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Dysregulated co-stimulatory molecule expression in a Sjögren's syndrome mouse model with potential implications by microRNA-146a

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Abstract

Sjögren's syndrome (SjS) is an autoimmune condition that primarily affects salivary and lacrimal glands, causing loss of secretion. We have previously shown that microRNA-146a (miR-146a) is over-expressed in the salivary glands and peripheral blood mononuclear cells (PBMC) of SjS-prone mice (C57BL/6.NOD-*Aec1Aec2*, B6DC) and in PBMC of SjS patients. The purpose of this research was to identify a target molecule of miR-146a and identify subpopulations of cells affected by altered miR-146a in the salivary glands of SjS-prone mice. *In silico* analyses identified costimulatory molecule CD80 as a potential target of miR-146a. Luciferase assay of the human CD80 3'untranslated region demonstrated miR-146a directly inhibited CD80 protein expression as indicated by reduced luciferase reporter expression and an examination of B6DC salivary glands revealed a reduction in CD80 protein. More interestingly, the specific reduction in CD80 protein was detected from the salivary gland epithelial cell population and in interstitial dendritic cells in

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Disclosure

The authors declare that they have no competing interests.

Authorship contributions

A.E.G conceived the study, carried out the molecular genetic studies, participated in sequence alignment, participated in design of the study, carried out acquisition and interpretation of data, performed statistical analysis, and drafted the manuscript. Y.J.P. participated in study design and carried out acquisition and interpretation of data and revising the manuscript for important intellectual content. G.N. and M.O. participated in the acquisition and interpretation of data and helped to draft the manuscript. J.O.J. carried out data acquisition and interpretation. C.M.S. contributed to the design of the study. Q.Y. and S.C. made substantial contributions to study's conception and design, coordination, and they helped to draft the manuscript. All authors read and approved the final manuscript.

the glands as well. The reduction in CD80 protein levels in salivary gland epithelial cells were negatively associated with elevated miR-146a expression. Therefore, this study provides the first indication that salivary gland epithelial cells may be critically involved in SjS progression by altering CD86:CD80 protein ratio in response to miR-146a upregulation.

Keywords

Sjögren's syndrome; microRNA; co-stimulation; miR-146a; CD80; salivary glands; epithelial cells

1. Introduction

Sjögren's syndrome (SjS) is an autoimmune disorder that affects approximately four million adults in the United States, making it one of the most common autoimmune conditions [1]. SjS manifests as loss of secretion in the eyes and/or mouth due to the autoimmune response to the lacrimal and/or salivary exocrine glands. In particular, loss of saliva secretion results in effects ranging from difficulty speaking and eating, oral candidiasis, and extensive tooth decay to chronic sialadenitis [1]. The pathophysiology of SjS includes inflammatory cell aggregates, which are largely composed of CD4⁺ T cells and B cells. And these cell aggregates are closely associated with ductal epithelial structures of the exocrine glands [2]. Current research has yet to identify the cause for formation of these immune cell foci in the exocrine glands. However, the trigger may involve how resident antigen presenting cells and exocrine epithelium participate in glandular inflammation [3].

Recent studies have shown links between microRNA (miRNA) and autoimmune diseases such as with rheumatoid arthritis, systemic lupus erythematosus, and SjS [4-10]. MiRNAs are short endogenous non-coding RNAs capable of inhibiting the translation of targeted messenger RNA (mRNA) transcripts into protein. Our research on SjS pathogenesis in the SjS-prone mouse model (C57BL/6.NOD-*Aec1Aec2*, B6DC) has documented several disease characteristics related to SjS salivary and lacrimal gland pathophysiology [11-14]. Notably, our lab has identified significantly elevated miR-146a expression in the salivary glands of B6DC mice during the asymptomatic stage of SjS-like disease, which remains elevated following disease progression [15]. The observed increase in miR-146a prior to clinical onset could indicate underlying inflammation and may contribute to disease progression in the salivary glands. However, cell populations contributing to increased miR-146a and how miR-146a could affect the pathophysiology of autoimmune SjS remains to be elucidated.

MiR-146a is a well-known modulator of differentiation and function in a variety of innate and adaptive immune cells [16-20]. Research has demonstrated that miR-146a is highly up-regulated following toll-like receptor (TLR) or inflammatory cytokine signaling, as well as during the maturation of immune cells into an active state [16, 18-21]. MiR-146a is generally believed to be up-regulated to dampen inflammatory processes by down-regulating target cytokine expression [22]. Although current lines of research suggest that miRNAs provide different functional outcomes in a cell depending on the existing transcriptome and accessibility of the mRNA target sequences. One well-studied outcome of miR-146a expression is the attenuation of innate immune signaling cascades through the down-

regulation of adaptor molecules interleukin-1 receptor-associated kinase (IRAK)-1 and tumor necrosis factor (TNF) receptor-associated factor (TRAF)-6. Our research on miR-146a's innate immune functions using the human THP-1 monocyte cell line have shown miR-146a mimics decreased cytokine production in response to lipopolysaccharide (LPS) stimulation of TLR-4 and also showed enhanced phagocytosis of *Escherichia coli* bacteria[15].

In addition, some of the most critical aspects of the innate immune response are the processes of antigen presentation and costimulation of antigen-specific T-cells, typically in response to a pathogen. Autoimmunity is associated with antigen presentation and costimulation of self-reactive T-cells without proper down-regulation by regulatory T-cells (Tregs)[23]. The initiation and maintenance of any naïve T-cell response requires at least two signals. The first signal is delivered through binding of the antigen-specific T-cell receptor to its major histocompatibility complex (MHC) ligand displaying the cognate antigen. And the second signal is delivered through binding of CD28 T-cell receptor to costimulatory molecules (either CD86 or CD80) on the surface of the antigen-presenting cell. Research suggests that CD86 mediates the initiation phase of T cell responses. In contrast, CD80 contributes more towards maintenance through CD28 receptor activation or termination of the ongoing T-cell response through cytotoxic T lymphocyte antigen (CTLA)-4(CD152) signaling[24-29]. Other research supports a more regulatory function of CD80 by showing that interaction with programmed death-ligand 1 (PD-L1) can directly suppress T-cell activation[30, 31].

Interestingly, our previous research indicates that over-expression of miR-146a in human THP-1 monocytes did not inhibit MHC class I or II molecules, CD86, or intercellular adhesion molecule (ICAM)-1 (CD54) surface expression in response to interferon- γ (IFN γ) [15]. As a continuation of the study focusing on the impact of miR-146a on innate immunity involving antigen presentation and costimulation, we investigated in this study a potential target of miR-146 in the salivary glands of SjS-prone mice, which may contribute to immune dysregulation. In addition, we narrowed down the subset of cells in the glands responsible for the altered expression of miR-146a and its target molecule.

2. Materials and Methods

2.1. Bioinformatics

TargetScan predicts biological targets of miRNAs by searching for the presence of conserved target sites from annotated human and orthologous mRNA UTRs that match the seed region of a particular miRNA. We utilized TargetScan 5.1 (<http://www.targetscan.com>) analysis to initially identify potential innate immune targets of miR-146a. Human CD80 3' UTR was selected for further analyses based on favorable context score. The potential CD80 3' UTR sequences were identified from both TargetScan analysis as well as from the National Center for Biotechnology Institute (NCBI) nBLAST® alignments to the human CD80 3' UTR. NCBI BLAST® search of the published nucleotide collection (www.ncbi.nlm.nih.gov/Blast) was also used to obtain homologue information for human CD80 3' UTR. The MAFFT 7.110 (<http://mafft.cbrc.jp/alignment/server/>) multiple sequence alignment program, which utilizes L-INS-I alignment method, was then used for sequence

comparison among species. Visualized consensus sequence and percentage identity were created from the JalView 2.0 program applet (<http://www.jalview.org/>). AREsite prediction in human CD80 3'UTR was carried out by searching the website (<http://rna.tbi.univie.ac.at/AREsite>). RNAhybrid 2.2 (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) was also used to predict base pairing interactions and minimum fold energy (MFE) between the mature miR-146a target sequence and CD80 mRNA 3'UTR target sequence for mice and humans.

2.2. Cell Culture

Human cell lines were obtained from the American Type Culture Collection. HEK293 cell line was grown in Dulbecco's modified Eagle medium (DMEM) and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Life Technologies, Carlsbad, CA) and penicillin-streptomycin (Gibco, Life Technologies). THP-1 cell line were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS, 4.5 g/L glucose, 1mM sodium pyruvate, 10 mM N-2hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), and penicillin-streptomycin. Cultured mouse primary salivary gland epithelial cells were grown in DMEM/F-12 media containing GlutaMAX™ and supplemented with 5% FBS and penicillin-streptomycin. All cell lines and primary cultured cells were grown in a 5% CO₂ incubator at 37°C.

2.3. Cloning of CD80 3' UTR and luciferase reporter assay

The pMiR-Target plasmid (OriGene, Rockville, MD) was used for the generation of human CD80 3'UTR reporter constructs. Briefly, cDNA was reverse transcribed from RNA isolated from LPS/IFN γ -stimulated THP-1 cell line using oligo(dT) primers. Subsequently, the 1493 nucleotide CD80 3'UTR was PCR-amplified using forward and reverse primers 5'-aagaattccagtgctccgcagaagc-3' and 5'-tttgcggccgcttattaaagaagc-3'. CD80 3'UTR PCR products were ligated into the pMiR-Target plasmid downstream of the firefly luciferase coding sequence into EcoRI and NotI restriction sites. The CD80 3'UTR mutant construct was generated using QuickChange II XL Site-Directed Mutagenesis kit following manufacturer's instructions, mutating seed target sequence 5'-agttctca-3' to 5'-agcggcca-3'. The CD80 3'UTR and the mutated CD80 3'UTR sequences were confirmed by DNA sequencing.

For the luciferase reporter assay, HEK293 cells were plated 24 hours prior to transfection. Synthetic pre-miR-146a (40nM) or pre-miR negative control (40 nM) miRNA mimics (Applied Biosystems, Life Technologies) were co-transfected with either pmiR-3'UTR-hCD80wt or pmiR-3'UTR-hCD80mut reporter plasmids using Lipofectamine® 2000 transfection reagent (Invitrogen, Life Technologies). The luciferase activities were measured using the Luciferase Reporter Assay (Promega, Madison, WI) 48 hours post-transfection in a FLUOstar OPTIMA reader (BMG Labtech, Cary, NC) according to the manufacturer's recommendations. Red fluorescent protein expression levels were used as an internal control to normalize the relative expression of firefly luciferase activity.

2.4. Transient transfection and *in vitro* stimulation

In order to enrich expression of miR-146a *in vitro*, hsa-miR-146a synthetic precursor miRNA mimics (PM10722, Applied Biosystems, Life Technologies) were transfected in

THP-1 or HEK293 using Lipofectamine® 2000 transfection reagent (Invitrogen, Life Technologies). The non-targeting Pre-miR™ miRNA Precursor Negative Control #1 (AM17110, Applied Biosystems, Life Technologies) was used as a control. Transfection reagent was diluted 4 hours post transfection using complete media. Three days post-transfection, THP-1 cells were split and either unstimulated phosphate buffered saline (PBS) or stimulated with 1 µg/ml LPS (*Salmonella minnesota*) and 10 ng/ml IFN γ or for 48 hours.

2.5. Animals

C57BL/6J (B6) and C57BL/6.NOD-*Aec1Aec2* (B6DC) mice were bred and maintained under specific-pathogen-free conditions within the Animal Care Services at the University of Florida, Gainesville. B6DC mice exhibit dry mouth and dry eye conditions, resembling clinical manifestations of primary SjS without developing type I diabetes [12]. For our study, age matched 8 and 20-24 week-old female mice were utilized to represent pre-disease stage and the advanced disease stages, respectively. Animal studies were approved and performed in accordance with the University of Florida Institutional Animal Care and Use Committee. The mice were sacrificed using American Veterinary Medical Association approved procedures.

2.6. Salivary gland immunofluorescence staining

Submandibular salivary glands of B6 and B6DC mouse models ages 8 and 20 weeks were collected, fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Duplicate 5µm sections were placed onto slides which were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed using a pressure cooker for 10 minutes with the slides submerged in Tris-EDTA. The slides were then incubated with 0.5% Triton X-100 for 15 minutes, and Vectastain ABC kit (Vector Labs, Burlingame, CA). The primary antibodies used were rabbit anti-CD80 (1:100 dilution, Epitomics) anti-rabbit IgG Alexa Fluor 568 (1:400 dilution). Sections incubated without primary antibody were used to evaluate background labeling. Slides were mounted using Vectashield mounting medium with DAPI (Vector Labs). Images were taken using a Zeiss Axiovert fluorescent microscope at 20X magnification. Image analysis was performed using ImageJ software (V. 1.45s, <http://imagej.nih.gov/ij>), integrated density of CD80 was normalized by subtracting the background integrated density of duplicate section and dividing by number of DAPI particles (normalized integrated density = (integrated density CD80 – integrated density background)/ number of DAPI particles).

2.7. Western blot analysis

Cell cultures and mouse tissues were prepared and lysed (50 mM Tris-HCL pH 7.5, 2 mM EDTA, 500 mM NaCl, and 1% NP-40 with complete protease inhibitor cocktail tablets (complete Mini, Roche, Indianapolis, IN, USA)). Equal amounts of total proteins, measured by Bradford assay (Bio-Rad, Hercules, CA, USA), were loaded and separated on precast 4-20% Mini-PROTEAN TGX gels (Bio-Rad) in Tris-Acetate-EDTA buffer. Gel-separated proteins were subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad) using the Pierce Fast Semi-Dry Transfer system (Thermo Fisher Scientific, Waltham, MA, USA). Protein-transferred membranes were blocked in 5% milk tris-buffered-saline-

Tween-20 solution and incubated with respective primary antibodies for 1 hour at room temperature. Primary antibody dilutions used were 1:1,000 rabbit anti-CD80 (Epitomics, Abcam, Burlingame, CA, USA) and 1:1,000 rabbit anti-GAPDH (Sigma-Aldrich, Life Technologies). Chemiluminescent detection of primary antibodies was through using the Pierce Fast Western Blotting Kit SuperSignal West Pico Rabbit (Thermo Fisher Scientific) following manufacturer's recommendations.

2.8. Mouse primary salivary gland cell isolation

Submandibular salivary glands were collected from B6 or B6DC mice. Single cell suspensions were isolated by mechanical or enzymatic disruption as previously described [32]. In short, salivary gland tissues were finely chopped and enzymatically digested with Collagenase type II (Worthington Biochemical Corporation, Lakewood Township, NJ, USA). Single cell suspensions were washed twice and subsequently filtered using nylon mesh and erythrocytes removed with red blood cell lysis buffer (Miltenyi Biotec, San Diego, CA, USA) prior to *ex vivo* magnetic cell isolation and flow cytometry. For the analyses of salivary gland epithelial cells, salivary gland epithelial populations were negatively selected by removing CD45⁺ leukocytes using anti-mouse CD45 magnetic MicroBeads (MiltenyiBiotec). In short, salivary gland cell suspension was incubated with anti-mouse CD45 magnetic microbeads. These labeled cells were then passed through a magnetic separation column and washed three times, collecting the flow through for further flow cytometry and quantitative reverse transcription and PCR analyses.

2.9. Flow cytometry

Following cytokine/LPS stimulation of transfected THP-1 cell line *in vitro*, cells were collected, washed, and labeled with anti-human CD80-PE (eBioscience, Affymetrix, San Diego, CA, USA). The equivalently labeled antibody isotype control was included as well. Cell surface expression was measured using FACSCalibur flow cytometer and analyzed using FlowJo version 7.6 software (Tree Star, Ashland, OR, USA). During the *ex vivo* analysis of mouse salivary gland antigen presenting cell populations, salivary gland cells were collected, washed, Fc-blocked (anti-mouse CD16/32), and labeled with anti-mouse CD80-PerCP-Cy5.5, CD86-PE-Cy7, CD11c-FITC, CD11b-PacificBlue, F4/80-Alexa647, and CD19-APC-Cy7 (Biolegend, San Diego, CA, USA). In addition, during the analysis of mouse salivary gland epithelial cells for expression of CD86 and CD80, salivary gland epithelial cells were negatively selected for using CD45 magnetic microbeads and were washed, Fc-blocked, and labeled with anti-mouse CD80-PE, CD86-FITC, and CD326-APC (BD Biosciences). Cell surface expression of mouse salivary gland cell populations were measured using a FACS Aria III cell sorter (BD Biosciences, San Jose, CA, USA) and FACSCalibur (BD Biosciences) flow cytometers, respectively. For all experiments, dead cells were excluded by forward light scatter and appropriate isotype controls were included as well. Cell staining was analyzed using FlowJo version 7.6 software.

2.10. Quantitative reverse-transcription and polymerase chain reaction (qRT-PCR)

Total RNA, including small RNAs, were extracted using miRVana miRNA Isolation Kit (Ambion, Life Technologies) according to the manufacturer's recommendations. For mature

miRNA and mRNA expression determination total RNA were reverse-transcribed using TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems) with a either TaqMan® miRNA-specific primer or random primers (Applied Biosystems). Reactions were performed with primers specific for miR-146a (ref #001097) or CD80 (ref# 4331182) in the ABI PRISM® apparatus. MiRNA expression was normalized based on the values of control mouse U6 (ref# 001973) RNA expression. The comparative Ct method (relative expression level (fold change) = $2^{-(Ct_{\text{sample}} - Ct_{\text{control}})}$) was used for quantification of transcripts.

2.11. Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software package version 5.0 (Graph Pad Software, La Jolla, CA, USA). Two-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-tests were used where indicated for THP-1 cell line *in vitro* experiments to compare quantitative variables from at least three independent experiments. Mann-Whitney two-tailed t-tests were used to where indicated for *ex vivo* experiments to compare quantitative variables from at least three independent experiments. P-values less than 0.05 were considered significant.

Results

3.1. CD80 is a direct target of miR-146a

TargetScan predicts biological targets of miRNAs by searching for the presence of conserved target sites from annotated human and orthologous mRNA UTRs that match the seed region of a particular miRNA. TargetScan 5.1 analysis identified the human CD80 3'UTR as a potential target of miR-146 (context score -0.43, context score percentile 98%). We used the human [GenBank: NM_005191.3] and mouse [GenBank: NM_009855.2] mRNA 3' UTR sequences to predict MFE of the interaction between CD80 and miR-146a using the RNAhybrid 2.2 program (Fig. 1A). The human CD80 3'UTR contains a perfect seed match at position 839-846 of the 3' UTR, with predicted MFE of -20.7 kcal/mole (Fig. 1A). The mouse CD80 3' UTR seed sequence at position 1010-1017 of the 3' UTR contains a point mutation in position two of the seed match, altering the position to a G:U wobble base pair. The predicted MFE of the interaction between the mice CD80 3' UTR to the miR-146a was -15.6 kcal/mole (Fig. 1A).

The human CD80 mRNA 3'UTR was tested directly for targeting by miR-146a using a luciferase assay (Fig. 1B). Luciferase protein activity from the pmiR-3'UTR-hCD80 construct transfected cells was inhibited up to 50% by the presence of miR-146a mimics when compared to negative control miRNA mimics (Fig. 1B). Mutation of target sequence prevented targeting by miR-146a. Additionally, we identified the single predicted miR-146a/146b target site in CD80 3'UTRs from among 25 mammalian species (Fig. 2). These 25 aligned CD80 3'UTR sequences revealed high levels of conservation ranging from approximately 53 nucleotides upstream to 23 nucleotides downstream of the predicted miR-146 target sequence.

We next tested for alterations in CD80 protein expression in human-derived THP-1 monocytes following miR-146a transfection by western blotting (Fig. 3A) and flow cytometry (Fig. 3B). Without immune activation, CD80 total protein levels were reduced an average of 60% in the miR-146a mimic transfected THP-1 cells compared with non-targeting control miRNA transfected cells (Fig. 3A). Following LPS and IFN γ immune activation, surface CD80 MFI on miR-146a mimic transfected cells were significantly reduced an average of 46% and 39.5% when compared to mock and non-targeting control-transfected cells, respectively (Fig. 3B).

3.2 SJS-prone B6DC mice salivary glands show altered co-stimulatory molecule expression

Because B6DC mouse model exhibited elevated miR-146a expression in the salivary glands, we sought to determine how CD80 protein expression may be affected *ex vivo*. We observed reduced total CD80 protein expression level, but not CD86 total protein expression, in 20-week-old B6DC mice salivary glands in comparison to age-matched healthy control B6 mice (Fig. 4A). There were no apparent alterations in the spleen total protein expression levels of CD80 or CD86 isolated from 8- or 20-week-old B6DC mice spleens when compared with B6 mice (Fig. 4B). Immunofluorescence staining of B6 and B6DC salivary glands indicated positive staining for CD80 throughout the salivary gland tissues and reduced CD80 protein expression in B6DC mice in comparison to B6 mice (Fig. 4C).

3.3 Salivary gland antigen presenting cell populations exhibit reduced co-stimulatory molecule expression

To help identify populations of cells that were affected by altered co-stimulatory molecule expression in salivary glands, we further evaluated resident populations of potential antigen-presenting cells: B-cells, dendritic cells (DCs), and macrophages (M Φ s) in 24-week-old mice for their surface expression of co-stimulatory molecules CD80 and CD86 by flow cytometry (Fig. 5A). Salivary gland B-cells, DCs, and M Φ s, altogether accounted for an average of $3.811\% \pm 1.057$ of B6 and $4.011\% \pm 0.7887$ of B6DC total salivary gland cells. The total numbers of B-cells (CD19+CD11c-) in salivary glands were significantly reduced 56% in B6DC mice (Fig. 5B). Overall, numbers of CD19+ plasmacytoid-like DCs (CD19+CD11c-cells) and interstitial M Φ (iM Φ , CD19-CD11c-CD11b+F4/80+) were low in B6DC mice in comparison to B6 mice (Fig. 5B). Therefore, further evaluations of costimulatory molecule expression were performed with B-cells, conventional DCs (cDC, CD11c+CD11b-F4/80-), myeloid DCs (mDC, CD11c+CD11b+F4/80-) mDCs, and interstitial DCs (iDC, CD11c+CD11b+F4/80+) populations due to sufficient numbers of cells for analyses (Fig. 5C). The number of CD86+ and CD80+ cells (Fig. 5D) and their surface MFI (Fig. 5E) were significantly lower in B-cells when compared to B6 mice (Fig. 5E). Our analyses of salivary gland iDCs by flow cytometry for CD80 and CD86 surface expression indicated a 49.4% reduction in CD80+ cell number (Fig. 5D) and an average reduction of 15% for CD80 surface MFI for B6DC iDCs in comparison to B6 mice (Fig. 5E).

3.4. Salivary gland epithelial cells over-express miR-146a and have reduced protein expression of CD80

Because salivary gland epithelial cells can also participate in antigen presentation and costimulation [3, 33-36], we tested salivary gland epithelial cells that were negatively selected for using CD45 magnetic microbeads for expression of miR-146a and altered CD80 or CD86 expression in B6DC mice at age 24 weeks. Salivary gland epithelial cells (CD326+) altogether accounted for $91.20\% \pm 1.93$ of B6 and $86.70\% \pm 4.51$ of B6DC for CD45-salivary gland cells (Fig. 6A). Our analyses of CD45-B6DC salivary gland epithelial cells by flow cytometry for CD80 and CD86 surface expression indicated a 40.9% reduction in CD326+CD80+ cell number (Fig. 6B) and a reduction of 15% for CD80 surface MFI for B6DC in comparison to B6 mice (Fig. 6C). In contrast, CD326+CD86+ number and CD86 MFI expression level were not significantly altered in B6DC when compared to B6 mice (Fig. 6B and 6C). Furthermore, we observed an average of 2.4 fold increase in miR-146a expression from CD45-salivary gland cells as measured by qRT-PCR with comparison to B6 mice (Figure 6D). Linear regression analysis was performed to evaluate associations between miR-146a expression level and CD80 MFI in B6DC mice salivary gland epithelial cells. In B6DC mice salivary gland cells CD80 MFI showed a negative relationship to the relative expression level of miR-146a (slope = -1.985 ± 0.4569 , $R^2 = 0.6537$, $P = 0.0015$) (Fig. 6E).

Discussion

The dysregulation of miRNA-146a could contribute towards SjS disease progression through its ability to target various immune mediators. Because miR-146a was elevated in salivary glands of B6DC mice prior to onset of SjS-like symptoms and remains elevated following onset of symptoms [15], we predict that miR-146a would be involved in SjS progression. Our previous *in vitro* analyses indicated that overexpression of miR-146a did not significantly alter surface expression of MHC class I or II, CD86, or ICAM-1 proteins following IFN γ stimulation of human THP-1 cells [15]. Interestingly, the critical costimulatory molecule CD80 was a predicted target of miR-146a. Our study identified CD80 as a direct target of miR-146a and described alterations in CD80 expression in SjS-prone mice salivary gland cells, which is negatively associated with miR-146a expression.

Of particular interest to our project, the human and mouse CD80 3'UTRs contained a predicted miR-146 target site. Although the mouse CD80 3'UTR contains a point mutation in position two of the seed match, altering the position to a G:U wobble base pair, this alteration does not exclude targeting by miR-146a. Research has shown that functional imperfect miRNA seed matches occur in nature, including G:U wobble base pairs, and some of these imperfect miRNA seed matches are shown to function *in vitro* and *in vivo* [37, 38]. The likelihood that the mouse CD80 3'UTR is a functional target is also supported by the RNAhybrid program analysis showing that the seed sequence contains six sequential Watson-Crick base pairing nucleotides and also has a predicted MFE of -15.6 kcal/mole (Fig. 1A).

In addition, the high conservation of the miR-146 target sequence among mammalian species and the surrounding nucleotide sequence in CD80 3'UTR suggests the reliability of CD80

mRNA as a direct target of miR-146a (Fig. 2). Interestingly, our *in silico* analysis of human CD80 3' UTR sequences indicated potential Adenine-Uridine(AU)-rich RNA-binding protein motifs (UUUAAA, CUUUA, GUUUG, AUUUA, UUUUA, GUUUA), which are largely conserved among the mammalian species, adjacent to the miR-146 target sequence (Fig. 2). A widely used classification system published by Shyu et al. [39], indicates that the region surrounding the miR-146a target sequence corresponds with criteria for a class I AU-rich regulatory element (ARE). Of note, miR-146a is the only miRNA predicted to target this conserved region. It remains to be determined whether this ARE functions to stabilize or destabilize CD80 mRNA or how RNA-binding proteins may enhance or interfere with the RNA-induced silencing complex machinery. At this time, mechanisms of CD80 mRNA stabilization or destabilization following inflammatory stimulation are largely undetermined.

Because of the prediction that CD80 is a target of miR-146a, the human CD80 3'UTR was tested directly *in vitro* using a luciferase reporter assay and our results confirmed miR-146a directly targeted human CD80 3'UTR, reducing total protein expression by up to 50% (Fig. 1B). Furthermore, exogenous addition of miR-146a to the human THP-1 cell line showed significant reductions in total CD80 protein expression and inhibited CD80 surface expression by an average of 42% (Fig. 3), which was also consistent with our mouse cell transfection study (data not shown). Our data is reinforced by a recent study that showed transduced expression of miR-146a consistently inhibited CD80 MFI expression in a human plasmacytoid DC cell line following either TLR7 or TLR9 activation, but showed no consistent differences in CD86 and HLA-DR expression after TLR9 stimulation [40]. Because miR-146a is significantly elevated in SjS patient PBMCs [15], further evaluations of miR-146a and its effect on CD80 expression in specific immune subsets of B-cells and T-cells will be performed in the future. It is plausible that CD80 expression on activated T_H17 cells is critical for regulation by T-regulatory cells and maintenance of immune tolerance [30].

Initially, evaluation of CD80 and CD86 total protein expression levels from 8- and 20-week-old mice revealed reduced CD80 protein expression by age 20 weeks in B6DC salivary gland tissues in comparison to B6 mice (Fig. 4A and 4C). Since expression of CD80 was not significantly altered in 20-week-old B6DC mice spleens (Fig. 4B) and miR-146a not significantly increased (data not shown) when compared with B6 mice, we expected the reduction in CD80 was target tissue-specific. Therefore, we evaluated CD80 and CD86 protein expression in populations of salivary gland APCs and epithelial cells. Since CD80 and CD86 are typically expressed on professional APCs, we evaluated CD80 and CD86 surface protein expression levels in salivary gland resident APC populations by flow cytometry. Salivary gland resident APCs tended to be similarly present in both B6 and B6DC mice and contributed to less than 5% of the total cells isolated from the salivary glands (Fig. 5). Of note, our data indicated only salivary gland iDCs showed a specific reduction in expression of CD80 from among the populations of antigen-presenting cells that were evaluated; suggesting that a subset of salivary gland APCs may contribute to the reduced expression of total CD80 previously observed in 20-week-old mice salivary gland lysates. Therefore, although it was a small subset of cells, the importance of salivary gland

APCs' miRNA and co-stimulatory molecule expression should not be discounted as it may represent other possible mechanisms of altered immune regulation in SjS pathogenesis.

Moreover, salivary gland epithelial cells are important contributors to SjS pathogenesis. Research has shown SjS patients' salivary gland epithelial cells express antigen-presenting molecules MHC class I and II, as well as, express CD86 and ICAM-1 co-stimulatory molecules [3, 33-36]. Of note, localized delivery of CTLA-4-Ig recombinant fusion protein to the salivary gland epithelial cells of B6DC mice prevented disease progression [41], indicating that costimulation from salivary gland epithelial cells are a requirement for SjS progression. Expression of CD86 is well documented to be highly expressed in salivary gland epithelial cells of SjS patients [33, 35, 36]. However, the expression status of CD80 in human SjS salivary gland epithelial cells is more controversial. Previously, use of immunohistochemistry showed expression of CD80 in SjS patient salivary gland epithelium [33, 34], however, re-evaluation of patient-derived salivary gland epithelial cells by flow cytometry with another antibody showed low to no expression [36]. This may be, in part due to previous use of an unreliable monoclonal antibody against CD80 [36, 42] and lower expression of CD80 relative to more abundant expression of CD86. In our study, we delineated the expression of CD80 in the salivary glands of the B6DC SjS-prone mouse model, for the first time, in association with elevated miR-146a.

We next explored how miR-146a may be associated with CD80 in the B6DC mouse model of SjS. As mentioned previously, miR-146a expression in B6DC salivary glands was significantly elevated in 8-week-old B6DC mice in comparison to B6 mice and remained elevated following disease progression [15]. Our current data indicated 24-week-old B6DC salivary gland epithelial cells, negatively selected for by CD45 magnetic microbeads, also maintained significantly elevated miR-146a expression (Fig. 6D). Furthermore, flow cytometry identified a significant reduction in CD80, but not CD86, surface expression in the B6DC salivary gland epithelial cells (Fig. 6C). We then used regression analysis to better characterize the relationship between miR-146a and CD80. As predicted, B6DC mice salivary gland epithelial cells' relative expression level of miR-146a was negatively associated with surface CD80 MFI (Fig. 6F). Thus, in B6DC mice salivary gland epithelial cells, miR-146a may indeed control CD80 expression levels. As indicated previously, an imbalance in CD80 relative to CD86 could be implicated in directing proinflammatory T-cell responses, which warrants further investigation.

Conclusions

An imbalance of CD86 to CD80 in salivary gland epithelial cells, following miR-146a over-expression from the early stage of disease, may be critically associated with disease progression in the salivary glands. This reduction in CD80 in the salivary gland epithelial cells could impact both the ability to activate Tregs and to inhibit effector T-cells directly. Importantly, full functionality of Tregs are shown to be biased towards CD80-CTLA-4 and CD80-PD-L1 interactions [26, 27, 31, 43], while CD86-CD28 interactions are prone to be inhibitory in Tregs [24, 25, 28, 29] and may contribute to SjS progression [35, 36, 41, 44]. Therefore, *in vitro* and *in vivo* testing to identify specific functional consequences of

miR-146a and the imbalance of CD86:CD80 in the salivary epithelial cells on T-cell responses are currently in progress.

Our study confirmed CD80 as a direct target of miR-146a, which suggests altered co-stimulation signaling that may be implicated in skewing immune responses towards activation of auto-reactive immune cells in the SjS target tissues. Our study provided the first evidence that miR-146a may play more diverse roles in altering costimulatory molecule CD80 expression and thus potentially altering T-cell regulation in autoimmune processes. The fact that altered expression of miR-146a and CD80 were observed in the target tissue of B6DC SjS-pronemouse model indicates for the first time that miRNAs may be critical in configuring the target tissue specificity of SjS.

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Abbreviations

SjS	Sjögren's syndrome
miRNA	microRNA
PBMC	peripheral blood mononuclear cells
B6	C57BL/6J
B6DC	C57BL/6.NOD- <i>Aec1Aec2</i>
TLR	toll-like receptor
IRAK-1	interleukin-1 receptor-associated kinase-1
TNF	tumor necrosis factor
TRAF-6	TNF receptor-associated factor-6
LPS	lipopolysaccharide
Treg	regulatory T cells
Teff	effector T cells
MHC	major histocompatibility complex
CTLA-4	cytotoxic T lymphocyte antigen-4
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-gamma
UTR	untranslated region
NCBI	National Center for Biotechnology Information

MFE	minimum fold energy
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
SEM	standard error of the mean
DC	dendritic cell
M	macrophage
APC	antigen presenting cell
AU	adenine-uridine
ARE	AU-rich elements
IDO	indoleamine 2,3-dioxygenase
DAPI	4',6-diamidino-2-phenylindole

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Highlights

- MicroRNA-146a directly inhibits CD80 protein expression.
- Salivary epithelial cells and interstitial dendritic cells in the salivary glands showed altered co-stimulatory molecule expression.
- The reduction in CD80 expression in salivary gland epithelial cells was negatively associated with elevated miR-146a expression.

Figure 1.

MiR-146a/b directly targets co-stimulatory molecule CD80. (A) Human and mouse CD80 3'UTR contain predicted miR-146 target sites. The sites of miR-146a target sequences are indicated by black arrows. The predicted base pair alignments of CD80 mRNAs and miR-146a and their MFE of the interacting nucleotides are indicated. Lines between nucleotides (|) indicate Watson and Crick base-pairing and star (*) indicate Guanine:Uridine wobble base pair interactions. (B) HEK293 cells were co-transfected with either hsa-miR-146a mimics (40nM) or non-targeting Ctrl miRNA mimics (40nM) along with reporter vectors pmiR-3'UTR-hCD80wt or pmiR-3'UTR-hCD80-mut. Luciferase activity was detected and normalized to red fluorescent protein levels. Values are the mean $\pm$ SEM for percentage of luciferase activity normalized to RFP and mock transfection treatments from three independent experiments. **** $P < 0.0001$, two-way ANOVA with Bonferroni post-tests. MFE, minimum fold energy; Ctrl, control; SEM, standard error of the mean; RFP, red fluorescent protein.

Figure 2.

The miR-146a/b target sequence in the CD80 3' UTR is highly conserved among various mammalian species. Percentages of identity to consensus sequence are indicated by the black bars above the consensus sequence and by gray-scale highlighting on text in the alignment. The target sequence seed region for miR-146a/b is indicated by a black box. UTR, untranslated region.

Figure 3.

MiR-146a reduces CD80 total protein and surface MFI expression. THP-1 cells were transfected with either hsa-miR-146a mimics, non-targeting control (Ctrl) miRNA mimics, or mock transfected and 3 days following transfection stimulated for 48 hours with or without 1 μ g/ml LPS (*Salmonella* *minnesota*) and 10 ng/mL IFN γ . (A) Western blot x-ray films of CD80 and GAPDH proteins from total protein lysates are shown. (B) Overlaid flow cytometry histograms of CD80 surface expression with and without immune stimulation. Filled line represents isotype control, dotted line represents mock, solid black line represents miR-146a mimic, and filled gray line represents Ctrl mimic treated cells as shown. Values in bar graphs are the mean $\pm$ SEM from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ by two-way ANOVA with Bonferroni post-tests. Ctrl, control; LPS, lipopolysaccharide; IFN γ , interferon-gamma; SEM, standard error of the mean.

Figure 4.

Salivary glands exhibit reduced CD80 protein expression in B6DC SjS-prone mice. (A) Salivary gland lysates or (B) spleen lysates of 8- and 20-week-old B6 and B6DC mice ($n = 5$ mice per group) were pooled and evaluated by western blot for expression of CD80 and CD86. GAPDH was used as a protein loading control. (C) Representative examples of B6 or B6DC mice submandibular salivary glands at 20X magnification at 8 and 20 weeks of age. Blue indicates DAPI nuclear staining and red indicates CD80 localization. Integrated density of CD80 was normalized by subtracting background integrated density and dividing by the number of DAPI particles. B6, C57BL/6J; B6DC, C57BL/6.NOD-*Aec1Aec2*.

Figure 5.

Salivary APCs have altered co-stimulatory molecule surface expression in B6DC Sjs-prone mice. (A) Representative density plots of salivary gland APCs and gating strategy are shown. B6 and B6DC (24 weeks-old, $n=6$ per group) mice salivary glands were mechanically disrupted and incubated with fluorescently labeled antibodies for cell type markers CD19, CD11c, CD11b, and F4/80 as well as for costimulatory molecules CD80 and CD86 and analyzed by flow cytometry. (B) Total number of APC populations identified from mouse salivary glands. (C) Representative histogram plot images of CD80 and CD86 expression in salivary gland APCs (light gray = isotype, dark gray = B6, and black = B6DC). (D) Bar graphs indicating number of CD86+ and CD80+ cells and (E) MFI. Bars represent mean $\pm$ SEM; * = $P < 0.05$; ** = $P < 0.01$ by Mann-Whitney two-tailed t-test. APCs, antigen presenting cells; DC, dendritic cell; Mφ, macrophage; MFI, mean fluorescence intensity.

Figure 6.

SjS-prone B6DC mice exhibit elevated miR-146a and reduced CD80 surface expression in the CD45-CD326+ salivary gland epithelial cells. (A) Gating strategy for selecting CD326+ salivary gland epithelial cells and measuring CD80 and CD86 surface expression by flow cytometry are shown. (B) Number of CD326+CD86+ and CD326+CD80+ (C) MFI CD86 and CD80 from 24 week-old B6 ($n = 5$) and B6DC ($n = 12$) mice salivary glands are shown. (D) CD45-negative salivary gland cells were used to measure miR-146a expression level by qRT-PCR of 24-week-old B6 ($n=5$) and B6DC ($n=12$) mice. RNU6b was used as an endogenous control for normalization. (F) Linear regression analysis showed associations between miR-146a expression level and CD80 MFI in the B6DC mouse salivary gland epithelial cells ($n=12$). Bar graph error bars indicate the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney two-tailed t-test. NS, not significant; MFI, mean fluorescence intensity, qRT-PCR, quantitative real-time PCR.