

Participation of Complement 3a receptor (C3aR) in the sensitization phase of Th2 mediated allergic contact dermatitis

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Abstract: The complement system has emerged as a bridge between innate and adaptive immune responses. An involvement of C3aR has been described during skin inflammation. The aim of the study was to investigate the role of C3a in a mouse model of allergic skin inflammation, such as allergic contact dermatitis (ACD) which is a clinical manifestation of contact sensitivity (CS). The sensitization phase was studied using the local lymph node test: Mice were sensitized on three consecutive days by application of non-irritant concentrations of toluene-2,4-diisocyanate (TDI; 0.5%) onto the ear skin. On day 5, auricular draining lymph nodes were obtained. The elicitation phase was investigated by sensitization with TDI on the depilated and tape-stripped abdominal skin and challenge with TDI on the ear skin and measuring of ear swelling *in vivo* and cytokine secretion in activated splenocytes *in vitro* respectively. Complement 3a

receptor deficient (C3aRKO) mice showed increased cytokine responses (interleukin[IL]-5, IL-6, IL-17, granulocyte macrophage-colony stimulating factor [GM-CSF]) in the sensitization phase of ACD to TDI. However, no differences in CS responses to TDI were observed in C3aR KO mice compared with WT controls in the elicitation phase of ACD as assessed by measuring of ear swelling *in vivo* and cytokines in skin and in activated splenocytes *in vitro*, namely IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, interferon- γ (IFN- γ), GM-CSF and tumor necrosis factor (TNF)- α . These findings provide a new insight into the participation of C3a in the sensitization phase of CS immune responses.

Key words: allergic contact dermatitis – anaphylatoxin – Complement 3a – Complement 3a receptor – T cell

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Introduction

Allergic contact dermatitis (ACD) is a common inflammatory skin disease in industrialized countries and one of the most common occupational disease (1). It is a T cell mediated skin inflammation caused by repeated skin exposure to contact allergens, namely chemicals called haptens (2). It is mediated by T cells, which are primed in lymphoid organs during the sensitization phase and are recruited in the skin upon re-exposure to the hapten (3).

The complement system is an ancient danger sensing system that recognizes exogenous threats, such as conserved microbial motifs as well as endogenous threats including altered-self molecules. It has emerged as a bridge between innate and adaptive immune responses (4). In general, the complement system operates with one of the three pathways – the classical, alternative, or mannose-binding lectin pathway. Each complement cascade results in the cleavage of C3, generating its functional peptides. C3a is an anaphylatoxin and interacts with a variety of hematopoietic (neutrophils, basophils and monocytes) and tissue cells including skin keratinocytes by the G protein-coupled C3aR (5–7). Depending on the disease model and microenvironment, C3a mediates both pro-inflammatory and anti-inflammatory activities. Pro-inflammatory

activities include chemotaxis of leukocytes, enhancement of vascular permeability and cytokine and chemokine synthesis from several cells including skin keratinocytes (5,6,8,9). Most of these pro-inflammatory activities are required for the development of skin inflammation in contact dermatitis (10). In contrast, some findings support the anti-inflammatory role of C3a: C3a inhibits LPS-induced TNF- α , interleukin (IL)-1 α and IL-6 secretion from human PBMCs (11), and C3aR deficient mice show an increased mortality to endotoxin-induced shock (12).

C3 deficient mice show impaired B cell immune responses to T cell dependent and independent antigens (Ag), when Ag dose is limiting (13). However, increased secretion of the Th2 cytokines IL-4, IL-5 and IL-10 were described in C3aR deficient mice using an ovalbumin-induced contact dermatitis model (14). Another study revealed a diminished IFN- γ and IL-17 production in murine T cells which were co-cultured with antigen presenting cells (APCs) from C3aR KO mice compared with C3aR WT mice in a model of experimental autoimmune encephalomyelitis (15).

Allergic contact dermatitis is a clinical manifestation of contact sensitivity (CS) (10). CS is the most widely used model of Ag-specific T cell-mediated immune reactions. In the sensitization phase, a hapten (contact allergens, drugs, metals) is painted on the animal's body (usually on the abdominal skin). The elicitation phase is induced by a subsequent re-exposure of the same hapten at a remote skin site (usually the ear). This results in rapid recruitment of a few circulating sensitized Ag-specific T cells (Th1 or Tc1) into the skin (10). Next, activation of Ag-specific T cells by hapten

Abbreviations: ACD, allergic contact dermatitis; C3aR, Complement 3a receptor; CS, contact sensitivity; DNFB, 2,4-dinitrofluorobenzene; FITC, fluoro-isothiocyanate; GM-CSF, granulocyte macrophage colony stimulating factor; IFN, interferon; IL, interleukin; TDI, toluene-2,4-diisocyanate; TNF, tumor necrosis factor.

loaded local APCs leads to the production of effector cytokines. This local inflammation often peaks at 24 h and results in skin cell apoptosis via Fas and/or perforin-mediated cytolytic function (16). In a previous study, we could show a protective role of complement C3 in a murine model of ACD. We demonstrated an increase in hapten-induced CS responses in C3KO mice in the sensitization as well as in the elicitation phase. *In vitro* stimulation of splenocytes derived from immunized and challenged C3KO mice secreted higher amounts of IFN- γ when compared with their WT controls (17).

As C3 KO mice lack the ability to produce C3a as well as C3b we wanted to elucidate whether C3a or C3b is primarily responsible for our previous observations. Therefore, C3aR KO mice were used to investigate the role of C3aR in T-cell mediated skin inflammation, such as CS in this study. Our findings provide a new insight into the role of the C3 in the sensitization phase of CS immune responses. However, we could not attribute higher responses in the elicitation phase in C3aR KO mice compared with WT mice pointing to a minor role of C3aR mediated signalling.

Materials and methods

Mice

C3aR^{-/+} (heterozygote) BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and bred in University of Veterinary Medicine Hannover, Germany. C3aR deficient (C3aR^{-/-} KO) and wild type (C3aR^{+/+} WT) mice were identified by polymerase chain reaction according to the protocol given by Jackson Laboratory. Furthermore, C3aR^{-/-} KO and C3aR^{+/+} WT colonies were bred and used for experiments. No infection including *Staphylococcus aureus* was found by pathological examination (FELASA protocol) of some sentinel animals (C3aR KO). All animal experiments were carried out in agreement with the German Law on the protection of animal welfare and approved by ethical committee (reference no. 509.6-42502-03/711).

Antibodies and reagents

Toluene-2,4-diisocyanate (TDI), 2,4-dinitro-1-fluorobenzene (DNFB) and Fluoro isothiocyanate (FITC) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Soluble anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) antibodies were purchased from BD Pharmingen (Heidelberg, Germany).

Contact sensitivity induction

We used two CS models of Th1 (DNFB) and Th2 (FITC and TDI) immune reactions. CS was induced with several haptens as described previously (17,18). Briefly, mice were sensitized by painting a hapten on shaved, depilated and tape stripped abdominal skin. After several days, the challenge phase was induced by application of the same hapten on the left ear of each mouse. 25 μ l of 0.5% DNFB (acetone:olive; 4:1) was used on two consecutive days to sensitize mice, challenge was performed with 10 μ l of 0.25% DNFB on day 5. A percentage of 0.5% FITC (acetone:dibutylphthalate; 1:1) was applied on three consecutive days (day 1 100 μ l, days 2 and 3 50 μ l), mice were challenged on day 5 with 20 μ l of 0.5% FITC. Mice were sensitized with TDI (in acetone) on four consecutive days (day 1 100 μ l 5%, days 2–4 50 μ l of 5%), boosted on day 21 with 50 μ l 5% TDI and challenged with 20 μ l of 0.5% TDI on day 28. Ear thickness was measured using a cutimeter (model 7309; Mitutoyo, Neuss, Germany) and calculated as follows: ear thickness in μ m after 24 h of hapten challenge – ear thickness (μ m) before application of hapten.

For testing effects of C3aR deficiency in the sensitization phase of ACD, a local lymph node test was studied in analogy to the OECD guidelines as described previously (19,20). In brief, 20 μ l of non-irritant concentration of TDI (0.5%) were administered onto the ear skin for three consecutive days. On day 5, auricular draining lymph nodes were obtained. Lymph node cells (LNCs) were cultured in anti CD3+ anti CD28 in different concentrations as indicated in the figures for 72 h. Cytokines were quantified by the Th1/Th2 10plex FlowCytomix following the manufacturer's instructions (Bender Med Systems GmbH, Vienna, Austria).

Ear skin preparation

For determination of biochemical parameters, mice ears were homogenized and IL-1 β , IL-4 and IL-6 levels were determined in the supernatants using commercially available ELISA according to the manufacturers recommendation (R&D systems, Wiesbaden, Germany) as described previously (21).

In vitro cytokine synthesis and analysis

Single-cell suspensions of spleen cells (splenocytes), CD3+ T cells and auricular lymph node cells from TDI immunized mice were prepared in complete Iscove's medium supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin and 100 μ g/ml streptomycin. CD3+ T cells were obtained by negative selection using the pan T Cell Isolation Kit from Miltenyi Biotec, Bergisch Gladbach, Germany according to the manufacturers recommendations. Cells were cultured in the above medium at 2×10^6 /ml in 24-well plates in the absence or presence of anti CD3+ antiCD28. Supernatants were collected after 48 h, centrifuged and frozen at -20°C until cytokines were quantified by the Th1/Th2 10plex FlowCytomix following the manufacturer's instructions (Bender Med Systems GmbH).

Statistical analysis

For statistical analyses, the software Sigma stat for Windows (Systat Software, San Jose, CA, USA) was used. Statistical analyses were performed using the student's *t*-test for normally distributed values. For one not normally distributed data pair (GM-CSF: medium control of C3aR KO mice versus C3aR WT mice), the Mann–Whitney Rank Sum Test was performed. A *P*-value <0.05 is considered as statistically significant. In the figures: $*P < 0.05$.

Results

Increased cytokine responses in C3aR KO mice in the sensitization phase of contact sensitivity

As CS develops in two distinct phases, namely the sensitization and the challenge phase, the cytokine expression profile of lymphocytes and APCs from the draining lymph node of C3aR KO and WT mice was studied after the sensitization phase using the local lymph node assay. Mice were sensitized on three consecutive days by application of non-irritant concentrations of TDI (0.5%) onto the ear skin. On day 5, auricular draining lymph nodes were obtained. Lymph node cells (LNCs) were cultured in the absence or presence of anti CD 3+ anti CD28 as indicated for 72 h before Th1 (IFN- γ), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17) cytokines were measured by Flow Cytomix ($n = 6$ mice per group). We detected no differences in IFN- γ secretion in C3aR KO mice compared with C3aR WT mice (data not shown). However, significantly more IL-5 and IL-17 secretion were observed in LNCs from C3aR KO mice compared with C3aR WT mice (Fig. 1a,c). As the cleavage products of C3 (C3a and C3b) interact with APCs to mediate several immune functions, 'classical' pro-inflammatory

cytokines (IL-1 α , IL-6, GM-CSF and TNF- α) secreted by APCs were also measured. Significant differences in the secretion of IL-6 (Fig. 1b) and GM-CSF (Fig. 1d) were detectable, but there was no difference in IL-1 α and TNF- α secretion (data not shown).

No differences in contact sensitivity responses to TDI in C3aR deficient (C3aR KO) mice as compared with WT controls in the elicitation phase of contact sensitivity

To explore the effect of C3aR in T cell-mediated skin inflammation, we sensitized C3aR KO and WT mice on four consecutive days and on day 21 by painting TDI on the abdominal skin of mice. On day 28, both groups were challenged with TDI on their left ears ($n = 24$ mice per group). In a former study, we examined whether C3 KO mice show prolonged ear swelling or delay in resolution of swelling and measured time dependent ear thickness after 24, 48 and 72 h. We showed a maximal ear thickness 24 h after hapten challenge which gradually decreased (17). Therefore, we investigated ear thickness 24 h after challenge in this study. As shown in Fig. 2a, there were no differences in skin inflammation in C3aR KO as compared with WT mice. To see whether our observations can be continued for other Th2 as well as Th1 contact allergens mice were sensitized and challenged with FITC ($n = 12$ mice per group, Th2) and DNFB ($n = 15$ –18 mice per group, Th1) respectively. Again, no differences in ear swelling were observed in C3aR KO compared with WT mice upon immunization with FITC (Fig. 2b) or DNFB (Fig. 2c).

To address the role of C3a in the skin, we analysed inflammatory mediators from immunized (sensitized and challenged with TDI) C3aR KO and WT mice. IL-1 β , IL-4 and IL-6 levels were analysed by ELISA ($n = 6$ mice per group). These cytokines were induced in C3aR KO mice as well as in WT mice upon immuni-

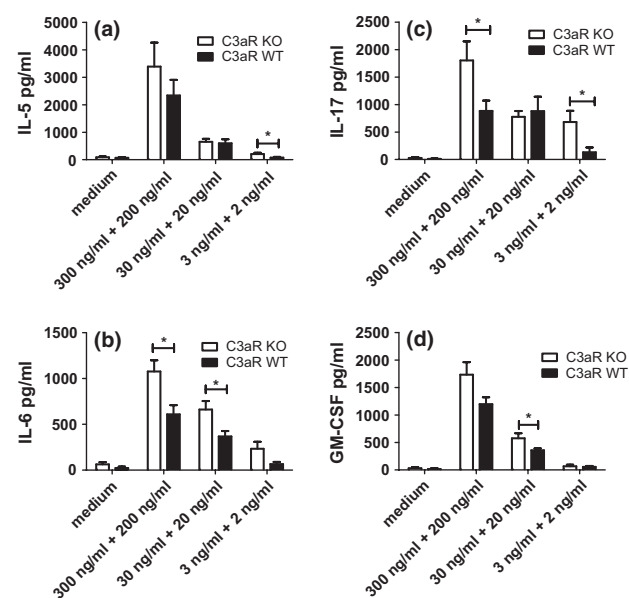


Figure 1. Increased cytokine responses in C3aR KO mice in the sensitization phase of contact sensitivity. Mice were sensitized on three consecutive days by application of non-irritant concentrations of TDI (0.5%) onto the ear skin. On day 5, auricular draining lymph nodes were obtained. Lymph node cells (LNCs) were cultured in the absence or presence of anti CD 3+ anti CD28 as indicated for 72 h before cytokines were measured by ELISA ($n = 5$ –6 mice per group). Data are presented as mean + SEM. * $P < 0.05$.

zation with TDI compared with levels before immunization. However, no differences between KO and WT mice were observed (data not shown).

We next focused on the mechanisms of ear inflammation in C3aR KO and WT mice in TDI (Th2) and DNFB (Th1) induced CS. Splenocytes ($n = 22$ mice per group), spleen derived T cells ($n = 6$ mice per group) and auricular lymph node cells ($n = 9$ –18 mice per group) from TDI immunized mice (C3aR KO and WT) were cultured in the presence of anti CD3+ anti CD28 for 48 h before Th1 (IFN- γ), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17) cytokines as well as 'classical' pro-inflammatory cytokines (IL-1 α , IL-6, GM-CSF and TNF- α) secreted by APCs were measured by

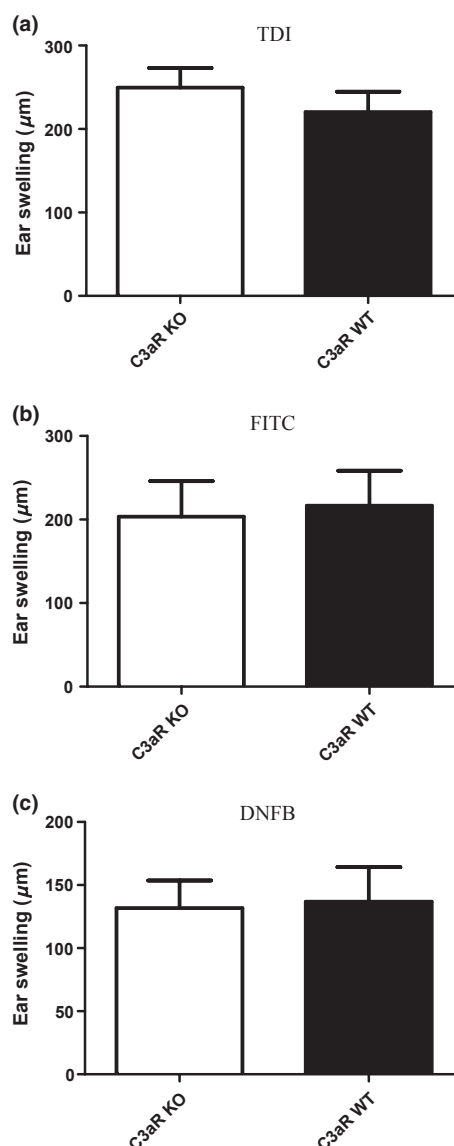


Figure 2. No differences in CS responses to TDI (a; $n = 24$ mice per group), FITC (b; $n = 12$ mice per group) or DNFB (c; $n = 15$ [WT] or 18 [KO] mice per group) in C3aR KO mice compared with WT controls in the challenge phase. C3aRKO and WT control mice were sensitized and challenged with TDI, FITC or DNFB respectively. After 24 h, the ear swelling was measured and calculated as described in methods. Data are presented as mean + SEM.

ELISA. We detected no significant differences in neither in splenocytes nor in T cells nor in lymph node cells from C3aR KO mice compared with their WT littermates (data not shown).

Moreover, we saw no differences in cytokine secretion (IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN- γ , GM-CSF and TNF- α) neither in splenocytes ($n = 5-6$ mice per group) nor in lymph node cells ($n = 5-8$ mice per group) from DNFB immunized mice (data not shown).

Discussion

The complement system has emerged as a bridge between innate and adaptive immune responses. An involvement of C3 and C3aR has been described during skin inflammation. In a previous study, we demonstrated an increase in hapten-induced CS responses in C3 KO mice *in vivo* and *in vitro*. An enhanced ear swelling was observed in C3 KO mice compared with WT mice in response to Th1 (2,4-dinitrofluorobenzene: [DNFB] and dinitrochlorobenzene: [DNCB]) as well as Th2 (fluoro-isothiocyanate [FITC] and TDI) sensitizer. *In vitro* stimulation of splenocytes derived from immunized C3 KO mice secreted higher amounts of IFN- γ compared with their WT controls. Moreover, we demonstrated that C3 KO mice developed oral tolerance to haptens to a lower degree than WT mice (17). As C3 KO mice lack the ability to produce C3a as well as C3b we wanted to narrow our previous observations and elucidate whether C3a or C3b is primarily responsible for our previous observations. Therefore, C3aR KO mice were used to investigate the role of C3aR in T-cell mediated skin inflammation, such as ACD in this study.

The present results demonstrate that production of both T cell (IL-5, IL-17) and APC (IL-6, GM-CSF) derived cytokines is enhanced in C3aR KO mice compared with WT mice in response to sensitization with the Th2 sensitizer TDI. In contrast, no alterations between C3aR KO and WT mice were observed in the elicitation phase of ACD with TDI – neither *in vivo* in ear swelling nor *in vitro* in splenocytes, T cells or lymph node cells respectively. No differences in ear swelling upon sensitization and challenge with FITC, another Th2 sensitizer and the Th1 sensitizer DNFB were observed either. These results suggest that C3aR plays a role in allergic sensitization to haptens rather than in eliciting ACD. Thus, the increase in TDI-induced *in vivo* and *in vitro* responses in the elicitation phase of CS in C3 KO mice in our former study (17) is more likely attributed to C3b than C3a. However, in the C3 KO mouse the complete complement cascade downstream of C3 is blocked. Thus, theoretically effects of C5a/C5aR or C5a/C5L2 or the MAC C5b-9 might also play a role in combination with the C3aR KO. Moreover, the genetic background of mice used in our previous study was C57BL/6 whereas the mice used in this study were on a BALB/c background. This could have influenced the results as well and should be addressed in further studies.

In a former study, we examined time dependency of ear swelling in C3 KO and WT mice and measured ear thickness after 24, 48 and 72 h. We showed a maximal ear thickness 24 h after hapten challenge which gradually decreased (17). Therefore, we investigated ear thickness solely 24 h after challenge in this study and might have missed changes in the early phase, i.e. 6 or 8 h after hapten challenge.

Kawamoto et al. (14) showed in C3aR KO mice epicutaneously sensitized with ovalbumin (OVA) a significantly increased secretion of Th2 cytokines, namely IL-4 and IL-5, upon *in vitro* stimu-

lation of splenocytes with OVA compared with WT littermates. These findings are in line with our results as we found an enhanced *in vitro* secretion of IL-5 in C3aR KO mice compared with WT mice in the sensitization phase of ACD. However, we failed to detect any differences in IL-5 secretion in splenocytes from C3aR KO mice compared with WT mice in the elicitation phase of ACD. The different results obtained in this study and ours may relate to methodical differences and substance of immunization as the genetic background of mice was the same (BALB/c): Kawamoto et al. used an OVA model for their study. They sensitized C3aR KO and WT mice *in vivo* and stimulated splenocytes *in vitro* with OVA. We sensitized and challenged mice with TDI, a well established *in vivo* Th2 sensitizer which is unfortunately not suitable for *in vitro* testing because of limited solubility in cell culture medium. Therefore, we re-stimulated splenocytes with anti CD3+ anti CD28 and might have missed specific allergen-induced differences in our model. Although significant, the differences in IL-5 secretion between C3aR KO and WT mice upon stimulation with 3ng/ml anti-CD3 and 2ng/ml anti-CD28 were rather small (208 pg/ml $\pm$ 43 pg/ml vs 78 pg/ml $\pm$ 25 pg/ml) and therefore their biological relevance needs to be investigated more in detail in further studies.

IL-17 expressing CD4+ T cells have been recognized as a new subset of Th cells, namely Th17 cells and have been associated with host defense against infectious agents, autoimmune diseases and skin inflammation (22). Th17 cells have been shown to be released by subsets of skin-infiltrating chemical-reactive T lymphocytes and may modulate ACD responses (23). A more recent study revealed that IL-17+ T cells infiltrate ACD reactions and predominantly distribute at the site of heavy spongiosis. Moreover, the authors showed that skin IL-17+ T cells were heterogeneous. Hapten-responsiveness was restricted to Th1/IL-17 (IFN- γ +IL-17+) and Th0/IL-17 (IFN- γ +IL-17+IL-4+) and to a lesser extent to Th2/IL-17 cells in their study. We worked in a Th2/IL-17 related milieu using TDI as a sensitizer and genetically Th2 related BALB/c mice. Therefore, our observation that no differing CS reactions in C3aR KO mice compared with C3aR WT mice were elicited despite higher IL-17 levels in the sensitization phase in C3aR KO mice compared with WT mice might be in line with observations made by Pennino et al. showing a predominant relevance of Th1/IL-17 and Th0/IL-7 cells. Furthermore, Pennino et al., (24) showed that IL-17 releasing T cells promoted IL-6, IL-8 and HBD-2 secretion from keratinocytes. A former study by Albanesi et al. (23) showed that stimulation of keratinocytes with IL-17 led to an enhanced IL-6 and GM-CSF secretion. Another group investigated the interaction of keratinocytes and T cells in ACD and found an up-regulation of GM-CSF in keratinocytes upon allergen stimulation as well (25). We could confirm the increased IL-17 secretion in lymph node cells from C3aR KO mice compared with WT mice. The combined induction of IL-17, IL-6 and GM-CSF secretion in C3aR KO mice compared with WT littermates observed in our study could be explained by amplification of IL-6 and GM-CSF secretion through IL-17 as previously shown by Albanesi et al. and Pennino et al. (23,24). As allergen-induced emigration and maturation of dendritic cells (DCs) are pivotal steps in sparking off ACD, our results with enhanced secretion of APC cytokines (IL-6 and GM-CSF) should be studied more in detail in further studies.

Taken together, C3a/C3aR might participate in conjunction with other complement effector functions down-stream of C3/C3b in the protection of Th2 induced ACD.

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Conflict of interest

The authors have no conflicts of interest to declare.

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