Local gene therapy and the identification of therapeutic targets in Sjögren’s syndrome
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TACI-FC GENE THERAPY IMPROVES AUTOIMMUNE SIALADENITIS BUT NOT SALIVARY GLAND FUNCTION IN NOD MICE

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ABSTRACT

Objective: Sjögren’s syndrome (SS) patients show aberrant expression of the B cell-related mediators B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) in serum and salivary glands (SGs). We studied the biological effect of neutralizing these cytokines by local gene transfer of the common receptor transmembrane activator and CAML interactor (TACI) in an animal model of SS.

Material and Methods: A recombinant serotype 2 adeno-associated virus (rAAV2) encoding TACI-Fc was constructed and its efficacy was tested in the SGs of non-obese diabetic (NOD) mice. Ten weeks later, SG inflammation was evaluated and serum and SG tissue were analyzed for inflammatory markers including immunoglobulins (Ig) and cytokines.

Results: AAV2-TACI-Fc gene therapy significantly reduced the number of inflammatory foci in the SG, due to a decrease in IgD+ cells and CD138+ cells. Moreover, IgG and IgM levels, but not IgA levels were reduced in the SG. Overall expression of mainly pro-inflammatory cytokines tended to be lower in AAV2-TACI-Fc treated mice. Salivary flow was unaffected.

Conclusion: Although local expression of soluble TACI-Fc reduced inflammation and immunoglobulin levels in the SG, further research will have to prove whether dual blockade of APRIL and BAFF by TACI-Fc can provide a satisfying treatment for the clinical symptoms of patients.
INTRODUCTION

Sjögren’s syndrome (SS) is a common systemic autoimmune disorder characterized by lymphocytic infiltration of the exocrine glands. Patients, mostly females in their 4th and 5th decade, suffer from dry eyes and dry mouth and may also experience systemic symptoms associated with vasculitis, arthralgias and peripheral neuropathy. Current treatment options are very limited and therapeutic approaches effective in other autoimmune diseases such as rheumatoid arthritis (RA) have been largely ineffective in SS patients. Rituximab, a B cell depleting agent, has had modest but promising effects in clinical trials, indicating an important role for B cells in the disease. This role is further supported by the recruitment of activated and memory B cells in salivary gland (SG) infiltrates, the presence of circulating autoantibodies to the nuclear antigens Ro and La, germinal center (GC) formation, and an increased risk for SS patients to develop B cell non-Hodgkin’s lymphoma.

The cytokines B cell activating factor (BAFF or BlyS) and a proliferation-inducing ligand (APRIL), exert different effects, but are both involved in B cell activation and survival. Both cytokines are aberrantly expressed in SS patients, which might explain the activation and survival of pathogenic B cells in this condition. The potential pathogenic role of BAFF in SS is further supported by animal models. BAFF transgenic (Tg) mice develop a systemic lupus erythematosus (SLE)-like disease, a condition with many similarities to SS. These mice show elevated numbers of mature B cells and effector T cells, and high levels of rheumatoid factor, anti-DNA autoantibodies as well as immunoglobulin deposition in the kidneys. In addition, with age these mice show increased inflammation of the SGs and decreased salivary flow, resembling SS in humans. APRIL transgenic mice display a different phenotype and suggest a different and not yet elucidated role for APRIL, compared to BAFF, in the pathogenesis of SS; young APRIL Tg mice have no signs of B cell hyperplasia, but they show enhanced survival of CD4+ T cells without an increase in T cell number, enhanced IL-2 production of CD8+ T cells ex vivo and enhanced T cell dependent IgM and T cell independent IgM and IgG responses. At a later age, these mice develop progressive hyperplasia and prolonged survival of B1 B cells in mesenteric lymph nodes and Peyer’s patches and disorganization of affected lymphoid tissue.

Blockade of APRIL and BAFF by intraperitoneal (ip) injection of soluble human transmembrane activator and CAML interactor (TACI), the common receptor for BAFF and APRIL, coupled to an immunoglobulin heavy chain (TACI-Fc), has been shown to delay disease onset in SLE-prone NZB/W F1 mice. Also, in several SLE-prone mouse strains, a single injection of adenovirus serotype 5 (Ad5) encoding murine TACI-Fc resulted in prolonged survival, depletion of plasma cells, marginal zone (MZ) and follicular B cells, and decreased IgM and IgG serum levels. These findings have led to the development of clinical studies with human TACI-Fc (atacicept) in patients with SLE, multiple sclerosis (MS) and RA. In contrast to RA and SLE, TACI-Fc has never been tested in mice and humans with SS. Therefore, we decided to evaluate the biological effects of adeno-associated virus (AAV)-based transfer of the gene encoding TACI-Fc into the SG in an animal model of SS.
MATERIAL AND METHODS

Construction, expression and biological activity of plasmid

The murine extra-cellular domain (ED) of TACI was synthetically designed using 20 partially overlapping 40-mer oligonucleotides spanning the TACI-ED sequence (Phe5-Thr129) and synthesized using regular polymerase chain reaction (PCR) under general conditions. This gene was cloned into the rAAV plasmid containing a cytomegalovirus (CMV) promoter, the Fc-region of mouse IgG1 and the inverted terminal repeat (ITR) sequences for AAV serotype 2 (AAV2; the shuttle vector is previously described in). The resulting plasmid (pAAV2-CMV-mTACI-mIgG1) was transfected into human embryonic kidney (HEK 293) cells and protein secretion into the supernatant was quantified by performing a regular ELISA for mouse TACI (R&D systems, Minneapolis, MN, USA) and by a modified ELISA in which the capture antibody was directed against TACI (R&D systems) and a biotin-labeled detection antibody was directed against mouse IgG (Bethyl Laboratories, Montgomery, TX, USA). The size of the secreted fusion protein was confirmed by western blotting under reduced conditions using a 10% SDS gel and a labeled (IRDye 800 CW) anti-mouse IgG (Li-Cor, Lincoln, NE, USA).

Vector production

rAAV serotype 2 vectors (rAAV2) were generated as previously described. The titer of DNA physical particles in rAAV stocks was determined by quantitative (q)-PCR using primers for the CMV-promotor region and the vectors were stored at -80°C. On the day of vector administration to non-obese diabetic (NOD) mice, the vector was dialyzed for 3 hours (hrs) against saline. Construction and vector production of the control vector rAAV-LacZ encoding β-galactosidase was previously described.

Animals, vector administration and detection

Female NOD mice (Jackson Laboratory, Bar Harbor, ME, USA) were kept under specific pathogen-free conditions in the animal facilities of the National Institute of Dental and Craniofacial Research (NIDCR). Animal protocols were approved by the NIDCR Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. Vectors were delivered into the submandibular SG by retrograde instillation as previously described. In short, 10 week old female NOD mice were anesthetized with a mild anesthesia (a combination of ketamine and xylazine) and 50 µl containing 1x10^11 vector particles was administered to each submandibular gland by retrograde ductal instillation using a thin cannula (Intermedic PE10, Clay Adams, Parsippany, NJ, USA). Mice were sacrificed at 20 weeks of age. At time of sacrifice, submandibular SGs were removed and cut in 4 equal parts. One part was homogenized and total genomic DNA was isolated using DNeasy blood & tissue kit (Qiagen, Venlo, the Netherlands). Vector was detected using q-PCR on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).
Saliva and serum collection and fusion protein detection
Saliva was collected at 20 weeks of age under anesthesia (procedure described above). Saliva secretion was induced by subcutaneous (sc) injection of pilocarpine (0.5 mg/kg BW; Sigma-Aldrich, St. Louis, MO, USA) and stimulated whole saliva was collected for 20 minutes (min) from the oral cavity by gravity with a hemocrit tube (Drummond Scientific Company, Broomall, PA, USA) placed into a pre-weighed 0.5 ml micro centrifuge tube. Saliva volume was determined by weight and expressed as μl/gram body weight*20 min. Blood was collected by heart puncture during sacrifice. Blood was left to clot on ice for 3 hours and centrifuged at 2,500 x g for 25 minutes at 4°C to obtain serum. Murine TACI was measured in both SG protein homogenates and serum using a commercial ELISA-kit (R&D systems).

Histological assessment and immunohistochemistry
One cross-section of the submandibular SG was embedded in paraffin and sections were cut at 5 μm. Three sections were stained with hematoxylin and eosin (H&E) according to standard protocol. Focus score (FS) was determined by averaging the number of aggregates (>50 lymphocytes) per 4 mm² of SG tissue per mouse and were scored blindly by 2 different examiners. Other slides were stained with anti-CD138 (clone 281-2, BD, Breda, the Netherlands), anti-BAFF (clone L17835/b, Alexis Biochemicals, San Diego, CA, USA), and anti-APRIL (Abcam, Cambridge, MA, USA) after heat-induced antigen-retrieval with citrate. Another cross-section of the submandibular SG was collected frozen in OCT compound. Sections (5 μm) were stained with anti-CD4 (clone L3T4, eBioscience, San Diego, CA, USA), anti-CD8 (clone 53-6.7, eBioscience), anti-CD19 (clone 1D3, BD), and anti-IgD (clone 11-26c, eBioscience), followed by goat anti-rat-HRP (Southern Biotechnology, Birmingham, AL) and developed with AEC substrate (Dako, Glostrup, Denmark; Detailed staining procedure as previously described30). Images of the foci in the salivary gland sections were taken, 18 high-power fields (taken with a 40X objective lens) for each cell subset per mouse, and were analyzed using the Qwin analysis system (Leica, Cambridge, UK) as previously described31, evaluating foci and directly surrounding salivary gland tissue in one analysis. Positive staining for the cellular markers was expressed as the number of positive cells/mm² and the staining for the cytokine markers as integrated optical density (IOD)/mm², an arbitrary unit representing the intensity of staining per mm²32.

Protein extraction and determination of immunoglobulin levels
One part of the submandibular SG of each mouse was crushed, placed in 2 mL tubes containing 1 mL HEPES lysis buffer (20 mM HEPES, 0.5 M NaCl, 0.25% Triton X-100 and 1 mM EDTA) and complete protease inhibitor (Roche, Mannheim, Germany), and was lysed shaking at 4°C overnight. The next day, samples were centrifuged at 1,500 x g at 4°C for 10 minutes and supernatant was collected. Total protein was determined with BCA™ protein assay kit (Pierce, Rockford, IL, USA). IgG, IgA and IgM were measured in serum and SG homogenates by commercially available ELISA kits (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer’s protocol. Values were corrected for total protein content for the SG protein homogenates.
Quantification of cytokines
Cytokines in serum and homogenates of SG were measured commercially using a multiplex sandwich-ELISA assay (Aushon Biosystem, Billerica, MA, USA). Values were corrected for total protein content in the SG protein homogenates.

Statistical analysis
Differences in cytokine levels between experimental groups were assessed using the non-parametric Wilcoxon’s rank-sum test or parametric Student’s t-test depending on data distribution. Differences in all the other experiments were assessed using Student’s t-tests. Correlations between vector copy number and FS were assessed using Spearman’s Rho test (non-parametric) and between BAFF and APRIL using Pearson correlation coefficient (parametric). Both were performed with SPSS for Windows (SPSS version 16.0.02, Chicago, IL, USA). All other analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA). A p value ≤ 0.05 was considered to be statistically significant.

RESULTS

In vitro AAV2-TACI-Fc transduction leads to expression of full length TACI-Fc fusion protein
A TACI-Fc construct was synthetically designed and cloned into an AAV2 serotype viral vector. We confirmed that the full length fusion protein was expressed and secreted in vitro after transfection of HEK293 cells with the viral construct. Both TACI and the Fc part could be detected in the supernatant after transfection by sandwich ELISA using rat anti-murine TACI or HRP labeled anti-rat antibodies and biotin-labeled goat anti-murine IgG as secondary antibodies (data not shown). In a western blot, secreted TACI-Fc migrated as a monomer with an expected molecular mass of approximately 40 kDa (Figure 1A). The concentration of the secreted protein in supernatant from TACI-Fc expressing cells was measured at 562 ng/ml, which is, based on previous work, within the expected range for in vitro transgene expression.

AAV2-TACI-Fc stably transduces the salivary gland and results in significant soluble receptor expression
The NOD mouse is one of the models used to study a SS-like disease. Infiltrates, resembling the morphology and composition of infiltrates seen in humans with SS, are detected in the SGs of these mice starting at 8 weeks of age and the number and size of the infiltrates increase thereafter. In addition, this mouse strain is known to spontaneously develop SG dysfunction over time. In our facility, SG inflammation in NOD mice becomes histologically evident at the age of 8 weeks, and progressively increases with age. In order to evaluate the therapeutic potential of soluble TACI-Fc to treat rather than to prevent the disease, which would be the most relevant for a clinical application in human SS, the vector was delivered to the mice by retrograde instillation of the submandibular SGs after the onset of disease at 10 weeks of age.
Vector delivery was confirmed by q-PCR detection of vector DNA in SG DNA isolated from treated mice at the end of the experiment (data not shown). Local expression of the fusion protein transferred to the SG by AAV2 is expected from 3 days after gene transfer into the SG of NOD mice (based on transduction with AAV-luciferase followed by in vivo imaging of luciferase activity, unpublished observations) and previous work in healthy mice has shown that it can still be detected up to one year after instillation.\textsuperscript{34} We determined TACI levels in protein homogenates from the SGs of treated mice and LacZ-treated mice 10 weeks after injection and found significantly higher TACI levels in TACI-Fc-treated mice compared with control mice (mean ± SD; 369.2 ± 110.7 versus 201.7 ± 172.2 pg/ml; \( p = 0.03 \); Figure 1B). Moreover, additional analysis of vector detection in the liver showed no spread to this organ and TACI-Fc protein could not be detected in serum. No effect on the incidence of diabetes was seen when LacZ treated mice were compared with mice that received TACI-Fc (data not shown).

Expression of TACI-Fc dampens salivary gland inflammation in NOD mice and reduces the number of infiltrating mature B and plasma cells.

To assess the effect of local expression of TACI-Fc on overall SG inflammation, the FS was determined. Initially, a pilot experiment was set up in which 4 NOD mice were treated with LacZ and 4 mice with TACI-Fc. The results showed a reduction in FS in the TACI-Fc treated mice (data not shown). To confirm this, and to analyze the effect of the treatment on the cells within the infiltrates on more detail, we set up a second experiment with 9 mice per treatment group. In this group we confirmed that local SG treatment with TACI-Fc reduced FS compared with controls (mean ± SD; 2.63 ± 0.57 versus 3.79 ± 0.78; \( p <0.005 \); Figure 2A). The decreased FS correlated with the detected number of vector copies (\( r = -0.833 \) and \( p = 0.01 \), data not shown).

**Figure 1. In vitro and in vivo expression of TACI-Fc.** (A) In vitro expression of TACI-Fc is shown using a SDS-PAGE gel under reducing conditions. The disulfide-linked homodimer protein migrates as a monomer with a molecular mass of approximately 40 kDa (Lane 2). Lane 1 is mock-transfected and lane 2 is TACI-Fc-transfected supernatant. (B) In vivo expression of TACI-Fc in homogenized SGs (\( N = 9 \) for LacZ and \( N = 8 \) for TACI-Fc) as measured by ELISA. Data shown are mean values +/- SD. \( p \)-Values were determined by a Student’s t-test and significant differences are indicated (*).
Focal infiltrates were characterized by quantitative immunohistochemical analysis. CD4+ and CD8+ T cell population numbers were similar for TACI-Fc and LacZ groups (p = 0.94 and p = 0.54 for CD4+ and CD8+ respectively; Figure 2B). CD19+ B cell numbers tended to be lower in the TACI-Fc-treated mice, nearly reaching significance (p = 0.06; Figure 2C). Moreover, non-switched mature B cells (IgD+, p = 0.02) and plasma cells (CD138+, p = 0.03; Figure 2C and D) were significantly decreased in TACI-Fc-treated SGs. BAFF and APRIL expression was found in ductal epithelial cells and infiltrating foci (Figure 3A and B). BAFF expression in the SGs of TACI-Fc-treated mice was significantly increased compared with LacZ-treated mice (p <0.005), APRIL expression in TACI-Fc-treated mice was unaltered compared to LacZ control mice (p = 0.18; Figure 3A and B).

TACI-Fc decreases IgG and IgM, but does not change IgA levels in the salivary gland

BAFF and APRIL stimulate B cells to produce immunoglobulins and, in autoimmune diseases, to produce autoantibodies9-10. Therefore, we investigated whether TACI-Fc affects immunoglobulin levels. Treatment with TACI-Fc resulted in significantly lower SG concentrations of IgG (1.45 ± 0.54 μg/ml) and IgM (0.32 ± 0.19 μg/ml) compared

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Figure 2. Decreased focus score, peripheral mature B cells and plasma cells. SGs (N = 9 for LacZ and N = 9 for TACI-Fc) were removed for histologic analysis to assess the overall inflammation by focus score (FS) (A), and for quantification of CD4+ T cells, CD8+ T cells (B), CD19+ cells, IgD+ cells (C) and CD138+ plasma cells (D). Data shown are the mean +/- SD (A) or the mean +/- SD positive cell counts per mm² tissue (B-D). The p-values were determined by Student’s t-tests and significant differences are indicated (*).
with SG levels of IgG (3.05 ± 1.80 μg/ml) and IgM (0.54 ± 0.21 μg/ml) in LacZ treated mice (p = 0.03 and p = 0.05 respectively), while IgA concentrations did not change (7.62 ± 5.00 versus 6.57 ± 2.43 μg/ml respectively; p = 0.60; Figure 4A). In serum, no significant difference in immunoglobulin levels was found between the TACI-Fc-treated and the control groups (Figure 4B). The reduced IgG levels after treatment of SGs with TACI-Fc resulted in an overall increased IgA/IgG ratio for treated mice compared with controls (Figure 4C).

Lower IL-2 levels in TACI-Fc-treated salivary glands
BAFF and APRIL have effects on both B and T cells, and might influence the cytokine balance. To investigate if local expression of TACI-Fc in the SG could change cytokine levels either systemically in the serum or locally in the SGs, serum samples and SG protein extracts were obtained from mice at time of sacrifice and cytokine levels were measured by multiplex analysis. TACI-Fc-treated SGs showed decreased levels of IL-2 (p = 0.05), a cytokine which modulates activated T cells and immunoglobulin synthesis. Moreover, levels of other cytokines (IL-1β, IL-4, IL-6, IL-10 and IL-17) were suppressed after TACI-Fc delivery, although this did not reach statistical significance. No differences were detected for IFNγ and IL-12p40 (Figure 5). Serum levels of cytokines were unaffected (data not shown).

Stimulated salivary flow is unchanged after treatment
To investigate if TACI-Fc mediated reduction in SG inflammation affected SG function, pilocarpine-stimulated salivary flow was measured at 20 weeks. Healthy mice and adult NOD mice of 10 weeks of age have a salivary flow of 4-5 ul/gram body weight*20 minutes. We found a decline in salivary flow in both the control group and the TACI-Fc treated mice at 20 weeks of age compared to 10 week old mice (2.18 ± 0.67 and 1.74 ± 0.71 versus 4.3 ± 1.4 μl/gram body weight*20 minutes respectively). The SFR of 20 week old treated mice did not differ from untreated controls (Figure 6).

DISCUSSION
SS patients have elevated serum levels of BAFF and APRIL, which may be involved in pathogenic B cell differentiation, survival and an increased risk of the development of lymphoma associated with the disease. There is no universally effective therapy, however some patients have benefited from the B cell depleting agent rituximab, validating the B cell compartment as a therapeutic target. Besides B cell depletion, another B cell-directed therapeutic approach is the neutralization of BAFF and APRIL by expression of soluble TACI, a common receptor. In this study, we are the first to evaluate the effects of soluble TACI in an animal model of SS. We show that expression of soluble TACI in the SG of NOD mice, which spontaneously develop a SS-like syndrome, reduces autoimmune SG inflammation via reduction of B and plasma cells, and immunoglobulins.

Expression of soluble TACI-Fc locally in the salivary glands of NOD mice resulted in a two-fold increase in detectable TACI in the SG. Additionally, we detected a significant
Figure 3. Increased BAFF detection in TACI-Fc-treated mice. SGs (N = 10 for LacZ and N = 9 for TACI-Fc) were stained and quantified for BAFF (A) and APRIL (B) expression. For each isotype and staining a representative picture, taken with a 40X objective lens. Arrows indicate a positive staining of ductal epithelial cells. Data shown are the mean +/- SD integrated optical density (IOD) per mm² tissue. The p-values were determined by Student's t-tests and significant differences are indicated (*).
increase in BAFF levels, while APRIL levels were unaffected. We did not measure free BAFF separate from soluble receptor-bound BAFF levels, but we attribute this increase to cytokine-soluble receptor binding and stabilization. This phenomenon, in which the therapeutic drug leads to a seemingly paradoxical increase of the targeted cytokine, has been described previously in a clinical trial with atacicept in RA patients. Herein, the atacicept-BlyS complex has been shown to accumulate in the high dose atacicept group. Additionally, in clinical trials with soluble TNF-receptors, the amount of circulating TNF in patients was seen to be increased up to 7-fold. Most of this TNF lacked TNF bioactivity.

Treatment with TACI-Fc lead to a decreased number of SG infiltrates in our initial pilot study, which was confirmed in our follow-up study. Analysis of the infiltrates showed that T cell numbers were not affected, but overall B cell numbers tended to be lower and treated mice had significantly reduced numbers of IgD⁺ non-switched mature B cells and plasma cells. This decrease in B cells in the SG can be explained by a shortened survival of B cells infiltrated in the gland or, since BAFF is also known to play a role in chemotaxis, reduced B cell recruitment to the SG due to the decreased availability of (free) BAFF. Since it is not known whether plasma cell differentiation takes place in the SG, the observed decrease of plasma cells within the SGs, can not be directly explained. It could be the direct result of a reduction in the recruitment of peripheral plasma cells into the SG, for instance by decreased bioavailability of APRIL after binding to TACI. Alternatively, since BAFF levels correlate with ectopic GC formation, a pathological feature commonly found in the SG of SS patients.

Figure 4. Decreased salivary gland IgG and IgM after TACI-Fc delivery. SG homogenates (N = 7 for LacZ and N = 8 for TACI-Fc) (A) and serum (N = 6 for LacZ and N = 9 for TACI-Fc) (B) from 20-week-old mice were analyzed for IgG, IgA and IgM. Data shown are mean +/- SD, the P-values were determined by Student’s t-tests and significant differences are indicated (*). Additional analysis showing the IgA:IgG ratio in the SGs (C).
Figure 5. Lower IL-2 levels in the salivary glands of TACI-Fc treated mice. Data shown are the mean values from replicate assays (pg/ml) (N = 9 for LacZ and N = 8 for TACI-Fc) after correction for total protein content. The p-value was determined by the Student’s t-test or the non-parametric Wilcoxon’s rank-sum test depending on data distribution for each cytokine and significant differences are indicated (*).
and NOD mice, the decrease could be the result of altered ectopic GC formation or interactions within these GCs in the SG resulting in decreased B cell to plasma cell switching. Future research will have to elucidate the exact mechanism. Taken together, these data suggest that expression of soluble TACI in the SG of NOD mice reduces the number of B cells, possibly by inhibiting proliferation and differentiation while T cell numbers were not affected.

The reduction in plasma cells was accompanied by decreased levels of IgG and IgM, but not IgA, in the SGs. These findings are supported by prior studies utilizing systemic administration of soluble murine and human TACI to murine models of SLE, which showed decreased B cells and plasma cells in the spleen as well as decreased serum levels of IgM and IgG. IgA is known to have an important role in mucosal immunity and the majority of plasma cells in the mucosal immune system of the SG express IgA. A lower percentage of IgA and a higher percentage of IgG-producing plasma cells resulting in a decreased IgA/IgG ratio has been found in the SGs of patients with SS. In our study, expression of soluble TACI in the SGs led to a statistically significant decrease in IgG levels and therefore an improved IgA/IgG ratio when compared with the control group, suggesting restoration of the mucosal immune balance. The APRIL-TACI axis is thought to be critical in the class switching to IgA, but not IgG and IgM. Soluble TACI binds to both APRIL and BAFF with varying affinities depending on dimerization/heteromerization of the ligands. Since soluble TACI affected IgM and IgG but not IgA, it can be speculated that soluble TACI affects BAFF more than APRIL, possibly due to a difference in binding affinity for the soluble receptor. This is supported by the significant increase in BAFF, but not APRIL, protein levels in treated SGs, suggesting that more BAFF is stably bound by soluble TACI compared with APRIL.

We detected an overall mild reduction in pro-inflammatory cytokines with IL-2 reaching statistical significance (p = 0.05). IL-2, a classical Th1 cytokine, is produced by activated T cells and leads to both activation of T cells and B cell differentiation. Besides B cell activation and stimulation, BAFF and APRIL were also found to induce IL-2 secretion by T cells. We did not analyze T cell activation specifically, but the

Figure 6. Salivary flow rates are unaffected by TACI-Fc treatment. Pilocarpine stimulated salivary flow was measured for 20 minutes for TACI-Fc and LacZ control treated mice at 20 weeks of age. Both groups showed equal reduction in SFR when compared to the 20 week old NOD mice that were not treated when compared with 10 week old mice. (n = 10 for NOD at 10 and 20 weeks, n = 9 for LacZ and n = 8 for TACI-Fc). Data shown is corrected for body weight (gram) and bars represent mean + SD.
decrease in IL-2 in the SGs of treated mice in our study implies inhibition of T cell activation state and cytokine secretion without a change in T cell numbers.

Although SG inflammation was reduced, stimulated salivary flow was not increased after treatment. This causes some major concerns for the use of TACI-Fc in a clinical setting, since dryness is one of the most debilitating symptoms of SS. The lack of effect on the salivary flow may be the result of the following. At first, it may be due to insufficient inflammatory suppression; although treated mice showed a reduced FS, there were still inflammatory foci present in the SG after 10 weeks of treatment. Second, the timing of our treatment may be a reason for lack of physiological improvement. A decline in salivary flow is usually not detected until 16-20 weeks of age; however, at the initiation of treatment, NOD mice already had evidence of inflammation and the processes leading to SG dysfunction may already have been initiated and the pathology involved may be beyond repair. Third, the lack of improvement in the SG function may also be due to unchanged levels of IFN\(\gamma\) and IL-12 in the SGs. In a previous publication our group has shown physiological and immunological effects of IFN\(\gamma\) on human salivary gland (HSG) cells\(^49\). Moreover, we have shown that overexpression of IL-12 in mice leads to salivary gland dysfunction\(^50\) suggesting important roles for IFN\(\gamma\) and IL-12 in the pathogenesis and function of the SGs. One last reason for the lack of efficacy of TACI-Fc could be related to the role of APRIL. Little is known about the role of APRIL in SGs of normal individuals and SS patients. And in contrast to the related autoimmune diseases RA and SLE (reviewed in\(^51\)) APRIL levels in SG of SS patients are detected in lower or similar instead of higher levels compared to healthy controls (J.L. Vosters et al, unpublished observations and\(^52\)). In addition, APRIL levels have been shown to inversely correlated with disease activity\(^53-54\) and BAFF levels\(^54\) in SLE. This raises the question whether the role of APRIL is solely pathogenic and, as a consequence, whether blocking APRIL is desirable in the treatment of SS. Our study does not prove that blocking APRIL prevented restoration of salivary flow, however future studies will have to show an effect on dryness before considering this therapy for humans with SS.
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REFERENCES

20. Ramanujam, M., et al. Mechanism of action of transmembrane activator and calcium modulator ligand interactor-Ig in