Original Article
Promotion of tumor immune escape by PI3K/Akt and MEK/ERK-mediated endothelial cells in colorectal cancer

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Abstract: This study aims to discuss the impact of PI3K/AKT and MEK/ERK signaling pathway on the promotion of tumor immune escape by colorectal cancer endothelial cells (CCVEC). SW480 freeze-thaw lysate is applied to load DC, to induce CTL with specific cytotoxicity of SW480. The co-culture system of SW480 human colorectal cancer cells and human umbilical vein endothelial cells HUVEC is constructed, and according to different intervening factors in the chamber above, the system can be divided into (1) SW480 and HUVEC co-culture group, (2) SW480 culture group, (3) DMSO group, (4) PI3K/AKT inhibitor LY294002 group: a final concentration of 10 nM and (5) MEK/ERK inhibitor PD98059 group: a final concentration of 20 nM. ELISA assay is used to test the influence of CTL tumor vascular endothelial cells on the secretion of IL-2 and INF-γ, FACS is used to detect the apoptosis rate of SW480, RT-qPCR and western blot is used to detect the expressions of B7-H1, FasL, TGF-β and COX-2 in SW480 cells. The results show colorectal cancer-associated endothelial cells can enhance the expressions of B7-H1, FasL, TGF-β and COX-2 in SW480 cells, and reduce the secretion of tumor-specific CTL cells, IL-2 and INF-γ and the destruction capability. Inhibiting the signals of the endothelial cells related to colorectal cancer, PI3K/AKT and MEK/ERK could reverse this phenomenon above. So it is concluded that CCVEC can promote tumor immune escape of colorectal cancer, and PI3K/AKT and MEK/ERK signals play an important role in this process.

Keywords: Colorectal cancer, PI3K/AKT, MEK/ERK

Introduction
As the No. 3 gastrointestinal cancer in the world, colorectal cancer has an increasing trend in incidence rate year by year, becoming a serious threat to human life and health disorder [1]. Although comprehensive therapy based on surgery, chemotherapy and radiotherapy lead to great progress in the treatment of colorectal cancer, the tumor metastasis and recurrence is still one of the important reasons for treatment failure and even death of patient [2, 3]. In recent years, studies have shown that tumor immune escape plays an important role in recurrence and metastasis of colorectal cancer, but the exact mechanism is not fully understood [4].

Tumor immune escape refers to that tumor cells evade surveillance and destruction of the immune system through multiple mechanisms, leading to the phenomenon of tumorigenesis and progression [5]. Cell immune plays a major role in the body's anti-tumor immune responses, and the generation of CD8+ CTL is the key for anti-tumor effectiveness. In the tissues of colorectal cancer, on the one hand, CD8+ tumor-infiltrating T lymphocytes in the tumor tissue can inhibit tumorigenesis and metastasis, and on the other hand, colorectal cancer affects CTL cells' proliferation, activation and destruction capabilities by high expressed TGF-β, FasL, PGE2 and other immunosuppressive molecules [4, 6]. Therefore, the inhibition of tumor immune escape will provide new means for colorectal cancer therapy [4].

Endothelial cells (EC) lining the body's circulatory system not only maintains the integrity of the blood vessels and lymphatic vessels, but also compose the costimulatory molecules of CD134L, ICOSL etc., stimulating the activation
of CD4+ memory T lymphocytes, promoting the secretion of Th1 and Th2 cytokines and regulating the immunity of body cells [7-9]. Studies have shown that tumor-associated endothelial cells may play an important role in the occurrence of tumor immunity exempt status [10-12]. The study of Berg et al. has shown that when the NK cell kills liver cancer cells, liver sinusoidal endothelial cells can induce the immune tolerance of specific CD8+T cells to promote tumor recurrence [10]. John et al. have found by the study in glioma that high-expressed FasL in tumor endothelial cells is negatively correlated with CD4+/CD8+TIL, and high-expressed FasL contributes to formation of immune exemption microenvironment in glioma [11]. Nummer finds in the exploration of the impact of endothelial cells of pancreatic cancer on Treg cells that, high-expressed address elements in endothelial cells can promote the aggregation of Treg cells and the formation of immune exemption status [12]. The above phenomenon indicates the presence of immune escape in endothelial cells of the tumor microenvironment.

As two major signal transduction pathways in Ras downstream, PI3K/AKT and MEK/ERK signals are involved in the regulation of gene expression, mediating the mitosis and inhibiting apoptosis [13]. At the same time, both the signals above not only regulate endothelial cell proliferation, migration and angiogenesis, but also play an important role in promoting tumor immune escape [14, 15]. However, whether there is tumor immune escape in endothelial cells related to colorectal cancer and whether PI3K/AKT and MEK/ERK signals mediate the process are still unknown, and therefore this study is to investigate these issues.

Materials and methods

Materials

C57 mice were purchased from Animal Center of Wuhan University, housed in SPF level; human colorectal cancer cell line SW480, and human umbilical vein endothelial cells HUVEC were saved in this room; Transwell chamber was purchased from Corning Corporation; DMEM/F12 medium, RPM1-1640 medium and fetal bovine serum were purchased from GIBIC company; murine IL-2, IL-4 and GM-CSF factors were purchased from Protech Company; PI3K/ AKT inhibitor LY294002 and MEK/ERK inhibitor were purchased from sigma company; murine IL-2 and INF-γ ELISA assay kit was purchased from Wuhan Boster Biotechnology Company; nylon wool column was purchased from Wako Company of Japan; flow apoptosis kit was purchased from Unitech Bio. BD flow cytometry, real-time quantitative fluorescence RT-PCR instrument was ABI7900, SYBR Green/Flourescein qPCR Master Mix was from Fermentas company, Ex TaqTM, DL2000 DNA Marker and DL15000 DNA Marker were from TAKARA company, each primer was synthesized by GenScript Biotechnology.

Cell culture

Human umbilical vein endothelial cell HUVEC, colorectal cancer cell line SW480 and SW480 human umbilical vein endothelial cell HUVEC were cultured by RPMI-1640 medium containing 10% FBS, and cultured in 37°C incubator with 5% CO₂. The medium was changed every two days, and passaging once every 3-4 days.

SW480 cell vaccine

SW480 cells were collected, washed twice with PBS; in serum-free RPMI-1640 medium resuspended cells, the density is 6 × 10⁶/ml, rewarming at 37°C after being frozen in liquid nitrogen, which was repeated three times, filtered by 0.2 μm filter and stored at -80°C standby.

Dendritic cells culture

Bilateral femur of mice is taken under sterile condition; needle washes marrow cavity; the cells were collected, lysed by erythrocyte lysis, and washed twice with PBS, added with RPMI-1640 medium containing 10% FBS to adjust the cell density to be 1 × 10⁷/ml. The RPMI-1640 medium containing 10 μg/ml GM-CSF and 5 μg/ml IL-4 was suspended in six-well plates, which was half changed every two days, cultured six days, and inverted microscope to see morphological change, the collected cell line FACS cytometry was used to detect the expression of DC surface marker, CD11c.

Mouse spleen-derived T lymphocytes culture

The above mouse spleen was taken, cut into pieces, milled, filtered by 200 mesh filter, red blood cell lysate was used to eliminate red
blood cells, 3 ml RPMI-1640 medium added, washed twice by centrifugation, monocytes separated by Ficoll-Hypaque, cell density was adjusted to $10^9$/ml; sterile nylon wool column was taken 5 ml syringe, 37°C preheating overnight, RPMI-1640 balance nylon wool column, the cell suspension was applied to the column, with constant temperature equilibrium 30 min, then eluted with RPMI-1640, 1.5 ml/min, the supernatant, the supernatant was centrifuged, and then the precipitate was mouse spleen-derived T lymphocyte.

**SW480 cell vaccine loading antigen**

The seventh-day DC cells were collected and cultured, the cell density was adjusted to be $2 \times 10^6$/ml. They were mixed according to the ratio DC:SW480 = 3:1, and incubated for 24 h at 37°C.

**SW480 cell specificity CTL**

T lymphocytes were taken, and the cell concentration was adjusted to $1 \times 10^6$/ml, seeded in six-well plates, with each well 1 ml, RPMI-1640 was added, containing IL-2 (500 u/ml), PHA (100 μg/ml) and 10% FBS, and then cultured for 5 days (final concentration), stimulated by DC cells loading SW480 antigen, and incubated by the ratio, lymphocytes:cancer vaccines = 20:1.

**Establishment of Transwell co-culture system**

According to the methodology in references, SW480 and HUVEC co-culture system is established, the cell densities of SW480 and pre-treated endothelial cells were adjusted to be $1 \times 10^7$/ml and $1 \times 10^5$, and the lower chamber was SW480 cell, 1 ml, the upper chamber was pre-processed or untreated HUVEC cells, 0.2 ml. Based on different groups of pretreated factors for endothelial cells, each set has three wells: (1) SW480 and HUVEC co-culture group; (2) independent culture group of SW480; (3) the upper chamber DMSO group; (4) PI3K/AKT inhibitor LY294002 group: a final concentration, 10 nM; (5) MEK/ERK inhibitor PD98059 groups: a final concentration of 20 nM. The upper chamber was removed 36 h after the co-culture, incubated for 18 h with the addition of CTL, and the target efficiency ratio = 1:50, the supernatant of the lower chamber and SW480 were collected for standby application.

**Detection of B7-H1, FasL, TGF-β and COX-2 mRNA expressions in SW480 cell by qRT-PCR**

PCR was amplified by ABI7900PCR instrument. The mRNA gene expressions of B7-H1, FasL, TGF-β and COX-2 and β-actin were taken as internal reference. The total PCR reaction system was 20 μl, SYBR Green/Flourescein qPCR Master Mix (2X) 10 μl, cDNA (10 × dilution) 4 μl, with 0.4 μl upstream and downstream primers respectively. Amplification procedure was as follows: B7-H1: 1 cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s; FasL: 1 cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s; TGF-β: cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s; COX-2: 1 cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. The expression amount of each gene was expressed by $2^{-\Delta\Delta Ct}$, where $[CT \text{(target gene)}-CT \text{(β-actin)}] \text{(experimental group)}-[CT \text{(target gene)}-CT \text{(β-actin)}] \text{(control group)}$. The primer of B7-H1, FasL, TGF-β and COX-2 were showed at Table 1.

**Detection of B7-H1 and FasL protein expressions in SW480 by western blot**

BCA assay was used to measure the protein concentration after the lysis of cell, SDS-PAGE electrophoresis was conducted after 20 μg denaturation, transferred to NC; TBS liquid of 5% nonfat dry milk was closed for 2 h, added with an anti-(1:1000) 4°C overnight, and ECL chemiluminescence could be seen after the membrane was washed 10 × 3 times by TBST; ray films were exposed and developed, and the relative expression of protein = A protein/β-actin.

### Table 1. The primer of B7-H1, FasL, TGF-β and COX-2

<table>
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<tr>
<th>Gene</th>
<th>Primer</th>
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<td>157</td>
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<tr>
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<td>R: 5'-TGCGTAAGTCTGTTCAGGTTG-3'</td>
<td>162</td>
</tr>
<tr>
<td>FasL</td>
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<tr>
<td>TGFB1</td>
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<td></td>
<td>R: 5'-GGGCGAAATGTCAATGACCG-3'</td>
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<tr>
<td>COX-2</td>
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<tr>
<td>β-actin</td>
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<td>176</td>
</tr>
<tr>
<td></td>
<td>R1663: 5'-GGGCGAAATGTCAATGACCG-3'</td>
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Figure 1. Culture and identification of dendritic cells.

Figure 2. The influence of endothelial cells associated with colorectal cancer on the expressions of B7-H1 (A) and FasL (B) mRNA expressions in SW480. SW480, SW480 group; SW480 + EC, SW480 + EC group; DMSO, SW480 + DMSO group; LY294002, SW480 + EC + LY294002 group; PD98059, SW480 + EC + PD98059 group. **P < 0.01 compared with SW480 group, ***P < 0.01 compared with SW480 + EC group.

Figure 3. Detection of protein expression of B7-H1 (A) and FasL (C) in SW480 by western blot, and statistical analysis of B7-H1 (B) and FasL (D) expression. SW480, SW480 group; SW480 + EC, SW480 + EC group; DMSO, SW480 + DMSO group; LY294002, SW480 + EC + LY294002 group; PD98059, SW480 + EC + PD98059 group. **P < 0.01 compared with SW480 group, ***P < 0.01 compared with SW480 + EC group.
Detection of the lower chamber IL-2 and the INF-γ secretion by ELISA

The cell suspension of lower chamber was taken and centrifuged, with the supernatant for standby application. The sample tubes of different concentrations were added to the corresponding reacting wells, at 37°C for 90 min, biotin-labeled by IL-2 and INF-γ antibody, the reacting at 37°C for 60 min, 0.01 M TBS and washed × 3, and then ABC was added; at 37°C for 30 min, 0.01 M, washed TBS × 5, reacting TMB 37°C × 30 min, and then TMB stop solution was added, reading.

Detection of SW480 cell apoptosis by Annexin V-FICT/PI assay

The EDTA-free trypsin was used for the digestion of lower chamber cells, centrifuged 2000 rpm × 5 min, PBS wash × 2, 200 μl × Binding Buffer resuspended cells, the density adjusted to be 1 × 10⁶/ml, 2 μl Annexin V added, jiggle mixing, and incubated for 15 min at 4°C dark condition; 4 μl PI was added for jiggle mix, incubated on ice for 15 min at 4°C dark, and the machine testing was conducted within 1 h.

Caspase-3 activation assay in SW480 cell

SW480 cell were seeded (1.5 × 10⁶ cells) well in 96-well plates. Briefly, caspase-3 activation was measured by Caspase-3 Activity Assay Kit (Sangong Biotech, Shanghai, China). 10 μL protein cell lysate per sample were added 80 μL reaction buffer with 10 μL substrate (Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) and incubated at 37°C for 4-6 h. Caspase-3 activation was measured with a Microplate Reader at an absorbance of 405 nm.

Statistical analysis

SPSS 18.0 statistical software was applied for statistical analysis. The results were expressed by mean ± standard deviation; student t test was used to compare the differences of groups, and the difference of P ≤ 0.05 was statistically significant.

Results

Culture and identification of dendritic cells

Mouse bone marrow cells were cultured for three days, showing that small colony forming; on the fifth day, cell colonies grew up and some cells grew in suspension. Under the microscope, the cells showed different lengths, flat cytoplasmic projections around the cell body, with irregular branch-like protrusions and folds. Flow cytometry test showed that the CD11c expression rate in cell surface was 91.7 ± 1.5%, as seen in Figure 1.

Detection of SW480 cell apoptosis by Annexin V-FICT/PI assay

The cell suspension of lower chamber was taken and centrifuged, with the supernatant for standby application. The sample tubes of different concentrations were added to the corresponding reacting wells, at 37°C for 90 min, biotin-labeled by IL-2 and INF-γ antibody, the reacting at 37°C for 60 min, 0.01 M TBS and washed × 3, and then ABC was added; at 37°C for 30 min, 0.01 M, washed TBS × 5, reacting TMB 37°C × 30 min, and then TMB stop solution was added, reading.

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The influence of endothelial cells associated with colorectal cancer on the expressions of B7-H1 and FasL mRNA expressions in SW480

The tumor microenvironment is composed of various cells, extracellular matrix and various immune molecules, which provides suitable conditions for tumor occurrence and development. As for the SW480 group, the endothelial cells related to colorectal cancer can promote the mRNA expressions of B7-H1 and FasL in SW480 cell in lower chamber, and inhibiting the signaling pathways of endothelial cells related to colorectal cancer, PI3K/AKT and MEK/ERK can reduce the transcription level of the molecules described above (Figure 2). The mRNA expressions of B7-H1 and FasL in SW480 + EC group were higher than those of SW480 group or DMOS group (Figure 2). Certainly, the mRNA expressions of B7-H1 and FasL were effectively suppressed in LY294002 group or PD98059 group, compared with the SW480 + EC group (Figure 2).

Detection of protein expression of B7-H1 and FasL in SW480 by western blot

The costimulatory molecules B7-H1 and FasL in the tumor microenvironment have the functions such as directly inducing tumor-associated CTL apoptosis and promote tumor immune escape. Therefore, we examine the FasL and B7-H1 expressions in SW480 cells. The results show that the inhibition of signaling pathways of endothelial cells related to colorectal cancer, PI3K/AKT and MEK/ERK by LY294002 and PD98059 can reduce the promotion of B7-H1 and FasL expressions in SW480 cells (Figure 3). As compared with those of SW480 group or DMOS group, the protein expression of B7-H1 and FasL in were effectively increased in SW480 + EC group. As expected, these protein expressions were effectively suppressed LY294002 group or PD98059 group, compared with the SW480 + EC group (Figure 3).

The influence of endothelial cells associated with colorectal cancer on the expressions of TGF-β mRNA in SW480

Our study reported that the mRNA expression of TGF-β was markedly activated in SW480 + EC group, compared with those of SW480 group or DMOS group (Figure 4). However, LY294002 or PD98059 could markedly suppress the TGF-β mRNA expressions in SW480 cell, compared with the SW480 + EC group (Figure 4).

The influence of endothelial cells associated with colorectal cancer on the expressions of COX-2 mRNA in SW480

Compared with SW480 group or DMOS group, the expressions of COX-2 mRNA was significantly induced in SW480 + EC group (Figure 5). However, EC significantly attenuate the increase in the expressions of COX-2 mRNA, compared with the SW480 + EC group (Figure 5).

The influence of the endothelial cells related to colorectal cancer on IL-2 and INF-γ secretions of CTL cells

In order to study the impact of endothelial cells related to colorectal cancer on CTL cells, we detect the change in secretions of cytokines IL-2 and INF-γ that are related to CTL cell activation and killer function. As for the independent culture group SW480, IL-2 and INF-γ secretions in CTL cells are reduced significantly in the lower chamber under the co-culture group of SW480 and HUVEC (Figure 6). From the inhibi-

![Figure 6. The influence of the endothelial cells related to colorectal cancer on IL-2 (A) and INF-γ (B) secretions of CTL cells. SW480, SW480 group; SW480 + EC, SW480 + EC group; DMSO, SW480 + DMSO group; LY294002, SW480 + EC + LY294002 group; PD98059, SW480 + EC + PD98059 group. **P < 0.01 compared with SW480 group, ***P < 0.01 compared with SW480 + EC group.](image-url)
PI3K/Akt and MEK/ERK

Figure 7. The influence of the endothelial cells associated with colorectal cancer on SW480 cell apoptosis (A) and statistical analysis of apoptosis rate. SW480, SW480 group; SW480 + EC, SW480 + EC group; DMSO, SW480 + DMSO group; LY294002, SW480 + EC + LY294002 group; PD98059, SW480 + EC + PD98059 group. **P < 0.01 compared with SW480 group, ***P < 0.01 compared with SW480 + EC group.
PI3K/Akt and MEK/ERK

The influence of PI3K/AKT and MEK/ERK signaling pathways in the endothelial cells related to colorectal cancer by LY294002 and PD98059, we have found a significant increase in IL-2 and INF-γ secretions in CTL cells (Figure 6).

The influence of the endothelial cells associated with colorectal cancer on SW480 cell apoptosis

FACS test shows DC-induced CTL loading SW480 cell lysates has a strong anti-tumor capability, and the endothelial cells associated with colorectal cancer can inhibit the killing of tumor-specific CTL on SW480 and the inhibition of PI3K/AKT and MEK/ERK signal in endothelial cells can enhance CTL killing on SW480 in co-culture system (Figure 7). It was evident from the results that cell apoptosis were memorably suppressed in SW480 + EC group, compared with SW480 group or DMOS group (Figure 7). The cell apoptosis was significantly enhanced by LY294002 and PD98059, compared with the SW480 + EC group (Figure 7).

The influence of the endothelial cells associated with colorectal cancer on caspase-3 activation in SW480 cell

Similarly, Figure 8 showed that caspase-3 activation was significantly inhibited in SW480 + EC group, compared with SW480 group or DMOS group. Interestingly, the caspase-3 activation was significantly induced by LY294002 and PD98059, compared with the SW480 + EC group (Figure 8).

Discussion

More and more evidence shows that tumor cells in the tumor microenvironment can be expressed or secreted; for example, Fas, B7-H1, TGF-β and COX-2 and other immuno-suppressive molecules influence the tumor-specific CTL cell killing in colorectal cancer [16]. Mesenchymal components, such as fibroblasts, endothelial cells, inflammatory cells and macrophages also played an important role in the tumor immune escape process, and the interaction between them constitutes the immune exemptions necessary for tumor formation and progression [17]. Although a large number of experiments have confirmed the presence of tumor immune escape phenomenon, the exact mechanisms such as the role of tumor stroma cells in tumor immunity escape has not been fully elucidated.

This study shows that, the endothelial cells related to colorectal cancer can promote the expressions of SW480 cell B7-H1, FasL, TGF-β and COX-2 [18]. High expressed B7-H1 in colorectal cancer can induce apoptosis of tumor-specific CTL and promote the aggregation of Treg cells in the foci [19]. However, the expressed FasL can induce TIL apoptosis, reduce the aggregation of TIL in the foci [20]. Tumor-derived TGF-β and COX-2 can inhibit the activation of T and promote the generation of CD4+ CD25+ Treg cells, recruiting MDSC into the nests [21, 22]. This suggests that the endothelial cells in tumor microenvironment may be involved in the induction of immune exemption status of colorectal cancer.

The balance between Th1/Th2 cell-associated factors is an important factor affecting the body’s anti-tumor immunity, in which the cytokines such as IL-2 and INF-γ secreted by Th1 cells play a predominant role in tumor cell immunity [23]. We have found that endothelial cells related to colorectal cancer can reduce the IL-2 and INF-γ secreting capability of DC-induced CTL cell that loads SW480 cell lysates and the endothelial cells related to colorectal cancer can inhibit tumor-specific CTL
killing on SW480. The endothelial cells related to colorectal cancer in the micro-environment tumor endow the tumor cells with the feature of inhibiting CTL activation and killing, resulting in immune escape phenomenon.

PI3K/AKT and MEK/ERK signals play a role in the processes of endothelial cell proliferation, migration and tumor angiogenesis [24]. The specific inhibitor is used in this study to block the signal above in endothelial cells, which may reduce the expressing abilities of endothelial cells related to colorectal cancer for tumor cells B7-H1, FasL, TGF-β and COX-2 and other immunosuppressive molecules. At the same time, the inhibitions of the endothelial cells signals related to colorectal cancer can promote the secretion of IL-2 and INF-γ by tumor-specific CTL cells, and the endothelial cells related to colorectal cancer can inhibit tumor-specific CTL killing on SW480. It is obvious that PI3/AKT and MEK/ERK signal mediates the promotion of the endothelial cells in colorectal cancer in tumor immune escape.

In summary, the present results show that endothelial cells related to colorectal cancer are involved in the immune escape of colorectal cancer tumor, and PI3/AKT and MEK/ERK signals play an important role in the processes above. The inhibition of endothelial cells signals related to colorectal cancer, PI3/AKT and MEK/ERK, may improve tumor immune microenvironment, and improve the anti-tumor immune therapy.

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Disclosure of conflict of interest

None.

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