Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells play a pivotal role in the development of active disease in experimental bullous pemphigoid model

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Abstract Bullous pemphigoid (BP), the most common autoimmune blistering disease, is caused by autoantibodies against type XVII collagen (COL17). We recently demonstrated that CD4+ T cells were crucial for the production of anti-COL17 IgG and for the development of the BP phenotype by using a novel active BP mouse model by adoptively transferring immunized splenocytes into immunodeficient COL17-humanized mice. Noncollagenous 16A (NC16A) domain of COL17 is considered to contain the main pathogenic epitopes of BP, however, the pathogenicity of COL17 NC16A-reactive CD4+ T cells has never been elucidated. To address this issue, we modulated the immune responses against COL17 in active BP model by using anti-CD40 ligand (CD40L) monoclonal antibody MR1, an inhibitor of the CD40–CD40L interaction, in various ways. First, we show the essential role of CD4+ T cells in the model by showing that CD4+ T cells isolated from wild-type mice immunized with human COL17 enabled naïve B cells to produce anti-COL17 NC16A IgG in vivo. Second, we show that the activation of anti-COL17 NC16A IgG-producing B cells via CD40–CD40L interaction was completed within 5 days after the adoptive transfer of immunized splenocytes. Notably, a single administration of MR1 at day 0 was enough to inhibit the production of anti-COL17 NC16A IgG and to diminish skin lesions despite the presence of restored anti-COL17 IgG at the later stage. In contrast, the delayed administration of MR1 failed to inhibit the production of anti-COL17 NC16A IgG and the development of the BP phenotype. These results

Abbreviations: BP, bullous pemphigoid; COL17, type XVII collagen; BMZ, basement membrane zone; NC16A, noncollagenous 16A domain; WT, wild type; hCOL17, human COL17; Tg, transgenic; CD40L, CD40 ligand; IF, immunofluorescence; OD, optimal density.
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1. Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering disorder. Clinically, tense blisters, erosions and crusts with itchy urticarial plaques and erythema develop on the entire body. Histologically, subepidermal blisters associated with inflammatory cell infiltration in the dermis are observed. BP is induced by autoantibodies against type XVII collagen (COL17, also called BP180 or BPAG2), a hemidesmosomal protein which spans the lamina lucida and projects into the lamina densa of the epidermal basement membrane zone (BMZ) [1–6]. The juxtamembranous noncollagenous 16A (NC16A) domain is considered to contain the main pathogenic epitopes on COL17, although BP patients’ sera can also react with other parts [7–9].

Recently, we developed a novel active BP mouse model by adoptively transferring wild-type (WT) splenocytes immunized by human COL17 (hCOL17)-expressing transgenic (Tg) skin-grafting into Rag-2−/−/COL17m−/−,h+ (Rag-2−/−/COL17-humanized) mice that express hCOL17 in the skin and lack both T and B cells [10]. The recipient mice accepted transferred splenocytes and produced high titers of anti-hCOL17 IgG in vivo for more than 10 weeks after the adoptive transfer, while circulating anti-hCOL17 NC16A IgG titer decreased in a short period for unknown reasons [10]. They developed blisters and erosions corresponding to clinical, histological and immunopathological features of BP [10]. This new active BP model enables us to observe the dynamic immune reactions induced by pathogenic antibodies against hCOL17 molecule.

In BP, the presence of autoreactive CD4+ T cells has been reported [11–13]. Particular MHC class II alleles occur more frequently in BP patients [14]. These findings indicated the contribution of CD4+ T cells to the pathogenesis of BP. Generally, the production of IgG by B cells requires the help of CD4+ T cells [15–17]. Our previous study demonstrated that CD4+ T cells were crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype because both the depletion of CD4+ T cells from immunized splenocytes, and the administration of cyclosporin A significantly suppressed the pathogenic IgG production and diminished the disease severity [10]. However, the pathogenicity of COL17 NC16A-reactive CD4+ T cells has never been elucidated. To address this issue, we modulated the CD4+ T cell function in active BP model by administering anti-CD40L monoclonal antibody MR1 [18] in various ways, and observed the phenotypic changes of the treated mice.

CD40 ligand (CD40L) is a costimulator molecule which is transiently expressed on the surface of activated CD4+ T cells and which binds to CD40 on antigen-presenting cells including B cells. CD40–CD40L interaction is crucial for the proliferation and differentiation of B cells into immunoglobulin-secreting plasma cells and for the formation of humoral memory [19]. Immunosuppressive effects of anti-CD40L monoclonal antibody have been shown in some T-cell-mediated antibody-induced autoimmune animal models, such as experimental autoimmune myasthenia gravis [20], and pemphigus vulgaris [21, 22]. In this study, we demonstrate that COL17 NC16A-reactive CD4+ T cells play a pivotal role in the development of BP through the CD40–CD40L interaction at an early stage of the disease in active BP model, which suggests that COL17 NC16A-reactive CD4+ T cell is a promising therapeutic target for BP.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from Clea Japan. Rag-2−/−/COL17m−/−,h+ mice which carry the homozygous null mutations of both the Rag-2 and mouse Col7 genes and the transgene of human COL17 were generated by crossing Rag-2−/− mice (C57BL/6 background) with COL17−/−,h+ (COL17-humanized) mice (C57BL/6 background) as described previously [10]. All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

2.2. Induction of active BP by adoptive transfer of immunized splenocytes

Immunization of WT mice by hCOL17-expressing Tg skin graft was performed according to the method reported previously [10, 23]. After the confirmation of anti-hCOL17 IgG production at 5 weeks after skin grafting by indirect immunofluorescence (IF) analysis using normal human skin, splenocytes were isolated and pooled from several Tg skin-grafted immunized WT mice and administered into Rag-2−/−/COL17-humanized mice by intravenous injection into the tail vein at 1.5–2.0 × 108 splenocytes in 500 μL PBS per mouse [10, 24].

2.3. Evaluation of active BP model mice

Weekly, the recipient mice were examined for general condition and cutaneous lesions (i.e., erythema, blisters, erosions, crusts and hair loss). Extent of skin disease was scored as follows: 0, no lesions; 1, lesions on less than 10% of the skin surface; 2, lesions on 10–20% of the skin surface; 3, lesions on 20–40% of the skin surface; 4, lesions on 40–60% of the skin surface; 5, lesions on more than 60% of the skin surface, as previously described [10]. Serum samples were also obtained from recipient mice weekly and assayed by indirect IF microscopy and hCOL17 NC16A ELISA as previously described [10]. The ELISA index value was defined by the following formula: index = (OD450 of tested serum − OD450 of negative control) / (OD450 of positive control − OD450 of
negative control) × 100 [10]. Biopsies of lesional skin were obtained for light microscopy (H&E), and for direct IF using FITC-conjugated antibody against mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and C3 (Cappel; Valeant Pharmaceuticals, Costa Mesa, CA).

2.4. Isolation of CD4+ T cells or CD45R+ B cells from splenocytes in mice

To examine the pathogenic role of CD4+ T cells in active BP model, we isolated CD4+ T cells from splenocytes of Tg skin-grafted WT mice by using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). 0.5 to 8×10^7 CD4+ T cells were mixed with 2.0×10^8 naive splenocytes from WT mice and adoptively transferred to Rag-2−/−/COL17-humanized mice. In another experiment, CD45R+ B cells were isolated from Tg skin-grafted WT mice by using CD45R MicroBeads (Miltenyi Biotec). 0.4×10^8 of CD45R+ B cells were transferred to Rag-2−/−/COL17-humanized mice. The isolation of CD4+ T cells and CD45R+ B cells was confirmed by flow cytometric analysis on FACSaria (BD Bioscience Pharmingen) using monoclonal antibodies purchased from BD Biosciences Pharmingen: H129.19-FITC (anti-CD4) and RA3-6B2-PE (anti-CD45R/B220).

2.5. In vivo monoclonal antibody treatment

Rag-2−/−/COL17-humanized recipients that were adoptively transferred with immunized splenocytes were intraperitoneally injected with 500 μg hamster monoclonal antibody MR1 specific to mouse CD40L (Taconic Farms, Hudson, NY) or an equivalent amount of control hamster IgG (Rockland Immunchemicals, Gilbertsville, PA) at days 0, 2 and 6 after the adoptive transfer of immunized splenocytes as previously described [21], with some minor modifications. In a delayed treatment experiment, MR1 was injected at days 13, 16 and 19 after the adoptive transfer. Some recipient mice were injected with 500 μg of MR1 just once on one of days 1 to 5 after the adoptive transfer, respectively. To investigate the immune responses in active BP model modulated by early single administration of MR1, 1000 μg of MR1 was injected into recipient mice at day 0 soon after the adoptive transfer. All treated mice were carefully observed for at least ten weeks after the adoptive transfer.

2.6. ELISPOT assay

ELISPOT assay was performed as previously described [10, 24]. Polyvinylidene-difluoride-bottomed 96-well multi-screen plates (Millipore) were coated with 30 μg/mL of recombinant hCOL17 NC16A protein. Splenocytes isolated from the Rag-2−/−/COL17-humanized recipients were incubated on the plate at 37 °C in a 5% CO2 incubator for 4 h. IgG bound to the membrane was visualized as spots, using alkaline-phosphatase-conjugated anti-mouse IgG antibody. The number of spots was counted using the ImmunoSpot 55 Versa Analyzer (Cellular Technology Ltd., Shaker Heights, OH), and the frequency of anti-hCOL17 NC16A IgG-producing B cells was defined as the number of spots in 10^5 mononuclear cells.

2.7. Statistical analysis

Data expressed as mean±standard error of means were analyzed using Student’s t-test. We considered P values of less than 0.05 as significant.

3. Results

3.1. CD4+ T cells are required for the production of pathogenic antibody in active BP model

We previously reported that CD4+ — but not CD8+ — T cells are crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype in active BP model [10]. To further analyze the contribution of CD4+ T cells, we additionally conducted two experiments. First, mixed transfer into Rag-2−/−/COL17-humanized mice of 4 or 8×10^7 CD4+ T cells from WT splenocytes immunized by hCOL17-expressing Tg skin-grafting and 2×10^8 naive splenocytes from unimmunized WT mice produced high titers of anti-hCOL17 NC16A IgG and severe BP skin changes associated with linear deposition of IgG at the BMZ. In contrast, reducing the number of CD4+ T cells (0.5×10^7) failed to produce such titers and skin changes (n=3, respectively; Fig. 1). Second, we isolated CD45R+ B cells from immunized splenocytes and adoptively transferred 0.4×10^8 of those cells into Rag-2−/−/COL17-humanized recipients (n=3), which produced quite low levels of anti-hCOL17 NC16A IgG (mean index value of ELISA at day 9: 3.28) and no skin changes (not shown). These results show that the production of anti-hCOL17 NC16A IgG by B cells and the development of BP skin changes in active BP model depend heavily on immunized CD4+ T cells.

3.2. Anti-CD40L monoclonal antibody suppresses the production of anti-hCOL17 IgG and skin changes in active BP model

To investigate the precise mechanism of the activation of B cells by immunized CD4+ T cells in active BP model, we assessed the role of CD40–CD40L interaction. Rag-2−/−/COL17-humanized recipients were injected intraperitoneally with 500 μg of monoclonal antibody MR1 specific to mouse CD40L or an equivalent dose of hamster IgG as a control on days 0, 2 and 6 after the adoptive transfer of immunized splenocytes (n=6, respectively). All the control Rag-2−/−/COL17-humanized recipients produced high titers of IgG against BMZ of normal human skin, which reflects the presence of anti-hCOL17 IgG, and those against hCOL17 NC16A, as previously reported [10]. In contrast, the production of those antibodies was almost completely inhibited in all the mice that were injected with MR1, and the inhibitory effect persisted for more than 10 weeks (Figs. 2A, B). The control mice developed patchy hair loss associated with erythema around day 14 after the adoptive transfer. Then, blisters and erosions spontaneously developed in the depilated areas on the trunk (Fig. 3A). Disease severity, scored by the percent of skin surface with the BP phenotype [10, 25], gradually increased, plateauing 7 weeks after the transfer in the control mice (Fig. 3G). In contrast, none of the MR1-
treated mice developed any skin lesions (Figs. 3D, G). Histo-
pathological analysis of the skin revealed the dermal–epidermal
separation that is associated with mild inflammatory cell infil-
tration in control mice (Fig. 3B), whereas there were no histo-
pathological changes in MR1-treated mice (Fig. 3E). Direct IF
analysis of lesional skin revealed linear deposition of IgG
(Fig. 3C) at the BMZ in the control mice, whereas IgG deposi-
tion was absent or faint in the MR1-treated mice (Fig. 3F).
We also examined the number of splenocytes which produced
anti-hCOL17 NC16A IgG by enzyme-linked immunospot assay
at day 9. In the control, 226.5±25.0 cells in 10⁵ splenocytes
produced anti-hCOL17 NC16A IgG, whereas only 9.0±3.0
cells in 10⁵ splenocytes produced them in the mice treated
with MR1 (n=3, respectively; Fig. 3H). Thus, preventive and
repetitive administration of MR1 can continuously suppress
the production of anti-hCOL17 IgG and skin changes in active
BP model.

3.3. Anti-CD40L monoclonal antibody shows no effects
in mice with established active BP

To examine the effect of MR1 in mice with producing IgG
against hCOL17 and hCOL17 NC16A, 500 μg of MR1 or the
equivalent dose of normal hamster IgG were administered
into active BP model at days 13, 16 and 19 after the adoptive
transfer of splenocytes (n =4, respectively). There were no
significant differences in the titers of anti-hCOL17 or anti-
hCOL17 NC16A IgG, nor in disease severity in both groups
at more than 10 weeks after the adoptive transfer (Fig. 4).

Figure 1  Immunized CD4⁺ T cells can activate naïve B cells to produce anti-hCOL17 NC16A IgG in vivo. (A) CD4⁺ T cells isolated from
WT splenocytes immunized by hCOL17-expressing Tg skin-grafting were mixed with naïve splenocytes from untreated WT mice, and
were adoptively transferred into Rag-2⁻/⁻/COL17-humanized mice (n=3, respectively). Mice transferred with 4 or 8x10⁷ immunized
CD4⁺ T cells mixed with naïve splenocytes produce significantly higher levels of anti-hCOL17 NC16A IgG than with 0.5x10⁷ CD4⁺ T
cells mixed with naïve splenocytes (P<0.05, **P<0.01). Mice transferred with 0.5x10⁷ of immunized CD4⁺ T cells and naïve spleno-
cytes show no skin changes (B) or deposition of IgG (C). In contrast, mice transferred with 8x10⁷ immunized CD4⁺ T cells and naïve
splenocytes develop severe BP skin changes (D) associated with linear deposition of IgG at the BMZ (E).

Figure 2  Anti-CD40L monoclonal antibody strongly suppresses the production of anti-hCOL17 and anti-hCOL17 NC16A IgG and in ac-
tive BP model. Rag-2⁻/⁻/COL17-humanized recipients were injected intraperitoneally with monoclonal antibody specific to mouse
CD40L (MR1) or the equivalent dose of control hamster IgG on day 0, 2 and 6 after the adoptive transfer of immunized splenocytes
(n=6, respectively). All the Rag-2⁻/⁻/COL17-humanized recipients that were injected with control IgG produce significantly high ti-
ters of IgG against hCOL17 (BMZ of normal human skin) and hCOL17 NC16A, while the production of those antibodies is almost
completely inhibited in all mice injected with MR1 (A, B) P<0.01 from day 9 to day 70 in both graphs.
These findings show that delayed administration of MR1 fails to diminish the disease activity in established active BP mice.

3.4. Activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is completed within five days after the adoptive transfer of immunized splenocytes

Since the delayed administration of MR1 failed to diminish the disease activity, we considered that the timing of T–B interaction via the CD40–CD40L pathway after the adoptive transfer needed to be elucidated. Single injections of 1000 μg of MR1 at days 1 to 5 after the adoptive transfer of immunized splenocytes into the Rag-2−/−/COL17-humanized recipients were administered (n = 4, respectively). Injection of MR1 at day 1, day 2 or day 3 strongly inhibited the production of anti-h COL17 NC16A IgG in recipients (Fig. 5A). The effects of MR1 successively decreased if the treatment was initiated at day 4 or day 5. Anti-hCOL17 NC16A IgG titer and disease severity of the recipients treated at day 5 were similar to those in active BP model without MR1 treatment (mean index value of anti-hCOL17 NC16A IgG at day 9: 765.3 vs. 918.97, P > 0.05; mean disease severity at day 35: 3.00 vs. 2.16, P > 0.05) (Figs. 2B, 3G and 5). Thus, the activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is completed within 5 days after the adoptive transfer of immunized splenocytes in active BP model.

3.5. Anti-hCOL17 IgG restored after the early single administration of anti-CD40L monoclonal antibody do not contain anti-hCOL17 NC16A IgG, and only weak pathogenicity is shown

The results above suggested that the early short-term effect of MR1 was sufficient to inhibit the production of anti-hCOL17 NC16A IgG. To observe the phenotypic changes in active BP model without the presence of anti-hCOL17 NC16A IgG, we induced the transient immunosuppressive condition in Rag-2−/−/COL17-humanized recipients by single injections of 1000 μg of MR1 at day 0 (n = 6). The production of anti-hCOL17 IgG in treated mice gradually recovered to levels similar to those in the control mice without MR1-treatment at 7 weeks after the adoptive transfer (Fig. 6A), but the restored IgG did not contain anti-hCOL17 NC16A IgG (Fig. 6B). The disease severity of the treated mice slowly increased but was significantly lower than that of the controls (Fig. 6C). Each of the IgG subclasses (IgG1, IgG2b, IgG2c, IgG3) against hCOL17 showed similar titers between an MR1-treated group and an untreated group at 10 weeks after the adoptive transfer (not shown). Although 3 out of 6 treated mice showed distinct deposition of C3, they...
developed only mild skin changes (Fig. 6D). Thus, anti-hCOL17 IgG restored after the transient blockade of CD40–CD40L interaction contain no anti-hCOL17 NC16A IgG and show only weak pathogenicity. This strongly suggests that hCOL17 NC16A-reactive CD4+ T cells play a crucial role in the development of BP lesions in active mouse model.

4. Discussion

This study has demonstrated the pivotal role of COL17 NC16A-reactive CD4+ T cells in BP induction for the first time by using active BP mouse model. We first demonstrated the pathogenic role of CD4+ T cells in active BP model by showing that CD4+ T cells immunized by hCOL17-expressing Tg-skin grafting could activate unimmunized B cells to produce anti-hCOL17 NC16A IgG. We also showed that immunized CD45R+ B cells needed the coexistence of activated CD4+ T cells to produce those IgG. These results suggest that the interaction between activated hCOL17-reactive T cells and B cells is essential for the production of anti-hCOL17 IgG. Administrations of anti-CD40L monoclonal antibody have previously demonstrated the strong suppression of humoral immune responses against autoantigens in some T-cell-mediated antibody-induced autoimmune animal models [20–22, 26]. Therefore, we considered that anti-CD40L monoclonal antibody may be utilized for the modulation of immune responses in active BP model.

Blockade of CD40–CD40L interaction by anti-CD40L monoclonal antibody (MR1) continuously suppressed the production of anti-hCOL17 NC16A IgG and the development of the BP phenotype in active BP model when MR1 was repetitively administered close to the time of adoptive transfer of immunized splenocytes. Although the production of anti-hCOL17 IgG detected by indirect IF study using normal human skin was not completely suppressed by MR1 treatment, ELISA revealed an absence of anti-hCOL17 NC16A IgG, resulting in the prevention of BP skin changes. Enzyme-linked immunospot assay demonstrated quite a small number of anti-hCOL17 NC16A IgG-producing B cells in the spleens of the MR1-treated mice.

Because the crucial role of B cell activation via CD40–CD40L interaction was elucidated at the initial stage of active BP model, we then tried to examine the effects of MR1 at the late stage of active BP model. Since the model starts to produce anti-hCOL17 and anti-hCOL17 NC16A IgG within a week after the adoptive transfer if no immunosuppressive treatment is added [10], we injected MR1 at days 13, 16

**Figure 4** Delayed treatment with anti-CD40L monoclonal antibody shows no effects in mice with established active BP. MR1 or control hamster IgG was injected into active BP model at days 13, 16 and 19 after the adoptive transfer of immunized splenocytes (n=4, respectively). There are no significant differences in the titers of anti-hCOL17 IgG (A) or anti-hCOL17 NC16A IgG (B), and in disease severity (C) between the groups. P > 0.05.
and 19 after the adoptive transfer (delayed treatment). No therapeutic effects were observed in mice with delayed treatment. This result indicates that the CD40–CD40L interaction is not required once the disease is established in active BP model. Similarly, delayed MR1-treatment was unable to suppress the titer of pathogenic antibody in an established pemphigus vulgaris model [21]. Meanwhile, delayed treatment can prevent relapses of ongoing diseases or can halt disease progression in models of multiple sclerosis [27], lupus nephritis [28, 29] and myasthenia gravis [20]. A possible mechanism of those therapeutic effects is the inhibition of epitope spreading. In experimental autoimmune encephalomyelitis, anti-CD40L monoclonal antibody treatment acts in part by inhibiting the expansion and/or differentiation of Th1 effector cells specific to relapse-associated epitopes [27]. Epitope spreading has also been reported in BP patients [30–32] and in an hCOL17-expressing Tg skin-grafting mouse model [33] although it is still unclear whether antibodies against hCOL17 – other than those against the NC16A domain – are pathogenic. Hence, the efficacy of anti-CD40L antibody treatment on epitope spreading in BP seems an interesting line of investigation.

Furthermore, we revealed that the activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is established within 5 days after the adoptive transfer of immunized splenocytes. *Rag-2<sup>−/−</sup>*/COL17-humanized recipients were injected with MR1 just once between days 1 and 5 after the adoptive transfer of immunized splenocytes (n = 4, respectively). (A) MR1-treatments at day 1, day 2 or day 3 significantly suppress the titers of anti-hCOL17 NC16A IgG at day 9 compared with those at day 5 (**P < 0.01). The effect of MR1 gradually decreases if the treatment is initiated late. The IgG titers at day 9 of the mice treated at day 5 are similar to those in active BP model without MR1 treatment (Fig. 2B) (mean index value: 765.3 vs. 918.97, P < 0.05). (B) Skin changes are strongly suppressed if MR1-treatment is initiated before day 3 after the adoptive transfer. Disease severity of the recipients treated at day 5 is similar to those in active BP model without MR1 treatment (Fig. 3G) (mean disease severity at day 35: 3.00 vs. 2.16, P > 0.05).

Figure 5  Activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is established within 5 days after the adoptive transfer of immunized splenocytes. *Rag-2<sup>−/−</sup>*/COL17-humanized recipients were injected with MR1 just once between days 1 and 5 after the adoptive transfer of immunized splenocytes (n = 4, respectively). (A) MR1-treatments at day 1, day 2 or day 3 significantly suppress the titers of anti-hCOL17 NC16A IgG at day 9 compared with those at day 5 (**P < 0.01). The effect of MR1 gradually decreases if the treatment is initiated late. The IgG titers at day 9 of the mice treated at day 5 are similar to those in active BP model without MR1 treatment (Fig. 2B) (mean index value: 765.3 vs. 918.97, P < 0.05). (B) Skin changes are strongly suppressed if MR1-treatment is initiated before day 3 after the adoptive transfer. Disease severity of the recipients treated at day 5 is similar to those in active BP model without MR1 treatment (Fig. 3G) (mean disease severity at day 35: 3.00 vs. 2.16, P > 0.05).
model. In conclusion, this study suggests that COL17 NC16A-reactive CD4^+ T cells play a pivotal role in the pathogenesis of active BP model via the CD40–CD40L interaction.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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