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Notch1 primes CD4 T cells for T helper type I differentiation through its early effects on miR-29

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ABSTRACT

The transmembrane receptor, Notch1 plays an important role during the differentiation of CD4 T cells into T helper (Th) subsets in the presence of appropriate cytokines, including differentiation into Th1 cells. MicroRNAs have also been shown to be important regulators of immune responses, including negatively regulating cytokine production by Th1 cells. The miR-29 family of microRNAs can act to inhibit *tbx21* and *ifng* transcription, two important pro-inflammatory genes that are abundantly expressed in Th1 cells. Here we show that Notch1 may prime CD4 T cells to be responsive to Th1-polarizing cues through its early repressive effects on the miR-29 family of microRNAs. Using a combination of cell lines and primary cells, we demonstrate that Notch1 can repress miR-29a, miR-29b, and miR-29c transcription through a mechanism that is independent of NF-κB. We further show that this repression is mediated by canonical Notch signaling and requires active Mastermind like (MAML) 1, but this process is superseded by positive regulation of miR-29 in response to IFNγ at later stages of CD4 T cell activation and differentiation. Collectively, our data suggest an additional mechanism by which Notch1 signaling may fine-tune Th1 cell differentiation.

1. Introduction

Naïve CD4 T cells are activated in response to encountering antigens. Upon activation, these cells will proliferate and differentiate into T helper (Th) cells, facilitated by the cytokines present at the time of stimulation. Based on the expression of unique transcription factors and characteristic cytokines, Th cells can be broadly categorized into defined subsets, with Th1, Th2, and Th17 being the three most-studied to date (Luckheeram et al., 2012). For Th1 cells, the transcription factor, T-bet, which is encoded by the gene, *tbx21*, is a critical regulator of cell fate, and its expression ultimately leads to production of interferon gamma (IFNγ), a signature Th1 pro-inflammatory cytokine (Afkarian et al., 2002; Szabo et al., 2000). T-bet is a direct transcriptional target of the transmembrane receptor, Notch1, and is controlled by non-canonical Notch1 signaling during Th1 differentiation (Minter et al., 2005; Bailis et al., 2013).

The Notch family of transmembrane receptors, can be activated by its ligands, Delta-like- (DLL)-1,3,4 and Jagged (Jag)-1,2 (Artavanis-Tsakonas et al., 1999). Notch signaling plays an important role in cell fate determination. There are four Notch receptors (1–4) expressed in mammalian cells; however, only the expression of Notch1-3 has thus far been described in T cells. Following interaction with cognate ligands, Notch receptors undergo a series of proteolytic cleavage events. The final, S3 cleavage is mediated by a multi-protein γ-secretase complex, and generates the transcriptionally active, intracellular domain (NICD) (Bray, 2006). NICD can translocate to the nucleus to interact with its DNA-binding partner, CBF1-Suppressor of Hairless-Lag1 (CSL) (Mumm and Kopan, 2000). NICD binding to CSL results in further recruitment of transcriptional co-activators such as mastermind like-1 (MAML-1) and p300 (Petcherski and Kimble, 2000; Maillard et al., 2003). CSL-

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dependent Notch signaling is referred to as canonical Notch signaling. There are also reports indicating NICD can interact with proteins other than CSL, both in the nucleus and cytosol, to signal through non-canonical mechanisms (Andersen et al., 2012; Minter and Osborne, 2012).

Studies have identified a crucial role for Notch signaling in Th cell differentiation, both through canonical and non-canonical signaling (Minter and Osborne, 2012; Amsen et al., 2015; Sun et al., 2008; Maekawa et al., 2003). One of the first studies implicating Notch in Th1 differentiation, demonstrated that blocking the actions of γ-secretase, prevented Notch cleavage and attenuated the expression of T-bet and IFNγ in developing Th1 cells (Minter et al., 2005). Expression of Th cell master transcription factors and proinflammatory cytokines must be tightly regulated. In the case of Th1 cells, dysregulated IFNγ expression can lead to chronic autoimmune disorders through its actions on other immune cells, resulting in a hyper-immune response (Pollard et al., 2013).

There are various cell-intrinsic mechanisms that have evolved to fine-tune cytokine expression, including those mediated by the actions of microRNAs (miRs). MiRs are short, single stranded non-coding RNAs that act as post-transcriptional inhibitors of gene expression. They silence their target mRNAs through complementary base pairing and by forming an RNA- induced silencing complex (RISC). Based on their complementarity, the target mRNA is either degraded or its translation into protein is greatly reduced. The miR-29 family of miRNAs consist of miR-29a, -b and -c, and directly targets the mRNA of Th1 signature molecules, *tbx21*and *ifng* (Steiner et al., 2011; Ma et al., 2011; Smith et al., 2018). Interestingly, in response to IFNγ, miR-29 expression increases, thereby forming a negative feedback loop (Smith et al., 2012). Furthermore, NF-κB, a non-canonical binding partner of Notch, can inhibit the expression of miR-29 (Ma et al., 2011; Mott et al., 2010; Wang et al., 2008), indicating miR-29 expression can be regulated through multiple mechanisms.

We previously showed Notch1 signaling contributes to Th1 differentiation, including regulation of T-bet and IFNγ production, as well as to the induction of NF-κB in activated T cells (Minter et al., 2005; Shin et al., 2006). Here, we investigated whether Notch1 may also prime T cells for Th1 differentiation by affecting the expression of miR-29, and whether this proceeds through an NF-κB-dependent mechanism. In this study, we demonstrate that Notch1 can directly inhibit the expression of miR- 29 in Th1 cells, during the early stages of Th1 differentiation. Furthermore, we show that Notch1 represses miR-29 through an NF-κB-independent, CSL (CBF1-Suppressor of Hairless- Lag1)-mediated canonical signaling pathway. However, we also found that the early repressive effects of Notch1 on miR-29 are masked by IFNγ − induced expression of miR-29, during late stages of Th1 differentiation. Thus, we suggest a mechanism by which Notch1 and IFNγ may act in opposition to maintain a balance of miR-29 expression and fine-tune Th1 cell fate.

2. Materials & methods

2.1. Mice

C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred, housed, and utilized in accordance with guidelines set forth by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. Mice aged 8–12 weeks were used.

2.2. Plasmids and cell lines

The pGL3-miR-29b1a promoter vectors were a kind gift from J. Mott (Mott et al., 2010). The N1ICD parental pcDNA3 expression plasmids were a kind gift from A. Capobianco (Jeffries and Capobianco, 2000) and subsequently cloned into pEGFP vector as described (Shin et al., 2006). MAML-1 and DNMAML-1 vectors were provided in collaboration with L. Miele and generated as described (Hao et al., 2010). Generation of DO11.10 T cell hybridoma cell lines with empty vector or N1ICD were described previously (Shin et al., 2006).

2.3. T cell isolation, activation, and in vitro polarization

CD4 T cells were isolated from bulk splenocytes by magnetic bead separation using anti-CD4 magnetics particles (BD Biosciences, San Jose, CA) according to the manufacturer's instruction. Cells were activated in vitro by plating 1×10^6 cells/ml/well of a 12-well plate precoated with anti-CD3ε and anti-CD28 (Biolegend, San Diego, CA) and crosslinked with anti-hamster IgG (Sigma-Aldrich Corp., St. Louis, MO). Cells were cultured in a 1:1 mixture of RPMI 1640:DMEM supplemented with 10% fetal bovine serum, L-Glutamine, Sodium Pyruvate, Penicillin/Streptomycin and β-mercaptoethanol. The following conditions were used for polarization. Th1: 10μg/ml of anti-IL-4 and 1 ng/ml of recombinant mouse IL-12. Th2: 10μg/ml anti-IFNγ and 1 ng/ml of recombinant mouse IL-4. For inhibitor assays, the CD4 T cells were pretreated with 25 uM of GSI PF-03084014 (Medchem Express, Monmouth Junction, NJ) for 30 min. For NF-κB inhibition studies, the DO11.10 T cell hybridoma cell line was treated with 5μM of Bay11-7085 (Sigma-Aldrich Corp., St. Louis County, MO).

2.4. Protein detection by FACS analysis and immunoblotting

Activated CD4 T cells were harvested at indicated time points and restimulated with plate-bound anti-CD3ε for 5 h with Golgi Stop (BD Biosciences). Intracellular staining was performed to detect Notch1, T-bet, and IFNγ using the FoxP3 staining buffer set, following the manufacturer's instructions (eBioscience, Santa Clara, CA). Data were acquired on an LSR Fortessa (BD Biosciences) and analyzed using FlowJo Software (FlowJo LLC, Ashland, OR). For immunoblotting, cells were lysed in RIPA buffer (150 mM NaCl, 1% IGEPAL-CA630, 0.1% SDS, 0.5% Sodium deoxycholate, 50 mM Tris pH 8.0) with protease and phosphatase inhibitors (Bimake, Houston, TX). Lysates were resolved using SDS-PAGE and proteins were transferred to nitrocellulose membrane (GE Amersham, Pittsburgh, PA). Cleaved, intracellular domain of Notch1 was visualized with anti-cleaved Notch1 antibody (Val1744) (D3B8, Cell Signaling Technology, Danvers, MA) and anti-rabbit secondary antibody conjugated with horse radish peroxidase (GE Amersham)

2.5. Quantitative real time PCR

Stimulated CD4 T cell were harvested at indicated time points and total RNA was extracted using Quick-RNA Mini Prep kit (Zymo Research, Irvine, CA) following the manufacturer's instruction. Total RNA was reverse-transcribed with oligo-dT or stem loop primers using MuLV Reverse Transcriptase. Stem loop primers for miRNA quantification were designed as described. Quantitative Real Time PCR was performed using 2× SYBR Green master mix (Bimake, Inc., Houston, TX). Transcripts were quantified using the $2^{-\Delta\Delta CT}$ method.

2.6. Luciferase assay

NIH3T3 cells were co-transfected with pGL3-29b1a, N1ICD, MAML-1 or DNMAML-1 and control vectors using Xtremegene transfection reagent (Sigma-Aldrich Corp.). Luciferase assay was performed after 48 h of transfection according to the manufacturer's instruction. Firefly luciferase activity was measured using Luminometer $20/20^n$ (Turner Biosystems-Promega, Madison, WI) and was normalized to Renilla luciferase activity to control for variances in transfection efficiency.

2.7. Chromatin immunoprecipitation

Cells were treated with 1% formaldehyde to crosslink proteins to