression of pro-inflammatory markers iNOS, IL-6, TNF-α, and MCP-1 were significantly decreased, whereas anti-inflammatory markers...
CD206, IL-10, IL-1RA, and arginase 1 were significantly increased. Our data suggest that activation of Notch1R results in proinflammatory M1 macrophage differentiation and an increased inflammatory response. However, when Notch1R was inhibited by either a pharmacological inhibitor like DAPT or by siRNA technology, differentiation toward M2 significantly increased along with anti-inflammatory markers, suggesting the significant role Notch1R plays in the areas of monocyte differentiation, macrophage polarization, and inflammation.

MATERIALS AND METHODS

THP-1 cell culture. THP-1 cells, a human monocytic cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in 25-cm² flasks with RPMI 1640 (ATCC) media that was supplemented with 10% fetal bovine serum and 0.05 mM β-mercaptoethanol. Media was changed every other day.

Apoptotic-conditioned media model. To induce stressed conditions, apoptotic-conditioned media (ACM) was used as we previously reported (28). In brief, H9c2 cardiomyoblasts were plated for 24 h in 60-mm tissue culture dishes with 5 × 10⁵ cells in each plate. After 24 h, 400 μM of H₂O₂ was added to each plate for 2 h. Following 2 h of treatment, the media was removed, filtered, and labeled as ACM. The ACM was stored in 15-ml conical tubes at 4°C for future use.

DAPT treatment. THP-1 monocytes were plated on 48-well plates with 20,000 cells per well for 24 h. Cells were divided into the following groups: control (RPMI media), ACM (RPMI media + 40% ACM), ACM + DAPT (RPMI media + 40% ACM + 30 μM DAPT), and control + DAPT (RPMI media + 30 μM DAPT). Forty-eight hours posttreatment, cell smears were prepared for immunohistochemistry to identify macrophage differentiation based on cell surface markers, and supernatant was preserved for cytokine analysis.

Notch 1 receptor siRNA. Notch1R expression was knocked down in THP-1 monocytes using Notch 1 siRNA following manufacturer’s instructions (No. sc-36095, Santa Cruz Biotechnology, Santa Cruz, CA). In brief, 1 × 10⁶ monocytes were placed into 1.5-ml sterile tubes, each containing 800 μl of transfection medium (No. sc-36868, Santa Cruz Biotechnology). Thirty minutes postincubation, solution A/B mixture [solution A: 6-μl siRNA or scrambled duplex + 100-μl transfection medium (No. sc-36868 Santa Cruz Biotechnology); and solution B: 6-μl transfection reagent (No. sc-29528, Santa Cruz Biotechnology) + 100 μl of transfection medium (No. sc-36868, Santa Cruz Biotechnology)] was added to the tubes, and monocytes were subsequently plated and incubated for 6 h at 37°C. Following washing, the cells were divided into the following groups: control (scrambled siRNA), ACM (scrambled siRNA and RPMI media +

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Fig. 1. Treatment with N-[N-((3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) decreases M₁ macrophage differentiation and enhances M₂ macrophage yields in stressed THP-1, a human monocytic cell line. Representative images of polarized macrophages stained with anti-Notch1R shown in green (a, e, i, and m), anti-inducible nitric oxide synthase (iNOS) (M₁ macrophages) or anti-CD206 (M₂ macrophages) shown in red (b, f, j, and n), 4',6-diamido-2-phenylindole (DAPI) shown in blue (c, g, k, and o) and merged images (d, h, l, and p) (A and B). White boxes in merged images are enhanced images depicting colocalization of Notch1R and iNOS or CD206. Scale bar = 50 μm. The percentage of M₁ and M₂ macrophages also positive for Notch1R expression was knocked down in THP-1 monocytes using Notch 1 siRNA following manufacturer’s instructions (No. sc-36095, Santa Cruz Biotechnology, Santa Cruz, CA). In brief, 1 × 10⁶ monocytes were placed into 1.5-ml sterile tubes, each containing 800 μl of transfection medium (No. sc-36868, Santa Cruz Biotechnology). Thirty minutes postincubation, solution A/B mixture [solution A: 6-μl siRNA or scrambled duplex + 100-μl transfection medium (No. sc-36868 Santa Cruz Biotechnology); and solution B: 6-μl transfection reagent (No. sc-29528, Santa Cruz Biotechnology) + 100 μl of transfection medium (No. sc-36868, Santa Cruz Biotechnology)] was added to the tubes, and monocytes were subsequently plated and incubated for 6 h at 37°C. Following washing, the cells were divided into the following groups: control (scrambled siRNA), ACM (scrambled siRNA and RPMI media +

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AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00896.2013 • www.ajpheart.org
40% ACM), ACM + siRNA (RPMI media + 40% ACM), and siRNA. As aforementioned, 48 h posttreatment, cells and supernatant were preserved for future analysis.

**Immunohistochemistry of Notch1R and macrophage markers.** In brief, cell smears prepared on glass ColorFrost slides, as previously reported, were fixed with 4% parafomaldehyde (28). Blocking was then performed for 1 h at room temperature with 10% normal goat serum (Vector, Burlingame, CA). Primary antibodies, anti-Notch 1 (1:30, No. ab44986, Abcam, Cambridge, MA), anti-iNOS (1:200, to identify M1 macrophages, No. ab129372, Abcam), or anti-CD206 (1:50, to identify M2 macrophages, No. ab64693, Abcam) were then added to the smears and incubated for 24 h at 4°C. Following incubation, the smears were washed and appropriate secondary antibodies, Fluorescein Avidin DSC (1:62.5, No. FMK-2201, Vector) or Alexa 568 (1:50, No. A11019, Invitrogen, Grand Island, NY), were added for 1 h at room temperature. The smears were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector) and coverslipped. Quantitative data were obtained by taking four representative areas in control and experimental groups via enzyme-linked immunosorbent assay (ELISA) analysis. AU, arbitrary units. *P < 0.05 vs. Con, #P < 0.05 vs. ACM; S no significance to Con. n = 4 to 5 for MCP-1 and TNF-α. n = 6–8 for IL-6, IL-10, and IL-1RA.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis. Western blot experiments were carried out as previously reported (27). In brief, proteins were separated on an 8–10% polyacrylamide gel and semidy transferred to a PVDF membrane. Membranes were blocked with 5% milk followed by incubation with primary antibodies [Notch 1, (1:1,000, No. 3268s, Cell Signaling, Danvers, MA), cleaved (activated) Notch 1 (C-Notch 1, 1:1,000, No. 41475, Cell Signaling), Hes-1 (1 µg/ml, No. ab49170, Abcam), or β-actin (1:1,000, No. 4967L, Cell Signaling)] at appropriate concentrations for 1 h at room temperature or at 4°C overnight. Following primary antibody incubation, membranes were incubated with appropriate secondary antibodies for 1 h. Resulting bands were scanned, and intensities were determined using NIH ImageJ software. Data, relative to β-actin, are expressed as arbitrary units.

**Statistical analysis.** One-way analysis of variance (ANOVA) followed by Tukey’s test was used for data analysis. All data are shown as means ± SE with statistical significance given when P < 0.05.

**RESULTS**

**Notch 1 receptor expression and macrophage differentiation augmented post-DAPT treatment.** Macrophages were generated in vitro from THP-1 monocytes cultured in the presence of ACM. Representative images of differentiated macrophages stained with anti-Notch1R shown in green (a, e, i, and m),

![Graphs](https://example.com/graphs/)

**Fig. 2.** Treatment of stressed THP-1 cells with DAPT decreases proinflammatory cytokine expression while enhancing anti-inflammatory protein secretion. Isolated culture media was used to quantify secreted proinflammatory cytokines [IL-6 (A), MCP-1 (B), and TNF-α (C)] and anti-inflammatory cytokines [IL-10 (D) and IL-1RA (E)] in control and experimental groups via enzyme-linked immunosorbent assay (ELISA) analysis. AU, arbitrary units. *P < 0.05 vs. Con, #P < 0.05 vs. ACM; S no significance to Con. n = 4 to 5 for MCP-1 and TNF-α. n = 6–8 for IL-6, IL-10, and IL-1RA.
anti-iNOS (M₁ macrophages) or anti-CD206 (M₂ macrophages) shown in red (b, f, j, and n), DAPI shown in blue (c, g, k, and o), and merged images (d, h, l, and p) are depicted in Fig. 1, A and B. Quantitative data suggest that Notch1R is significantly upregulated on differentiated M₁ macrophages following exposure to ACM relative to control cells (P < 0.05, Fig. 1C). However, Notch1R expressing M₁ macrophages were significantly reduced when additionally treated with DAPT (P < 0.05, Fig. 1C). Of note, no statistical significance was obtained for Notch1R expression on M₁ differentiated macrophages between the control group and the DAPT-treated cells not cultured in ACM (Fig. 1C). Assessment of Notch1R expression on polarized M₂ macrophages revealed no deviation in the number of receptors among any of the control or treatment groups (Fig. 1E).

The percentages of THP-1-differentiated M₁ and M₂ macrophages were also analyzed. Consistent with Notch1R expression, the percentage of M₁ macrophages was significantly elevated following ACM treatment compared with control, which was abrogated in the presence of DAPT (P < 0.05, Fig. 1D). However, when not cultured in “inflammation mimicry” media, M₁ macrophage concentrations were unchanged between control and control + DAPT groups (Fig. 1D). Interestingly, M₂ macrophage concentrations were significantly elevated in the ACM + DAPT group compared with the ACM group (P < 0.05), whereas no deviation in M₂ macrophage outcomes were noted between the control and control + DAPT groups (Fig. 1F).

Proinflammatory cytokine expression altered in THP-1 cells following DAPT treatment. Activated macrophages, whether M₁ or M₂, have unique cytokine profiles. Previous data suggest M₁ macrophages secrete various proinflammatory cytokines including IL-6, MCP-1, and TNF-α (16). To this end, media from each group was removed following experimental design time parameters and used to assess secreted cytokine concentrations. ELISA revealed that THP-1 monocytes cultured in ACM, compared with controls, secreted significantly higher quantities of IL-6, MCP-1, and TNF-α, which is consistent with the M₁ macrophage phenotype (P < 0.05, Fig. 2, A–C). However, THP-1 cells exposed to ACM, additionally treated with DAPT, had significantly reduced proinflammatory cytokine concentrations relative to the ACM group (P < 0.05, Fig. 2, A–C). Notably, when cultured in absence of ACM but with DAPT, IL-6, MCP-1, and TNF-α expression was unaffected relative to control cells (Fig. 2, A–C).

Fig. 3. Treatment with DAPT diminishes Notch 1 activation and signaling. A, top: representative blots from all control and experimental groups showing Notch 1, cleaved Notch 1 (C-Notch 1), and β-actin control expression. A, middle: quantified Notch 1 expression demonstrating downregulated Notch 1 expression following DAPT treatment. A, bottom: quantified activated Notch 1 expression, which suggests C-Notch 1 is significantly diminished following DAPT treatment. B, top: representative blots depicting Hes-1 and β-actin expression. B, bottom: densitometric analysis of Hes-1 expression from all control and experimental groups. *P < 0.05 vs. Con, #P < 0.05 vs. ACM. © no significance to Con. n = 4 to 5.
Treatment with DAPT increases anti-inflammatory cytokine secretion from THP-1 cells. As we previously identified, treatment with DAPT yielded higher concentrations of M2 macrophages during inflammation mimicry using ACM. As such, levels of anti-inflammatory cytokines, which are characteristically secreted from M2 macrophages, were assessed for all control and experimental groups. No significant deviation in IL-10 and IL-1RA expression was reported between control and ACM groups (Fig. 2, D and E). However, secreted IL-10 and IL-1RA were significantly augmented following DAPT treatment during stressed conditions compared with the ACM group ($P < 0.05$, Fig. 2, D and E). Additionally, no significant differences in IL-10 and IL-1RA expression were noted between control and control + DAPT groups, which is consistent with the lack of significant difference in M2 macrophage yields previously noted in Fig. 1F (Fig. 2, D and E).

Notch 1 signaling is augmented in THP-1 cells following treatment with DAPT. To demonstrate that DAPT treatment inhibits Notch 1 signaling in THP-1 cells exposed to inflammation mimicry media, levels of Notch 1, activated Notch 1 (C-Notch 1) and Hes-1, and a downstream effector in the Notch 1 signaling cascade were evaluated. Levels of Notch 1 and activated Notch 1 were unaffected in THP-1 cells exposed to ACM compared with controls (Fig. 3A). However, when treated with DAPT, whether exposed to ACM or not, levels of Notch 1 and C-Notch 1 were significantly decreased in THP-1 cells compared with ACM alone-treated cells ($P < 0.05$, Fig. 3A). Additionally, levels of Hes-1 were significantly enhanced in the ACM group compared with the control group ($P < 0.05$, Fig. 3B). Notably, following DAPT treatment in control and ACM-treated THP-1 cells, Hes-1 expression was significantly diminished relative to the ACM group ($P < 0.05$, Fig. 3B).

siRNA knockdown of Notch1R augments M1 and M2 macrophage differentiation in THP-1 cells. Widely accepted, DAPT is a promiscuous γ-secretase inhibitor with target substrates not limited to Notch alone. As proof of concept and to strengthen our findings obtained using DAPT, Notch 1 signaling was also knocked down using Notch1R siRNA. Alterations to Notch1R expression and M1/M2 differentiation were evaluated in THP-1 monocytes following siRNA treatment. The number of M1 macrophages expressing Notch1R was significantly enhanced in the ACM group compared with control cells, both of which were treated with scrambled siRNA ($P < 0.05$, Fig. 4A). Notably, treatment of ACM-stressed THP-1 monocytes with Notch1R siRNA significantly blunted Notch1R expression on M1 macrophages compared with the ACM group ($P < 0.05$, Fig. 4A). However, siRNA treatment to cells not exposed to ACM had no differences in Notch1R expressing M1 macrophage concentrations between the control and siRNA groups (Fig. 4A). Equally important, M1 macrophage differentiation significantly decreased in stressed THP-1 cells treated with Notch1R siRNA compared with the ACM group ($P < 0.05$, Fig. 4B). Data also suggest Notch1R expressing M2 macro-

![Fig. 4. Knockdown of Notch 1 receptor using siRNA results in decreased M1 macrophage and increased M2 macrophage differentiation in stressed THP-1 monocytes. The percentages of Notch1R-positive M1 and M2 macrophages were quantified in THP-1 monocytes treated with scrambled or Notch1R siRNA (A and C, respectively). Quantitative analysis of M1 and M2 macrophage yields following treatment of THP-1 monocytes were also determined (B and D, respectively). *$P < 0.05$ vs. Con, #$P < 0.05$ vs. ACM, and $P$ no significance to Con. $n = 4$ to 5. ACM + siRNA, THP-1 cells treated with Notch1R siRNA and cultured in apoptotic-conditioned media; siRNA, THP-1 cells treated with Notch1R siRNA.](http://ajpheart.physiology.org/)
phage concentrations were not significantly altered between any of the control or treatment groups (P < 0.05, Fig. 4C). However, the overall percentage of CD206-positive M2 macrophages was significantly increased following Notch1R siRNA treatment (P < 0.05, Fig. 4D).

Proinflammatory cytokine expression decreases following Notch 1 knockdown in THP-1 cells. Although Notch1R siRNA significantly diminished Notch1R expressing M1 macrophages, the downstream consequences of such actions on proinflammatory secretion remains obscure. To that end, levels of IL-6, MCP-1, and TNF-α were evaluated, and analyzed data suggest a significant increase in all three secreted proinflammatory cytokines from stressed, scrambled siRNA-treated THP-1 monocytes (P < 0.05, Fig. 5, A–C). Importantly, treatment with Notch1R siRNA resulted in significantly reduced IL-6, MCP-1, and TNF-α expression in the ACM + siRNA and siRNA groups relative to the ACM group (P < 0.05, Fig. 5, A–C). However, when cells were treated with Notch1R siRNA and not exposed to ACM, proinflammatory cytokine secretion was unaffected relative to the control group (Fig. 5, A–C).

Expression of anti-inflammatory cytokines is augmented following Notch1R knockdown in THP-1 monocytes. Quantified IL-10 expression was significantly upregulated in the ACM + siRNA group relative to the ACM group (P < 0.05, Fig. 5D). However, IL-10 was significantly upregulated following siRNA Notch1R knockdown in THP-1 cells not exposed to ACM (P < 0.05, Fig. 5D), mimicking data obtained in THP-1 cells treated with DAPT. Additionally, IL-1RA expression was significantly elevated following Notch1R siRNA in ACM-treated THP-1 cells compared with ACM-treated cells (P < 0.05), whereas no difference was noted between control and siRNA groups (Fig. 5E).

Notch 1 signaling is decreased in THP-1 cells following siRNA knockdown of Notch1R. As with DAPT-treated THP-1 cells, levels of Notch 1, activated Notch 1, and Hes-1 were evaluated following siRNA knockdown of Notch1R to illustrate Notch 1 signaling interference. Data presented suggest Notch 1 and activated Notch 1 expressions were significantly downregulated following treatment with Notch1R siRNA compared with the ACM group (P < 0.05, Fig. 6A). Additionally, levels of Hes-1, which were significantly elevated in the ACM group relative to the control group, were significantly diminished following Notch1R siRNA compared with the ACM group (P < 0.05, Fig. 6B). Collectively, our data suggest that Notch1R interference via DAPT and siRNA hinders Notch 1 activation and downstream cascade signaling.

DISCUSSION

During an immune or inflammatory response, monocytes are the first cell type to migrate to the injury site. Upon arrival, monocytes undergo reprogramming, yielding two distinct phenotypes of proinflammatory and anti-inflammatory macrophages, M1 and M2, respectively, which is consequent to intracellular signals and the cellular microenvironment (2, 16, 19). M1 “classically activated” macrophages yield various cytotoxic effectors including reactive oxygen/nitrogen intermediaries and inflammatory chemokines/cytokines. Recent studies have suggested an association between predominant M1 macrophage differentiation and disease pathophysiologies in-

Fig. 5. Expression of proinflammatory and anti-inflammatory cytokines following knockdown of Notch 1 receptor on THP-1 cells. Isolated culture media was used to quantify secreted proinflammatory cytokines [IL-6 (A), MCP-1 (B), and TNF-α (C)] and anti-inflammatory cytokines [IL-10 (D) and IL-1RA (E)] in control and experimental groups via ELISA analysis. *P < 0.05 vs. Con, #P < 0.05 vs. ACM, and $no significance to Con. n = 6–8 for IL-6, TNF-α, and IL-10.
cluding that of cancer, atherosclerosis, and prediabetic phenotypes (4, 8, 15, 32). Conversely, activated M2 macrophages, which circumscribe all nonclassically polarized macrophages, are implicated in salutary mediation of inflammation by promoting resolution, remodeling, and repair, in part, through secretion of prototypical cytokines including IL-10, IL-1RA, and arginase-1 (2, 19). Studies are currently underway to exploit novel growth factors/small molecules, which dictate M1/M2 differentiation outcomes for therapeutic applications (6, 28, 30, 35). Notably, we recently published data identifying bone morphogenetic protein-7 (BMP-7) as a novel differentiating signal, which promotes enhanced M2 macrophage yields from THP-1 monocytes during inflammation mimicry in vitro (28). Although it has been suggested that the dynamic monocyte to macrophage differentiation paradigm is dictated by microenvironmental signals, the mechanisms, which mandate macrophage phenotypic plasticity, remain largely unknown. Within this context, the current study was undertaken to identify possible alternative intracellular signaling pathways, independent of BMP-7, which are involved in M1/M2 differentiation.

Widely accepted, the Notch signaling cascade dictates cell fate determination and tissue homeostasis in both physiological and pathological conditions. Evidence provided suggests inactivation of the Notch pathway mitigates atherosclerosis and vasculitis and enhances wound healing, whereas stimulation of the signaling cascade promotes pulmonary and cardiac inflammation as well as contributes to neoplasia and metastasis (1, 10, 23, 25). Although a correlation between Notch signaling and inflammation has been established, the exact mechanisms have yet to be elucidated. Importantly, recent evidence has been provided suggesting Notch signaling plays a critical role in fate determination during tumor-associated macrophage activation (34). Specifically, following forced Notch signaling and LPS stimulation, the M1 phenotypic outcomes were dramatically enhanced, whereas blockade of Notch signaling promoted M2 polarization, even in the presence of LPS and TNF-α (34). Taking into account the aforementioned studies, we hypothesized that the inflammatory response is propagated and heightened through activation of Notch1R, specifically, which in turn promotes M1 macrophage differentiation.

THP-1 monocytes were cultured in a specialized apoptotic-conditioned media, which has been previously reported, inducing inflammation mimicry conditions driving monocyte polarization and macrophage differentiation (28). Cells were additionally treated with DAPT, a γ-secretase inhibitor, which has

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**Fig. 6.** siRNA knockdown of Notch1R augments Notch 1 activation and signaling. A, top: representative blots from all control and experimental groups showing Notch 1, cleaved Notch 1 (C-Notch 1), and β-actin control expression. A, middle: quantified Notch 1 expression demonstrating downregulated Notch 1 expression following Notch1R siRNA treatment. A, bottom: quantified activated Notch 1 expression, which suggests C-Notch 1 is significantly diminished following siRNA treatment. B, top: representative blots depicting Hes-1 and β-actin expression. B, bottom: densitometric analysis of Hes-1 expression from all control and experimental groups. *P < 0.05 vs. Con, #P < 0.05 vs. ACM, and $no significance to Con. n = 5–7.
been used in countless studies to drive Notch signaling interference (9, 20, 33). To validate Notch signaling impediment, Western blot analysis was performed and our data shows that when treated with DAPT, a significant decrease in activated Notch 1 expression was achieved.

To determine whether or not Notch1R-activated signaling plays a critical role in macrophage differentiation outcomes, Notch1R expression was evaluated on M1 and M2 macrophages as well as concentrations of each macrophage subset. The current findings suggest that THP-1 monocyctic cells, when cultured in inflammation mimicry media, significantly differentiate toward the M1, the phenotype with a dramatic increase in Notch1R expression, which is concurrent with Kitajewski et al. (23) who suggest Notch 1 signaling plays a monumental role in activated macrophage propagation. Importantly, the addition of DAPT, a Notch inhibitor, significantly reduced the expression of Notch1R on differentiated M1 macrophages as well as decreased the overall M1 macrophage concentration. Although no difference was observed in Notch1R expression on M2 macrophages, as evidenced by colocalization of Notch1R and CD206, the number of M2 macrophages was significantly upregulated following ACM exposure and DAPT treatment. Our data suggest that Notch signaling plays a role in dictating macrophage differentiation during inflammation mimicry with predominant proinflammatory M1 macrophage phenotypic outcomes.

Concurrent with increased M1 macrophage concentrations consequent to ACM exposure, proinflammatory cytokine (IL-6, MCP-1, and TNF-α) secretion was significantly escalated. Our data are in corroboration with Hirata et al. (10) who suggest a correlation between elevated M1 macrophage concentration in epicardial adipose tissue obtained from coronary arterial disease (CAD) patients and upregulated IL-6, MCP-1, and TNF-α expression. Of importance, inhibition of the Notch pathway via DAPT significantly ameliorated proinflammatory cytokine expression while enhancing secreted anti-inflammatory cell signaling molecules, IL-10 and IL-1RA. The decreased levels of proinflammatory cytokines and the increased anti-inflammatory cytokine expression-yielded post-Notch pathway inhibition correlates with blunted M1 macrophage differentiation and amplified M2 macrophage activation. Overall, data presented suggest that interference of Notch signaling, specifically Notch1R, inhibits classical activation of M1 macrophages and drives monocytic differentiation yielding M2-polarized macrophages, which is supported by data obtained from the pro- and anti-inflammatory cytokine profiles.

γ-Secretases, including DAPT, are promiscuous enzymes, which target and cleave multiple substrates independent of Notch including LDL receptor-related protein (LRP), E-cadherin, and ErbB-4 (17, 29, 36). In lieu of the nonspecific inhibition of Notch1R via DAPT, additional studies were carried out in which the Notch1R was knocked down using siRNA to authenticate our data previously collected using DAPT. Consistent with the findings obtained using THP-1 cells treated with DAPT, M1 macrophage differentiation and Notch1R expression on M1 macrophages were drastically quenched, whereas M2 macrophage differentiation was enhanced in the ACM + Notch1R siRNA group relative to the ACM group. Additionally, knockdown of Notch1R and subsequent signaling inhibited IL-6, MCP-1, and TNF-α secretion while promoting anti-inflammatory cytokine release including IL-10 and IL-1RA.

Although the goal of the current article was to identify independent signaling cascades driving M1/M2 differentiation outcomes, we acknowledge that often there is cross talk between various cellular communication pathways. For instance, we previously published a correlation between exogenous BMP-7 application to THP-1 monocytes during stressed conditions and enhanced M2 macrophage differentiation and anti-inflammatory cytokine expression (28). Independent of our study, an investigation (7) was published suggesting BMP-7 restricts branching of the prostate epithelium with mechanistic implications of Notch signaling repression. Such data combined with the current findings may suggest that BMP-7 promotes anti-inflammatory macrophage differentiation, in part, through depressed Notch 1 signaling. Although speculative, future studies are warranted to identify upstream and downstream mediators driving M2 macrophage paradigm shifts via Notch signaling inhibition.

In conclusion, our presented data suggest that Notch1R plays a key role in macrophage differentiation during the inflammatory response by driving M1 macrophage activation and subsequent proinflammatory cytokine release. Additionally, treatment of THP-1 monocytes with DAPT or Notch-specific siRNA and subsequent Notch signaling interference resulted in significantly decreased M1 macrophage differentiation and Notch1R expression, enhanced M2 macrophage activation, and upregulated anti-inflammatory cytokine secretion. Future studies are warranted to determine the extent, if any, Notch2R, Notch3R, or Notch4R activation plays in monocytic differentiation and macrophage polarization. However, data presented identify a novel target, Notch1R, which may be used for future therapeutic applications in proinflammatory diseases.

ACKNOWLEDGMENTS

We are grateful to Crystal Rocher for technical assistance with THP-1 monocyte cell culture.

GRANTS

This paper is supported in part by Grants RO1-HL-090646, HL-117241, and RO1-HL-117241 (to D. K. Singla).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.D.S. and J.W. performed experiments; R.D.S. analyzed data; R.D.S. and D.K.S. interpreted results of experiments; J.W. prepared figures; D.K.S. drafted manuscript; D.K.S. approved final version of manuscript.

REFERENCES


