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# Cutting Edge: Enhanced IL-2 Signaling Can Convert Self-Specific T Cell Response from Tolerance to Autoimmunity[1]

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CUTTING EDGE

# Cutting Edge: Enhanced IL-2 Signaling Can Convert Self-Specific T Cell Response from Tolerance to Autoimmunity[1]

*Jason Waithman,[2]* Thomas Gebhardt,[2]* Gayle M. Davey,[†] William R. Heath,[†] and Francis R. Carbone[3]**

***Naive and memory T cells show differences in their response to antigenic stimulation. We examined whether this difference extended to the peripheral deletion of T cells reactive to self-Ag or, alternatively, the induction of autoimmunity. Our results show that although both populations where susceptible to deletion, memory T cells, but not naive T cells, also gave rise to autoimmunity after in vivo presentation of skin-derived self-Ags. The same migratory dendritic cells presented self-Ag to both naive and memory T cell populations, but only the latter had significant levels of the effector molecule granzyme B. Memory T cells also expressed increased levels of the high affinity IL-2 receptor chain after self-Ag recognition. Provision of IL-2 signaling using a stimulatory complex of anti-IL-2 Ab and IL-2 drove the otherwise tolerant naive T cells toward an autoimmune response. Therefore, enhanced IL-2 signaling can act as a major selector between tolerance and autoimmunity. The Journal of Immunology,* 2008, 180: 5789–5793.**

Activation of naive T cells can lead to one of two outcomes: either immunity or tolerance. Ag-experienced or memory T cells differ from their naive counterparts in their ability to respond to antigenic stimulation. These memory cells show rapid effector elaboration upon Ag re-encounter and, as a consequence, they are more effective at pathogen control (1–5). However, such enhanced responsiveness would also suggest that the memory population would inherently be more potent at inducing autoimmune damage in the case of self-Ag recognition. Nonetheless, naive and memory type T cells share a commonality in their susceptibility to tolerization (6, 7). Thus, both naive and memory T cells are deleted after exposure to self-Ag, suggesting that continuous Ag expression is the key to tolerance induction (8). Although the ultimate tolerization of both types of cells is not in dispute, the original experiments involved quite low T cell numbers that could have hidden differences between the naive and memory populations. As a consequence, we have re-examined peripheral T cell tolerance using T cell numbers that permit tracking of the responding self-Ag specific naive and memory populations. Our studies show that while both naive and memory T cells are susceptible to peripheral tolerance, the latter can induce autoimmunity and such a pathologic response can be controlled, in part, by IL-2 signaling.

## Materials and Methods

### *Mice and virus infections*

C57BL/6J (B6),[4] B6.SJL-Ptprc$^a$Pep3$^b$/BoyJ (B6.Ly5.1) × B6.(bm1)-TgN(TcrOT-I)243–2wehi (OT-I), B6.(bm1)-TgN(RIP-OVA)HL59–2Kwehi (RIP.OVA$^{high}$), K5.mOVA (9), and B6.C-H-2$^{bm-1}$ (bm1) × K5.mOVA mice were bred and maintained at the Department of Microbiology and Immunology, University of Melbourne or the Walter and Eliza Hall Institute of Medical Research, Parkville, Australia. Mice were inoculated with 500 PFU of WSN-OVA via s.c. foothock infection. Chimeric mice were generated as previously described (10).

### *Monoclonal Abs and CFSE labeling*

Cells were labeled with CFSE as described previously (10). The following Abs were used for flow cytometry: anti-mouse CD45.1-allophycocyanin (clone A20) or FITC (clone A20); anti-mouse CD8α-allophycocyanin or PECy-7 (clone 53-6.7); anti-mouse Vα2-PE (clone B20.1); anti-mouse CD44-FITC (clone IM7); anti-mouse CD69-PE (clone H1.2F3); anti-mouse CD62L-FITC (clone MEL-14); anti-mouse CD25-PE (clone PC61); anti-human granzyme B (clone GB12); anti-mouse CD43-PE (clone 1B11); anti-mouse H-2K$^b$-FITC (clone 5F1); anti-mouse CD11c-FITC (clone N418); and anti-mouse CD205-allophycocyanin (clone NLDC-145).

### *T cell preparation*

Memory OT-I cells were generated as previously described (6). Single-cell suspensions prepared from lymph nodes (LNs) (axillary, brachial, inguinal, cervical, and mesenteric) were incubated for 30 min with the following purified mAbs: anti-Mac-1α (clone M1/70); anti-F4/80 (clone F4/80); anti-erythrocyte (clone TER-119); anti-GR-1 (clone RB68C5); anti-I-A/I-E (clone M5114); and anti-CD4 (clone GK1.5). The Ab-coated cells were then removed by incubation with goat anti-rat IgG-coupled magnetic beads. Memory OT-I T cells were purified in the same manner from B6 mice that had at least 3 wk earlier

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[4] Abbreviations used in this paper: B6, C57BL/6J; DC, dendritic cell; LN, lymph node.

received 20–25 × $10^6$ effector OT-I × B6.Ly5.1 T cells. For experiments involving $CD8^+$ T cell stimulation by DCs in vitro, both naive and memory OT-I × B6.Ly5.1 T cells were purified by flow cytometry after labeling with anti-mouse CD45.1.

*Enumeration of naive and memory OT-I T cells isolated from the skin*

For analysis of $CD8^+$ T cells in the skin, mice were sacrificed and subsequently perfused with 10 ml HBSS. A 1-$cm^2$ area of skin was removed, chopped into small fragments, and incubated for 90 min at 37°C in Dulbecco's PBS containing collagenase (3 mg/ml) and DNase (5 $\mu$g/ml). Thereafter, the cell suspension was filtered through a nylon mesh and stained for CD8 and CD45.1 expression and analyzed by flow cytometry.

*Coculture of DC subpopulations with CFSE-labeled naive or memory OT-I T cells*

DCs were isolated as described previously (10). Serial dilutions starting at 2.5 × $10^4$ of each DC population were cocultured in vitro with 5 × $10^4$ CFSE-labeled naive or memory OT-I × B6.Ly5.1 T cells. Proliferation was measured as a loss of CFSE staining of $CD8^+V\alpha2^+PI^-$ cells (where PI is propidium iodide) as determined by flow cytometric analysis after 60 h of culture.

*Analysis of T cell deletion*

Naive or memory OT-I × B6.Ly5.1 T cells (5 × $10^6$) were adoptively transferred into recipient chimeric mice and after 6 wk the mice were analyzed. Cells pooled from the axillary, brachial, inguinal, cervical, and mesenteric LNs and splenocytes of each recipient mouse were stained with anti-mouse Ly5.1, V$\alpha$2, and CD8$\alpha$ and analyzed by flow cytometry with propidium iodide used to exclude dead cells. A known number of small nonfluorescent Sphero beads (BD Biosciences) were added to each sample to enable the number of cells to be determined.

*Treatment with IL-2-anti-IL-2 complexes*

IL-2-anti-IL-2 complexes were prepared by incubation (37°C for 30 min) of recombinant human IL-2 (Peprotech) with anti-human IL-2 Ab (MAB602, clone 5355; R&D Systems). Two hundred microliters of complexes containing 1.5 $\mu$g of IL-2 and 10 $\mu$g of an anti-IL-2 Ab per mouse were injected at days 0, 1, and 3 following the transfer of naive OT-I T cells into K5.mOVA and B6 mice, respectively.

## Results and Discussion

Although Ag-experienced or memory T cells can undergo peripheral tolerization, it remains unclear whether these cells are less susceptible to tolerance than their naive counterparts. To determine this, we used an in vivo system where a neo-self-Ag (a membrane form of OVA) was expressed in the skin under the control of the keratin-5 promoter. K5.mOVA mice make OVA in keratinocytes and drive proliferation followed by deletional tolerance of transferred naive OVA-specific transgenic T cells (9, 10). To examine memory responses, a population of memory OT-I T cells was produced by primary stimulation of $Ly5.1^+$ OT-I T cells in vitro with syngeneic $OVA_{257–264}$-pulsed splenocytes. After 4 days in culture, the cells were transferred into B6.Ly5.2 recipients, which were rested for 3 wk to permit development of memory $CD8^+$ OT-I T cells. These memory $CD8^+$ T cells were phenotypically characterized and compared with the endogenous repertoire of the recipient (Fig. 1*A*). The cells had a central memory rather than effector memory phenotype, with high level expression of CD44 and CD62L and low expression of activation markers CD69 and CD25.

Transfer of either naive or memory T cells into K5.mOVA mice resulted in a similar level of T cell proliferation (Fig. 1*B*). Moreover, when transferred into K5.mOVA mice the memory OT-I T cells were actively tolerized (Fig. 1*C*), as were their naive counterparts (10). In these experiments, graded numbers of memory OT-I T cells were transferred into either K5.mOVA or B6 recipients and the OT-I T cell expansion was measured 4 wk later as a proportion of total $CD8^+$ T cell population after infection with a recombinant influenza virus expressing the OVA

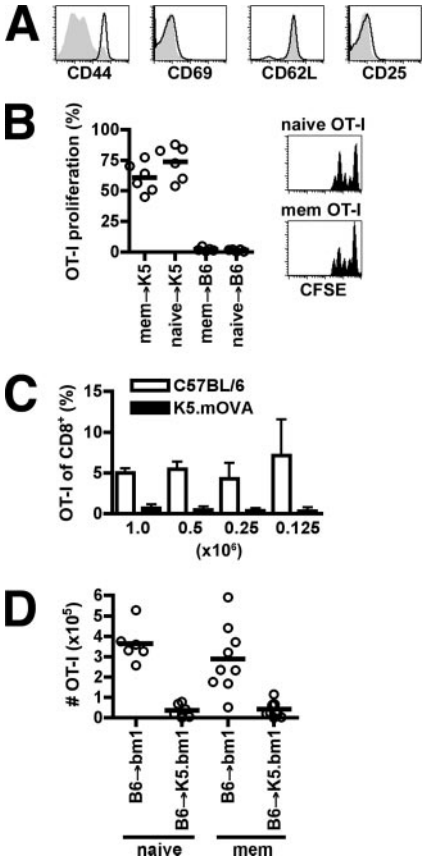


**FIGURE 1.** Peripheral deletion of naive and memory OT-I T cells in K5.mOVA mice. *A*, Flow cytometric analysis of $CD8^+CD45.1^-$ endogenous (gray histograms) and $CD8^+CD45.1^+$ OT-I (open histograms) T cells for their expression of CD44, CD69, CD62L, and CD25. *B*, CFSE-labeled naive or memory (mem) OT-I × Ly5.1 cells (1.5 × $10^6$) were adoptively transferred into K5.mOVA or B6 recipients and skin draining LN cells were analyzed after 42 h. *C*, Memory OT-I × Ly5.1 cells were transferred into K5.mOVA or B6 recipient mice. Four weeks later, recipients were immunized with recombinant influenza-OVA virus and the expansion of OT-I cells was determined using flow cytometry after a further 7 days. *D*, Naive or memory OT-I × Ly5.1 cells (5 × $10^6$) were adoptively transferred into B6→bm1 and B6→K5.mOVA × bm1 chimeric mice. The number of OT-I T cells in the LNs and spleen was determined by flow cytometry after six weeks. Individuals (open circles) and means (horizontal bars) are shown.

determinant. Elimination of transferred OT-I T cells was observed when presentation was limited to that from the bone marrow compartment, as exists in K5.mOVA × bm1 recipients of B6 bone marrow (Fig. 1*D*), arguing that T cell loss is not dependent on Ag recognition of the expressing keratinocytes. Thus, consistent with previous reports (6, 7), we found that both naive and memory T cells were susceptible to tolerance induction via a deletional mechanism effectively driven by bone marrow-derived APCs.

Memory cells have been shown to express higher levels of effector cell molecules upon Ag encounter (3–5). Consistent with this, we found that 7 days after transfer, Ag-experienced memory OT-I T cells in both the LNs and spleen had increased expression of the effector molecule granzyme B compared with naive T cell counterparts (Fig. 2*A*). Importantly, the detection of these effectors in the spleen contrasts to what is seen in the peripheral tolerance system used by Sherman and colleagues where deletion takes place during the first few cycles of cell division and is totally confined to the draining LNs (6, 11, 12).

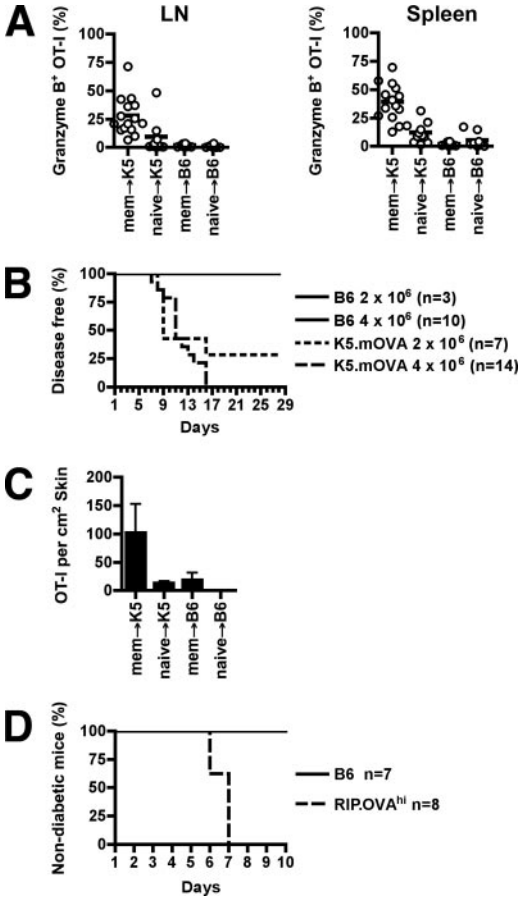


**FIGURE 2.** Memory OT-I T cells acquire effector function and cause autoimmune skin disease in K5.mOVA mice. *A*, Naive or memory OT-I × Ly5.1 cells ($1.5 \times 10^6$) were transferred into K5.mOVA or B6 recipients and 7 days later cells in skin-draining LNs were analyzed by flow cytometry for expression of granzyme B in $CD8^+CD45.1^+$ cells. Individuals (open circles) and means (horizontal bars) are shown. *B*, Memory OT-I T cells ($2$ or $4 \times 10^6$) were transferred into either K5.mOVA or B6 recipients and the percentage of mice showing no signs of disease (weight loss or skin lesions) is indicated. *C*, Naive or memory (mem) OT-I × Ly5.1 cells ($1.5 \times 10^6$) were adoptively transferred into K5.mOVA or B6 recipients and the $CD8^+CD45.1^+$ T cells contained in 1 $cm^2$ of skin after 7 days were enumerated by flow cytometry. *D*, Memory OT-I T cells ($4 \times 10^6$) were adoptively transferred into RIP.$OVA^{high}$ or B6 recipients. RIP.$OVA^{high}$ mice express secreted OVA in the $\beta$ cells of the pancreas under the control of the rat insulin promoter (RIP). All recipients were monitored daily for induction of diabetes as measured by glucosuria.

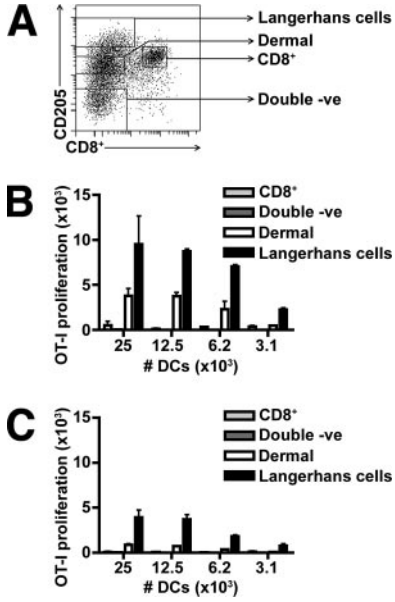


**FIGURE 3.** Skin-derived self-Ag is presented to naive and memory OT-I T cells by DCs of skin origin. *A*, $CD11c^+$ DCs from the skin-draining LNs of K5.mOVA mice were flow cytometrically sorted into $CD8^-CD205^{high}$ Langerhans cells, $CD8^-CD205^{int}$ dermal DCs (where int is "intermediate"), $CD8^-CD205^-$ double negative (−ve) DCs, and $CD8^+CD205^{int}$ DCs. *B* and *C*, Purified DC subsets were cocultured with CFSE-labeled naïve (*B*) or memory (*C*) OT-I T cells for 60 h and the $CD8^+V\alpha2^+$ OT-I T cells were analyzed by flow cytometry. Data are pooled from two independent experiments showing the mean ± SEM.

Thus, although memory OT-I T cells are ultimately deleted in the K5.mOVA mice, this release of T cells into the wider circulation, coupled with the enhanced effector response, has the potential to result in autoimmune disease. This was indeed found to be the case. Transfer of memory OT-I T cells resulted in disease as measured by the appearance of skin lesions and loss of weight (Fig. 2*B*). In addition, transfer of the memory OT-I T cells resulted in significantly more cells in the skin compared with the naive OT-I controls, consistent with the appearance of lesions in these animals (Fig. 2*C*). This contrasts with the lack of any overt autoimmunity with transferred naive OT-I T cells into K5.mOVA recipients (9, 10). Finally, differential disease induction was not confined to the K5.mOVA system. Transferred Ag-experienced OT-I T cells caused diabetes in RIP.$OVA^{high}$ mice (13) (Fig. 2*D*), which express OVA in the pancreatic islets and were otherwise resistant to disease when given naive OT-I T cells (14). Combined, these results show that, despite their susceptibility to deletional tolerance, memory T cells are nonetheless more autoaggressive compared with their naive precursors.

One possible reason for the differential disease outcome observed with naive vs memory T cells may be an altered stimulation by different types of APCs. It is now clear that dendritic cells (DCs) are a heterogeneous population with only certain subsets operating in any given response and that some may prove more potent at stimulating autoimmune responses (15). Thus, it was a possibility that the naive T cells were being stimulated by a different DC population than that used to activate the memory population. We have shown previously that in the K5.mOVA mice self-Ag is presented to naive T cells by skin-derived DCs and not by lymphoid-resident cells such as the $CD8^+$ DCs (10). To determine whether this was true in the case of presentation to the Ag-experienced T cells, we fractionated DCs in skin draining LNs from K5.mOVA mice using CD205 vs CD8 expression (Fig. 3*A*) and then tested the ability of the purified subsets to stimulate OT-I T cells in vitro. The results in Fig. 3, *B* and *C*, show that naive and Ag-experienced T cells have a similar pattern of response to the different subsets isolated by this approach, with the majority of presentation falling within the skin-derived DC populations. Of note, skin migrants showed a somewhat weaker stimulation of the memory population (Fig. 3*C*) compared with naive cells (Fig. 3*B*), something that is found in other systems (16).

Comparison of the disease-causing memory cells with their naive counterparts showed that the former had higher level expression of the high-affinity IL-2 receptor subunit CD25 when transferred into K5.mOVA mice (Fig. 4*A*). Given this, we

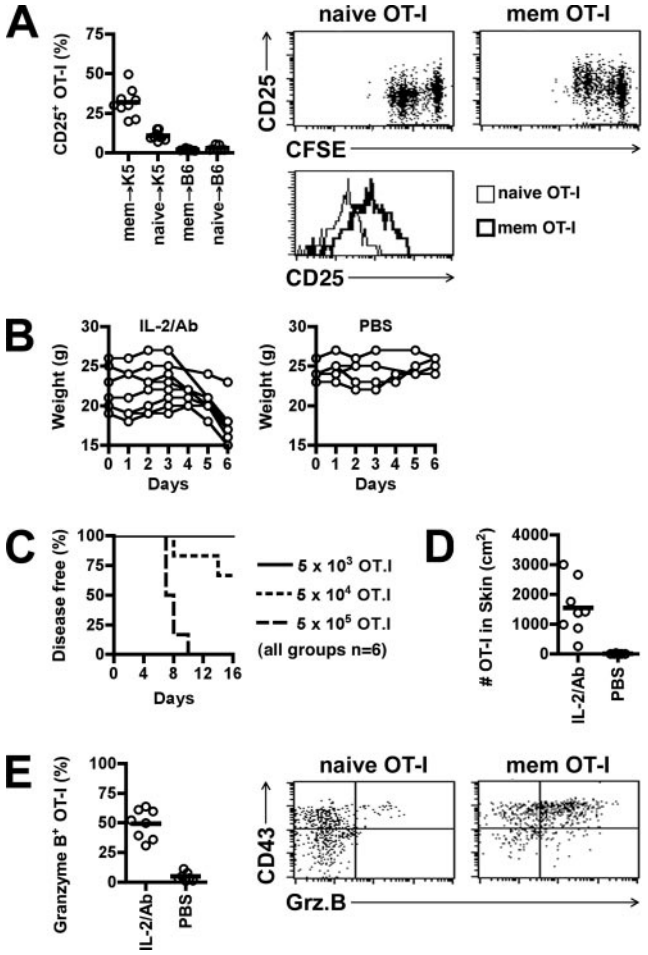


**FIGURE 4.** Enhanced IL-2 signaling induces autoimmunity. *A*, Naive or memory (mem) OT-I × Ly5.1 cells ($1.5 \times 10^6$) were transferred into K5.mOVA or B6 recipients. After 7 days, $CD8^+CD45.1^+$ T cells in skin-draining LNs were analyzed for expression of CD25. The histogram shown is gated on proliferating cells. *B–E*, Unless otherwise stated, $4 \times 10^6$ naive OT-I × Ly5.1 cells were adoptively transferred into K5.mOVA recipients on day −1. The mice were given either PBS or IL-2-anti-IL-2 complexes on days 0, 1, and 3. *B*, Shown is the weight of individual (open circles) mice. *C*, Disease incidence after transfer of decreasing numbers of OT-I T cells following complex treatment. *D*, After 6 days, the $CD8^+CD45.1^+$ T cells contained in 1 $cm^2$ of skin were enumerated. *E*, After 6 days, the CD43 and granzyme B (Grz.B) expression was determined for $CD8^+CD45.1^+$ splenic T cells. Individuals (open circles) and means (horizontal bars) are shown.

thought it possible that IL-2 signaling may prove critical to the induction of autoimmunity. It has recently been shown that anti-IL-2 Ab complexes can provide an intense form of signaling that bypasses the high affinity IL-2 receptor (17). We were therefore interested in determining whether this same Ab-cytokine complex would also result in converting the tolerance seen with the transfer of naive T cells into an autoimmune response. To this end, naive T cells were transferred into the K5.mOVA mice in addition to the complex of IL-2 and anti-IL-2 Ab. The combination of naive T cells and Ab-cytokine complex resulted in drastic weight reduction in contrast to the transfer of naive T cells alone (Fig. 4*B*). Autoimmunity developed in some animals following adoptive transfer of even low numbers of OT-I T cells (Fig. 4*C*). In addition, OT-I T cells infiltrated the skin (Fig. 4*D*) and had a markedly increased expression of CD43 and the effector molecule granzyme B (Fig. 4*E*). Autoimmunity was not evident in the absence of transfer of self-reactive OT-I T cells (data not shown).

Our results show that memory T cells mount a more aggressive response to self-Ag than their naive counterparts, with enhanced effector function combined with autoimmune disease. These T cells are nonetheless susceptible to tolerization. Tolerance and effector expression are not mutually exclusive. The combination has been reported in selected cases of self-Ag recognition albeit involving naive T cells (12, 18, 19), suggesting that recognition of self-Ag can lead to a range of outcomes, from tolerance to overt autoimmunity. For naive T cells, enhanced IL-2 signaling using the activating complex of IL-2 and anti-IL-2 Ab can convert what is otherwise a purely tolerogenic response into one that includes the destruction of self-tissues. It has been reported that constitutive expression of IL-2 can result in organ-specific autoimmunity (20), although conversely lower intrinsic IL-2 production on the NOD genetic background increases diabetes susceptibility (21). It is known that memory $CD8^+$ T cells are an immediate source of IL-2 following Ag-specific stimulation (22) and that IL-2 exposure leads to the induction of CD25 expression (23). Finally it has most recently been suggested that $CD25^+$ regulatory T cells suppress immune-induced pathology by deprivation of common $\gamma$-chain cytokines, including IL-2 (24). In light of all these findings, we hypothesize that it is the enhanced IL-2 responsiveness of memory vs naïve T cells that dictates their divergent stimulatory outcomes and the initiation of autoimmunity by the former population. Furthermore, these findings provide an obvious route to generation of more effective immunotherapies, where priming with tumor Ags might be combined with the IL-2 and anti-IL-2 Ab complex to facilitate aggressive naive T cell responses to cancer.

## Disclosures

The authors have no financial conflict of interest.

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