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EXTENDED REPORT

Th17 cells play a critical role in the development of experimental Sjögren’s syndrome

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ABSTRACT

Objective Although Th17 cells have been increasingly recognised as an important effector in various autoimmune diseases, their function in the pathogenesis of Sjögren’s syndrome (SS) remains largely uncharacterised. This study aims to determine the role of Th17 cells in the development of experimental SS (ESS).

Methods The ESS was induced in wildtype and IL-17A knockout (IL-17 KO) C57BL/6 mice immunised with salivary glands (SG) proteins. Phenotypic analysis of immune cells in the draining cervical lymph nodes (CLN) and SG was performed by flow cytometry and immunofluorescence microscopy. To determine the role of Th17 cells in ESS, immunised IL-17 KO mice were adoptively transferred with in vitro-generated Th17 cells and monitored for SS development. The salivary flow rate was measured, whereas inflammatory infiltration and tissue destruction in SG were assessed by histopathology.

Results SG protein-immunised mice developed overt SS symptoms with increased Th17 cells detected in CLN and within lymphocytic foci in inflamed SG. Notably, immunised IL-17 KO mice were completely resistant for SS induction, showing no evidence of disease symptoms and histopathological changes in SG. Adoptive transfer of Th17 cells rapidly induced the onset of ESS in immunised IL-17 KO mice with markedly reduced saliva secretion, elevated autoantibody production and pronounced inflammation and tissue damage in SG.

Conclusions Our findings have defined a critical role of Th17 cells in the pathogenesis of ESS. Further studies may validate Th17 cell as a potential target for treating SS.

INTRODUCTION

As a systemic autoimmune disease, primary Sjögren’s syndrome (SS) is characterised by progressive inflammation and tissue damage of salivary glands (SG) and lacrimal glands, leading to dry-mouth and dry-eye symptoms as a result of reduced secretion of saliva and tears.1-3 Histopathologically, SS is mainly featured with extensive lymphocytic infiltration and glandular destruction in exocrine glands.4 Early studies have depicted the swollen glands accompanied with enlarged cervical lymph nodes (CLN) in SS patients.5 Moreover, reduced cervical lymphadenopathy and glandular swelling were observed in SS patients after corticosteroid treatment,6 indicating the involvement of immune responses in CLN during the disease pathogenesis. Inflammatory infiltrates in SG consist of macrophages, dendritic cells (DCs), T cells and B cells with a predominant presence of CD4+ T cells.7-9 Although the aetiology of SS still remains unclear, studies using various animal models have indicated a pivotal role of T cell-mediated autoimmune inflammation in SS pathogenesis.10-11 Accumulated data have also shown a significant contribution of dysregulated cytokine network to the development of SS.12 Although studies on NOD/Ltj and C57BL/6.NOD-Aec1Aec2 mice with SS-like disease have clearly illustrated the important implication of Th1 and Th2 cytokines in the pathogenesis of SS,13-14 a pivotal role of IL-17 has been increasingly recognised in the disease process. In patients with SS, increased levels of circulating IL-17 have been observed, whereas IL-17-expressing cells are identified as a dominant population within inflammatory lesions in SG.15 Recent studies by Nguyen et al16 have elegantly demonstrated an important function of IL-17 during SS induction in SS-non-susceptible C57BL/6 mice.

As a key effector T cell subset distinct from the Th1 and Th2 CD4+ T lineages, IL-17-producing CD4+ T (Th17) cells have been identified to play an important role in immune response and autoimmune pathogenesis.17-18 Recent studies including our recent findings have highlighted a pathogenic role of Th17 cells in animal models for rheumatoid arthritis and systemic lupus erythematosus.19-20 Notably, the presence of Th17 cells in SG of patients with SS is associated with focus scores.21-22 Although lines of evidence indicate that overexpression of IL-17 exacerbates the disease progression of SS, it remains unclear whether Th17 cells play a pivotal role in the pathogenesis of SS.23-24 In this study, we first characterised the kinetic changes of Th17 cells in draining CLN and within lymphocytic infiltrates in SG of mice with experimental SS (ESS). To further define the function of Th17 cells in ESS, adoptive transfer of Th17 cells was performed in SG protein-immunised IL-17 knockout (KO) mice. Together, our findings identified a critical role of Th17 cells in the development of ESS.

MATERIALS AND METHODS

Induction of SS in mice

The ESS mouse model was induced in 8-week-old female wildtype (WT) or IL-17 KO C57BL/6 mice (kindly provided by Dr Yoichiro Iwakura from University of Tokyo) by immunisation with SG proteins using our previously described protocol with Q10. Q7 128
For SG protein preparation, the bilateral SG from naïve WT mice was collected for homogenisation in phosphate buffered saline and centrifuged at 12 000×g for 5 min at 4°C. The supernatant was collected with protein concentration determined by the bicinchoninic acid assay (Sigma-Aldrich) and emulsified in an equal volume of Freund’s complete adjuvant (Sigma-Aldrich) to a concentration of 2 mg/mL. For SS induction, each mouse received subcutaneous multijections on the back with 0.1 mL of the emulsion on days 0 and 7, respectively. On day 14, the booster injection was carried out with a dose of 1 mg/mL SG proteins emulsified in Freund’s incomplete adjuvant (Sigma-Aldrich). Mice immunised with either proteins extracted from pancreas or adjuvant alone served as controls. NOD/ShiLtJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal studies were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

Saliva and blood collection

After the animals were anesthetised, saliva secretion was induced by intraperitoneal injection of pilocarpine (Sigma-Aldrich) at a dose of 5 mg/kg body weight.3 Stimulated whole saliva was gravimetrically collected using a 20 µL sized pipet tip from the oral cavity for 15 min at room temperature. Blood samples were collected for serum preparation.

Phenotypic analysis of SG-infiltrating leukocytes

SG-infiltrating leukocytes were harvested as previously described.24 To avoid contamination of sequestered leukocytes with non-inflammatory circulating cells, extensive intracardial perfusion of ESS mice prior to organ extraction was performed. Sorting-purified CD4 T cells were subsequently examined by ELISPOT assay.

Detection of serum autoantibodies

Serum samples from WT and IL-17 KO mice were analysed for autoantibodies against SG proteins, M3 muscarinic receptor (M3R) and antinuclear antibodies (ANA). Peptides encoding murine M3R extracellular domains (VLYNTFCDSCIPKTVW NLGYS)23 or control peptides (SGSGSGSGSGSGSGSGSGSG) were synthesised chemically by a solid-phase approach and purified by high performance liquid chromatography (SBS Genetec Co., Ltd). Serum antibodies were measured by a standard ELISA assay.19

Histological assessment and immunofluorescence microscopy

Mouse SG tissues were prepared for sectioning with haematoxylin and eosin (H&E) staining. A widely used scoring system based on the size and the degree of lymphoid organisation of the infiltrates was adopted to assess the severity of tissue damage.26 For immunofluorescent microscopy, frozen sections were stained with monoclonal rat antimes a anti-CD4 (clone RM4-5), biotinylated rat antimes a IL-17 (clone TC11-18H10.1) and FITC-streptavidin (BioLegend) while nuclei were counterstained with Hoechst 33258 (CalBioChem). Rat IgG antibody was used for control staining.

Detection of apoptotic cells in situ

Terminal uridine nick-end labelling (TUNEL) assay was performed as previously described.27 Frozen sections were analysed with a confocal laser scanning microscope (LSM 710, Carl Zeiss).

Flow cytometric analysis

Surface markers were identified with the following monoclonal antibodies (BioLegend): anti-CD4 (clone GK1.5), anti-CD3 (clone 145-2C11), anti-CD8 (clone 53-6.7), anti-CD19 (clone 6D5), anti-CD45 (clone 30-F11), anti-GL-7 and anti-Fas (clone 15A7). The intracellular staining for IFN-γ and IL-17 was performed as previously described.28 Stained cells were analysed with a FACS Calibur flow cytometer (BD Biosciences).

Adaptive transfer of Th17 cells

Mouse spleenic CD4 T cells were cultured for Th17 differentiation as previously described.24 Purification of Th17 cells was performed using a mouse IL-17 secretion and enrichment assay kit (Miltenyi Biotec). Purified Th17 cells (1×10⁶) were intravenously transferred into IL-17 KO mice on the day of boosting immunisation while the control group was transferred with 1×10⁶ CD4 T cells from IL-17 KO mice.

Data analysis and statistics

Results are expressed as mean±SD. Data were analysed using an unpaired Student t test to determine the difference between groups using SPSS V16.0 (*p<0.05, **p<0.01 and ***p<0.001).

RESULTS

Enhanced Th17 cell response during the development of ESS in mice

 Upon immunisation with SG proteins, C57BL/6 mice developed a full disease profile of ESS, including reduced saliva secretion, elevated serum autoantibody production and tissue destruction with lymphocytic infiltration in submandibular gland (figure 1). To further examine the antigen specificity in ESS induction, mice were also immunised with pancreatic proteins (see online supplementary figure S1). We found that SG protein-immunised mice started to exhibit a significant reduction in salivary flow rate on day 7 postimmunisation while control groups immunised with either adjuvant alone or pancreatic proteins did not develop any sign of disease symptoms (figure 1A; see online supplementary figure S1A). Moreover, levels of autoantibodies against total SG antigens, M3R and ANA were significantly elevated in the sera of SG protein-immunised mice (figure 1B,C; see online supplementary figure S1B). Notably, SG protein-immunised mice displayed substantially enlarged SG and CLN during ESS development, whereas other secretory glands such as pancreas remained unaffected (figure 1D and data not shown). Flow cytometric analysis revealed both significantly increased frequency and total number of Th17 cells in CLN of SG protein-immunised mice compared with controls (figure 1E). In addition, the total number of CD8 T cells and γδ T cells was found slightly increased in the CLN of ESS mice (figure 1F). Notably, no obvious immune responses, including IFN-γ and IL-17 production, were detected in mice immunised with pancreatic proteins (see online supplementary figure S1C,D).

To ascertain the presence of Th17 cells among lymphocytic infiltration in SG, we performed histological analysis on the tissue sections of submandibular gland from ESS mice and controls. Large lymphocytic infiltrates become prominent in the submandibular gland of ESS mice 5 weeks postimmunisation, with multiple lymphocytic foci and extensive acinar destruction readily detected at 30 weeks postimmunisation (figure 2A). In contrast, leucocyte infiltration and tissue destruction were not detected in the SG of control animals immunised with either adjuvant or pancreatic proteins (see online supplementary figure S1E). Using a previously described scoring system,26 we
quantified the number of infiltrated lymphocytes and found significantly increased histological scores in submandibular gland of mice with ESS (figure 2B). Flow cytometric analysis identified CD4 T cells as the major cell subset among infiltrated CD45 leukocytes in SG at early stage, followed by gradually increased infiltration of B cells (figure 2C,D). IL-17-producing CD4 T cells were detected as the major source of IL-17 in the infiltrates by immunofluorescence microscopy (figure 2E), which was further confirmed by ELISOPT assay (figure 2F).

Enhanced Th17 responses occur in CLN and SG during ESS development

Next, we examined the kinetic changes of Th1 and Th17 responses in the draining CLN at various time intervals during ESS induction. Th17 cells were significantly increased 7 days prior to an enhanced Th1 response in CLN of ESS mice, with a peaked Th17 response detected on day 17 post first immunisation (figure 3). During ESS development, the enhanced Th17 response in the CLN rapidly diminished from day 24 onward while a persistent elevation of Th1 response was maintained till 30 weeks post first immunisation. Notably, a similar pattern of Th17/Th1 shift was also observed in CLN of NOD mice, a mouse model with spontaneous SS-like autoimmune exocrinopathy (see online supplementary figure S2). Together, these results identify an initially enhanced Th17 response prior to Th1 response during ESS development.

We further examined the kinetic changes of SG-infiltrating Th17 cells during ESS progression. Flow cytometric analysis revealed that both frequencies and total number of Th17 cells were persistently increased in SG up to 30 weeks postimmunisation (figure 3B).
A critical role of IL-17 in the development of ESS

To define the function of IL-17, the signature cytokine produced by Th17 cells, in the pathogenesis of ESS, we immunised IL-17 KO mice with SG proteins for ESS induction. Remarkably, the immunised IL-17 KO mice exhibited no clinical symptoms and histological changes in SG up to 24 weeks of age. As shown in figure 4A, the saliva flow rate remained unaffected in immunised IL-17 KO mice while immunised WT mice showed a significant reduction. Moreover, there were no signs of cervical lymphadenopathy and tissue destruction in the SG of immunised IL-17 KO mice (figure 4B–D). As expected, flow cytometric analysis detected no Th17 cells both in CLN and SG of IL-17 KO mice while WT mice showed significantly increased Th17 response upon ESS induction (figure 4E and data not shown). Furthermore, detailed analysis of various types of IL-17-producing leucocytes confirmed that Th17 cells were the predominant IL-17-producing cell subset in the LN of ESS mice (figure 4G).

Adoptive transfer of Th17 cells drives ESS development in IL-17 KO mice

To further define the role of Th17 cells in the pathogenesis of ESS, we examined whether adoptive transfer of Th17 cells...
Figure 3  
Kinetic changes of Th17 responses in CLN and SG during ESS development. (A) Frequencies of IFN-γ and IL-17-producing Th1 and Th17 cells in the cervical lymph nodes (CLN) of mice immunised for ESS induction were examined by flow cytometry analysis on days 0, 3, 10, 17, 24 and 30 weeks post first immunisation. Total cell numbers of Th1 and Th17 cells in CLN of ESS mice or control mice immunised with adjuvant alone on various time intervals post first immunisation were enumerated and compared with their respective numbers on day 0. (B) SG-infiltrating Th17 cells were analysed by flow cytometry at 5, 10, 15, 25 and 30 weeks post first immunisation. Total cell numbers were enumerated and compared with their respective numbers on 5 weeks. Data are derived from three separate experiments (mean±SD; n=10; *p<0.05; **p<0.01; ***p<0.001). ESS, experimental Sjögren’s syndrome; SG, salivary glands.

Figure 4  
IL-17 KO mice are resistant for ESS induction. (A) SG protein-immunised IL-17 KO mice exhibited normal salivary secretion while significantly decreased saliva flow rates were observed in immunised wildtype (WT) mice. (B) Representative micrographs showing glandular destruction in the SG of WT mice with ESS; normal histology was observed in the SG of immunised IL-17 KO mice at 24 weeks of age (n=15 for each group). (C) Acinar tissues in SG were assessed for histological scores. (D) Representative micrographs show the sizes of salivary gland and CLN of IL-17 KO mice and WT mice upon ESS induction (bar=1 cm). (E, F) Both frequencies and total numbers of Th1 and Th17 cells in the CLN of IL-17 KO mice and WT controls with ESS were analysed by flow cytometry. (G) Total numbers of various IL-17-producing cell populations in CLN of WT mice with ESS were enumerated by flow cytometry (n=10 for each group). Values are derived from three separate experiments (mean±SD; **p<0.01 and ***p<0.001). CLN, cervical lymph nodes ESS, experimental Sjögren’s syndrome; IL-17 KO, IL-17A knockout; SG, salivary glands.
could promote the development of ESS in SG-protein-immunised IL-17 KO mice. First, naïve CD4 T cells isolated from the spleen of WT mice were cultured under a Th17-polarising condition, followed by purification of IL-17-producing Th17 cells using a cytokine capture assay (figure 5A). One week after adoptive transfer of purified Th17 cells, immunised IL-17 KO mice began to show markedly reduced saliva rates, displaying a similar disease profile with decreased saliva secretion as WT ESS controls (figure 5B). Histological examination detected extensive acinar apoptosis and submandibular gland destruction with periductal foci of CD4+ T cell infiltrates in Th17 cell-transferred IL-17 KO mice (figure 5C). Notably, control groups of IL-17 KO mice transferred with or without CD4+ T cells from IL-17 KO mice did not show any significant reduction in salivary secretion with normal glandular structure observed in SG. Thus, these findings reveal a critical role of Th17 cells in driving the development of SS in mice.

Adoptive transfer of Th17 cells enhances Th1 and B cell responses in IL-17 KO mice
In Th17 cell-transferred IL-17 KO mice, we found significantly increased frequencies and total numbers of Th1 cells and GL-7+ Fas+ germinal centre (GC) B cells in the enlarged CLN by flow cytometric analysis (figure 6A–D). Moreover, immunofluorescence microscopy confirmed both increased numbers and sizes of GCs in the CLN of Th17 cell-transferred IL-17 KO mice (figure 6E). Elevated levels of serum autoantibodies against total SG antigens and M3R were detected in Th17 cell-transferred IL-17 KO mice but not in IL-17 KO mice transferred with CD4 T cells (figure 6F). Remarkably, similar levels of elevated serum autoantibodies were detected between Th17 cell-transferred IL-17 KO mice and WT ESS controls. Furthermore, we observed pronounced infiltration of CD4 T cells in the SG of Th17 cell-transferred IL-17KO mice, among which transferred Th17 cells were also detected (figure 6G). These results suggest that adoptive transfer of Th17 cells may drive the development of ESS via promoting Th1 and B cell responses.

DISCUSSION
In this study, we have revealed an enhanced Th17 response in draining CLN and prominent infiltration of Th17 cells in SG during ESS development. Although IL-17-deficient mice are completely resistant for ESS induction, adoptive transfer of Th17 cells can drive the development of ESS in IL-17 KO mice as featured by reduced salivary secretion and tissue destruction in the SG. Together, these results have demonstrated a critical role of Th17 cells in the pathogenesis of ESS.

Up to date, many mouse models with SS-like disease have been used for investigating SS pathophysiology. SG proteins such as laminin and exosomes have been characterised as autoantigens in initiating SS while most SS patients exhibit significantly increased autoantibodies against M3R.29–32 In this study, we successfully induced a full profile of SS-like disease with characteristic autoantibody production in SG protein-immunised C57BL/6 mice, which allow us to study immune dysregulations during ESS development in a normal mouse strain without genetic susceptibility. Notably, our observations that pancreatic protein-immunised mice did not exhibit any detectable immune responses and pathological changes in SG further confirm the antigen specificity against SG in ESS model. Earlier studies have suggested Th1 cytokine-mediated inflammatory responses in SS patients,33–36 but increasing evidence indicates the involvement of IL-17 in autoimmune inflammation during the pathogenesis of SS.37 Here, we reveal a significantly enhanced Th17 response occurring 7 days prior to the predominant Th1 response during ESS induction. Notably, similar kinetic changes of Th17

Figure 5  Adoptive transfer of Th17 cells induces ESS development in immunised IL-17 KO mice. (A) Purified CD4 T cells from wildtype (WT) mice were cultured in the Th17 cell polarisation media for 4 days. Th17 cells enriched by cytokine capture assay were further examined for purity by flow cytometry. (B) Changes in saliva flow rates were measured in SG protein-immunised IL-17 KO mice with adoptively transferred either Th17 cells from WT mice or CD4 T cells from IL-17 KO mice compared with control group immunised with adjuvant alone (n=14 for each group). Values (mean±SD) are derived from four separate experiments (**p<0.01). (C) The upper panel micrographs show histological assessment of tissue destruction in SG of IL-17 KO mice 3 weeks after transferred with either Th17 cells or control CD4 T cells (bars=10 μm). Micrographs in the middle panel show infiltrated CD4 T cells in the SG of Th17 cell-transferred mice, whereas the lower panel indicates the detection of apoptotic cells in SG by TUNEL staining. ESS, experimental Sjögren’s syndrome; IL-17 KO, IL-17A knockout; TUNEL, terminal uridine nick-end labelling; SG, salivary glands.

response followed by increased Th1 response were also observed in NOD mice with SS-like disease (see online supplementary figure S2). Moreover, adoptive transfer of Th17 cells resulted in markedly enhanced Th1 response in vivo, which suggests a pivotal role of Th17 cells in initiating inflammatory reactions and driving T cell-mediated disease pathologies during the development of ESS. Although the mechanisms involved in Th17/Th1 shift remain currently unclear, recent studies have indicated a role of IL-17-stimulated DCs in driving Th1 cell polarisation under autoimmune conditions.18

In patients with SS, the predominant IL-17 expression in CD4 T cells within lymphocytic infiltrates has been detected in the SG.15 IL-17-producing cells are also found within the lymphocytic foci in the SG of C57BL/6.NOD-AecIa1e2 mice with SS-like disease.19 However, the available data only suggested the possible involvement of Th17 cells in the disease progression but did not demonstrate Th17 cells as the major effector in the pathogenesis of SS. Here, we provide strong evidence that adoptive transfer of Th17 cells can effectively trigger the onset and progression of SS in IL-17 KO mice. Moreover, adoptive transfer of CD4 T cells from IL-17-deficient mice failed to induce the development of ESS in IL-17 KO mice, which further highlights a critical function of Th17 cell-derived IL-17 in driving the disease pathologies. Since increased peripheral Th17 cells in SS patients have been recently reported,15,40 it remains to be investigated whether peripheral Th17 cells could serve as a biomarker for disease activity.

During ESS development, we have detected markedly increased serum antibodies against SG antigens, M3R and ANA in IL-17 KO mice 2 weeks after Th17 cells transfer (figure 6), indicating an augmented antibody response mediated by Th17 cells in vivo. In addition, GC B cells in CLN are markedly increased in Th17 cell-transferred IL-17 KO mice. Thus, these data clearly indicate that adoptive transfer of Th17 cells effectively drives B cell activation and maturation, resulting in augmented antibody response in vivo. Collectively, our results are consistent with recent findings

**Figure 6** Enhanced Th1 and B cell response in IL-17 KO mice transferred with Th17 cells. (A) Representative micrograph shows the sizes of SG and CLN from wildtype (WT) naïve mice and SG protein-immunised IL-17 KO mice transferred with either CD4 T cells from IL-17 KO mice or Th17 cells from WT mice (bar=1 cm). (B, C) Flow cytometric analysis of Th1 cells (B) and germinal centre (GC) B cells (C) in the CLN of mice. (D) The total number of GC B and Th1 cells in CLN were enumerated for comparison among various groups (mean±SD; ***p<0.001). (E) Examination of GCs (PNA+ IgD−) in CLN in immunised IL-17 KO mice 3 weeks after adoptive transfer of control CD4 T cells and Th17 cells, respectively, by confocal microscopy (bar=50 μm). (F) Elevated levels of serum antibodies against SG antigens and M3R were detected by ELISA in IL-17 KO mice transferred with Th17 cells for 3 weeks, showing similar titres as those detected in WT mice with ESS. Values (mean±SD) are derived from four separate experiments (**p<0.01 and ***p<0.001). (G) IL-17-producing CD4 Th17 cells (arrows) in the submandibular gland of immunised IL-17 KO mice 15 weeks after Th17 cells transfer were detected (bar=20 μm). CLN, cervical lymph nodes; ESS, experimental Sjögren’s syndrome; IL-17 KO, IL-17A knockout; SG, salivary glands.
that Th17 cells act as an effective B cell helper in promoting B cell activation and autoinhibitory-induced arthritis.41

Recent studies have identified many proinflammatory molecules involved in promoting IL-17-mediated inflammatory cascades.12 42 B cell-activating factor (BAFF) transgenic mice develop SS-like disease with infiltration of large amounts of marginal zone B cells found in SG along with increased BAFF expression.43 Several studies have characterised the aberrant elevation of BAFF in the SG of patients with SS.44–46 We recently showed that BAFF and leptin exacerbate collagen-induced arthritis via promoting IL-17 response, suggesting a key effector function of IL-17 in autoimmune inflammation.19 47 Although SS is characterised by extensive lymphocytic infiltration with predominant presence of CD4 T cells, B cells and macrophages are also present in the inflammatory lesion in SG. Interestingly, SG epithelial cells in SS patients have been found to produce BAFF under inflammatory conditions.48 Furthermore, activated DCs with increased TNF-α production may contribute to disease onset, resulting in salivary dysfunction before the rise of pathogenic autoantibodies and evident lymphocytic infiltration in SG.49 50 Thus, further studies are needed to delineate the marginal zone B cells found in SG along with increased BAFF production in SS, which will provide new insights into understanding the pathophysiology of SS.

In summary, this study identifies a central role played by Th17 cells in driving the pathogenesis of ESS in mice. Future studies may validate the targeting of Th17 cells as a therapeutic strategy for the treatment of SS.

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Contributors

All authors have taken at least some part in the contribution to the idea, experiment design, data collection, data analysis, manuscript writing or manuscript revision.

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Competing interests

None.

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