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Danger Signaling through the Inflammasome Acts as a Master Switch between Tolerance and Sensitization

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Efficient priming of adaptive immunity depends on danger signals provided by innate immune pathways. As an example, inflammasome-mediated activation of caspase-1 and IL-1β is crucial for the development of reactive T cells targeting sensitizers like dinitrofluorobenzene (DNFB). Surprisingly, DNFB and dinitrothiocyanobenzene provide cross-reactive Ags yet drive opposing, sensitizing vs tolerizing, T cell responses. In this study, we show that, in mice, inflammasome-signaling levels can be modulated to turn dinitrothiocyanobenzene into a sensitizer and DNFB into a tolerizer, and that it correlates with the IL-6 and IL-12 secretion levels, affecting Th1, Th17, and regulatory T cell development. Hence, our data provide the first evidence that the inflammasome can define the type of adaptive immune response elicited by an Ag, and hint at new strategies to modulate T cell responses in vivo. The Journal of Immunology, 2008, 180: 5826–5832.

The prime goal of the immune system is to protect us from harm by pathogens. To fulfill this function, it relies on sequential activation of innate and adaptive immune systems. The latter is characterized by the generation of very large repertoire of cells, each one with a specific target that proliferates rapidly when encountering the right Ag, adapting efficiently to the host environment. However, it requires clues from the less specific but faster innate immune response (1). Previously regarded as a primitive mechanism for cleaning up pathogen debris, it is now recognized as a remarkably specific system that is essential for priming adaptive immunity. Pattern recognition receptors are key sensors of the innate immune system, which are capable of recognizing molecules that are unlikely to vary much, due to their essential function for the pathogen survival. Both the transmembrane receptors called TLRs and intracellular receptors called NOD-like receptors (NLR) belong to this family (2, 3).

Although very efficient and elaborate, this dual immune surveillance is prone to making errors, such as exaggerated responses to environmental substances leading to severe skin (contact hypersensitivity (CHS)) or lung (asthma) diseases. Environmental chemicals may be categorized in respect to their potency as sensitizers: whereas weak sensitizers will induce a feeble adaptive immune response in a selected number of people, potent sensitizers will induce strong CHS in 100% of humans. Although several reports have shown that local irritation is important for the development of CHS (4–7), the underlying molecular mechanisms are still largely unknown. Because Freund’s adjuvant is used to boost the adaptive immune response during vaccination and has recently been demonstrated to activate both NLRs and TLRs (8), we postulated that CHS also depends on danger signaling through similar receptors.

Others and we have recently demonstrated that CHS, due to small irritant chemicals such as dinitrofluorobenzene (DNFB), is dependent on the inflammasome (9, 10). This protein complex is composed of three proteins: 1) an NLR of the NALP family (NALP, neuronal apoptosis inhibitory protein, CIITA, HET-E, TP1, (NALP) LRR- and pyrin domain); 2) the adaptor protein apoptosis speckled-like protein with a caspase recruitment domain (ASC); and 3) caspase-1 (3). This complex regulates the activity of caspase-1 and, consequently, of the proinflammatory cytokines IL-1β and IL-18. We found that mice lacking NALP3, ASC or the IL-1 receptor, failed to develop CHS after exposure to certain contact sensitizers, suggesting that the inflammasome plays a key role in the priming of adaptive immunity.

DNFB belongs to a family of structurally related chemical compounds with high sensitizing properties that have been used as model haptens to study effector T cell development in vivo. Dinitrochlorobenzene (DNCB) and trinitrochlorobenzene (TNCB) belong to the same family and, like DNFB, induce a strong CHS response. Dinitrothiocyanobenzene (DNTB), by contrast, fails to induce a strong immune response in vivo, despite its high structural homology to DNFB. Interestingly, several reports suggest that DNTB may induce immunological tolerance to itself and to cross-reactive dinitrophenyl compounds, in particular to DNFB (11–14). We hypothesized that the potency of a contact sensitizer may be linked to its capacity to provide danger signals mediated by the inflammasome, rather than its antigenicity, and that the inflammasome is implicated in tolerance vs sensitization mechanisms. We therefore studied the effect of DNTB and DNFB on inflammasome signaling in vitro, as well as the effect of inflammasome modulation on sensitization and tolerance in vivo.
Materials and Methods

Cell culture and stimulation

Primary keratinocytes (human epidermal keratinocytes (Biocoba), were cultured in Epilife medium supplemented with Human Keratinocyte Growth Supplement (Biocoba) and 60 μM of Ca2+. HaCaT cells were cultured in DMEM: F-12 medium (1/1) (Invitrogen) supplemented with 5 μg/ml human insulin, 10 ng/ml cholea toxin, 0.4 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, and 10% FCS. Cell cultures were maintained at 37°C in humidified incubators with 5% CO2. Primary keratinocytes were pretreated with 0.1 ng/ml hTNF (Alexis) for 6 h, and stimulated with TNCB (0.5–10 μg/ml) (Fluka), DNCB (0.5–10 μg/ml), DNFB (0.5–10 μg/ml) (Fluka), DNTB (Lancaster Synthesis) (0.5–50 μg/ml) (a gift from Ian Kimber, Faculty of Life Sciences, The University of Manchester, Manchester, U.K.), urushiol, (1–5 μg/ml) and SDS (15 μg/ml), for 24 h with or without Z-Val-Ala-Asp-fluoromethylketone (Alexis) at 50 μM for 24 h before IL-1β secretion was determined by ELISA.

Analysis of contact hypersensitivity and tolerance in mice

Mice were handled according to institutional and Swiss Federal Veterinary Office guidelines. IL-1R and ASC-deficient mice were obtained from M. Kopf (Molecular Biomedicine ETHZ, Zürich-Schlieren, Switzerland) and V.M. Dixit (Genentech, San Francisco). For classical CHS studies: 6–7-week-old mice were sensitized by topical (external) applications of 20 μl of 0.5% TNCB, DNCB, DNFB, oxazolone, or 1% DNTB in acetone-olive oil (AOO) or AOO alone to the skin of the right ear at days 0 and 1. Elicitation was done at day 5 by topical application of 20 μl of 0.3% DNCB, DNCB, oxazolone, or 1% DNTB in AOO to the contralateral ear. Ear thicknesses were measured with a digital thickness gauge (Mitsutoyo) before and 24 h after hapten challenge. For tolerance induction, mice were shaved on the belly on day −1, then treated with 20 μl of 0.5% DNTB or DNFB on day 0 and 1, then sensitized on the right ear and challenged on the left ear as described above. For adoptive transfer, mice were shaved on the belly on day −1, then treated with 20 μl of 0.5% DNTB or DNFB on day 0 and 1, sacrificed on day 5. Single cell suspension from inguinal lymph nodes and spleen were washed in DMEM medium, counted, concentrated, and injected in the tail vein of strain-matched naive mice. Alternatively, single cell suspension obtained from the lymph node and spleen of DNTB-treated mice were depleted of CD4+/CD25+ cells using a two-step magnetic sorting (AutoMACS and Treg depletion kit, Miltenyi Bionetics) according to the manufacturer’s protocol. Recipient mice were sensitized on the right ear with 20 μl of 0.5% DNFB on day 1 and 2 after adoptive transfer, then challenged on the left ear on day 6, as described above.

SDS, IL-1β, zYVAD, and IL-1R agonist (IL-1RA) administration

Twenty microliters of either SDS in dimethylformamide (1% v/v) or dimethylformamide alone was applied 30 min before each sensitization with DNFB. Ten microliters of either recombinant mIL-1β (BD Biosciences) in PBS (200 ng/ml) or PBS alone was injected s.c. in the ear 30 min before each sensitization with DNFB. Twenty microliters of either acyloxy-Z-Val-Ala-Asp-chloromethylketone (Alexis) in DMSO (0.2 mM) or DMSO alone was applied on the right ear 30 min before sensitization with DNFB.

Cytokine detection by ELISA

Supernatants from primary keratinocyte cultures were analyzed by ELISA for IL-1β (BD Biosciences) according to the manufacturer’s instructions. ASC or wild-type littermates were treated with 20 μl of 0.3% DNFB on day 0 and 1 and were sacrificed on day 2. Single cell suspension of draining neck lymph nodes were obtained and cultivated in DMEM medium for 5 days. Supernatants were then analyzed by ELISA for IL-6 (R&D Systems), IL-10 (R&D Systems), and IL-12 (mouse IL-12/IL-23 p40 allele-specific DuoSet DY499, BD Biosciences) according to the manufacturer’s instructions.

Statistical analysis

Groups were compared using one-tailed Student’s or Aspin-Welch’s t tests.

Results

DNFB and DNTB provide similar epitopes yet differ in their capacity to activate the inflammasome and induce contact hypersensitivity

DNFB, DNCB, TNCB, and DNTB have been used as model hapten to study contact hypersensitivity and T cell reactivity in mice (11–14). As we had previously observed that TNCB activates the inflammasome in vitro and in vivo, we decided to study the effect of DNCB, DNFB, and DNTB on this signaling pathway. We found that primary keratinocytes exposed to DNFB, DNCB, and TNCB, but not DNTB, secrete significant amounts of active IL-1β, in a dose dependent manner, while the viability of the cells was not affected at these concentrations (Fig. 1A). This effect was inhibited by the caspase-1 inhibitor zYVAD, strongly suggesting an inflammasome dependent mechanism. Interestingly, the active component of poison ivy, urushiol, also potentely activates IL-1β secretion, as previously reported (15). Because detergents with irritant properties potentiate allergic responses (4–6) and have been reported to promote IL-1β secretion by keratinocytes (15), we included SDS in our assays and found that it could also trigger IL-1β secretion.
activation. Our results hinted that DNTB might fail to trigger danger signals necessary to induce CHS in vivo. Indeed, DNFB, DNBC, and TNCB induce strong sensitization (Fig. 1B), while DNTB is a weak sensitizer at best (11–14). The difference in the sensitizing potency of these molecules may be due to the antigenicity of these molecules, but our results rather suggest that it is linked to their capacity to provide danger signals through the activation of the inflammasome.

We found that DNFB-sensitized mice display a strong reaction to DNTB (Fig. 1B). Our data are consistent with previously published observations and tend to confirm that DNFB and DNTB provide epitopes that can be recognized by the same T cells, yet differ in their capacity to prime the adaptive immunity (11, 12, 16). Although the value of DNTB as a tolerogen is still debated (17, 18), this suggests that the level of inflammasome signaling induced by a given molecule may better predict its sensitizing potential than the antigenic properties itself.

Danger signaling mediated by the inflammasome allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization

IL-1β had been previously reported to play a key role for the CHS response to small irritants like DNFB, acting as an adjuvant promoting effector T cell response (19). We reasoned that whether the inflammasome is responsible for the activation of IL-1β, it should be possible to turn weak sensitizers into strong sensitizer by providing danger signals. Mice exposed twice on the right ear to DNFB on the controlateral ear (Fig. 2A) failed to mount an immune response when challenged with DNFB (day 5 on the right ear) do not develop CHS, except if danger signals, such as i.v. IL-1β or topical SDS, are provided at the time of sensitization. Application of either SDS or IL-1β alone, in the absence of any hapten (AOO), had no effect, while neither could further increase the sensitization capacity of DNFB. A, Blocking of danger signals provided by DNFB during sensitization, using i.v. IL-1Rα (Anakinra) or topical zYVAD prevents the development of CHS. Similar results are obtained when the sensitization is performed in ASC or IL-1R-deficient mice. C, SDS increases the sensitizing capacity of DNTB in ASC+/− but not ASC−/− littersmates. (n = 5 mice/group, unless indicated in the figure). The irritant effect DNFB alone is limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of DNFB (A and C).

FIGURE 2. Concomitant danger signaling allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization. A, DNFB-sensitized mice (days 0 and 1 on the left ear) challenged with DNFB (day 5 on the right ear) do not develop CHS, except if danger signals, such as i.v. IL-1β or topical SDS, are provided at the time of sensitization. Application of either SDS or IL-1β alone, in the absence of any hapten (AOO), had no effect, while neither could further increase the sensitization capacity of DNFB. B, Blocking of danger signals provided by DNFB during sensitization, using i.v. IL-1Ra (Anakinra) or topical zYVAD prevents the development of CHS. Similar results are obtained when the sensitization is performed in ASC or IL-1R-deficient mice. C, SDS increases the sensitizing capacity of DNTB in ASC+/− but not ASC−/− littersmates. (n = 5 mice/group, unless indicated in the figure). The irritant effect DNFB alone is limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of DNFB (A and C).
FIGURE 3. Exposure to topical sensitizers in the absence of inflammasome-mediated danger signals leads to tolerance. A, Exposure of BALB/c mice to DNTB 5 days before sensitization and challenge with DNFB results in tolerance to DNFB, as indicated by the decrease in ear swelling. Pretreatment with DNTB does not however impair the response to another contact sensitizer like oxazolone, suggesting strongly that the effect of DNTB on DNFB response is hapten specific. The irritant effect DNFB and oxazolone alone are limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of either DNFB or oxazolone (A). B, Induction of tolerance is a cellular mediated process as adoptive transfer of cells of the spleen and lymph nodes of DNTB, but not DNFB, treated BALB/c mice blocks the development of CHS to DNFB in the recipient mice. In IL-1R deficient mice, DNFB fails to induce CHS. Adoptive transfer of cells derived from DNFB-treated mice block development of CHS to DNFB in wild-type BALB/c mice, strongly suggesting that in the absence IL-1β signaling, DNFB induces tolerance. C, Induction of tolerance to DNFB by DNTB is also observed in C57BL/6 mice. Adoptive transfer of cells from DNFB treated ASC-deficient mice, but not WT C57BL/6 mice, blocks CHS to DNFB in naive wild-type recipient mice, suggesting that DNFB induces tolerance in mice that cannot activate the inflammasome. (n = 5 mice/group).

mechanism implicated in the diminished response to DNFB is tolerance, as previously detailed (11–14) and not a more general nonspecific immunosuppression.

Because we found that, in the absence of danger signaling, DNFB is no longer a potent sensitizer, we decided to test its capacity to induce tolerance in this setting. As ASC and IL-1R-deficient mice fail to mount a proper CHS to DNFB, we could not study tolerance directly in these strains (9, 10, 23, 24). However, we could expose ASC and IL-1R-deficient mice to DNFB and then transfer lymphoid cells in a wild-type recipient mouse, where sensitization and tolerance can be studied. Indeed, several groups have observed that DNTB-induced tolerance to DNFB can be transferred to a recipient syngeneic mouse, using adoptive transfer of lymph nodes and spleen cells (11, 14). We first confirmed these findings, showing that naive mice injected with lymph node and spleen extracted cells from DNTB-, but not DNFB-, treated mice failed to mount a CHS response when later sensitized and challenged with DNFB (Fig. 3B). The extent of tolerance induction was similar in mice directly pretreated with DNTB (Fig. 3A). Likewise, mice injected with cells from DNFB-primed IL-1R- or ASC-deficient mice showed a significant decrease in their capacity to mount an immune response to DNFB (Fig. 3, B and C, respectively), suggesting that tolerance develops in the absence of inflammasome-mediated IL-1β activation. Hence, we could demonstrate that the balance between sensitization and tolerance depends on danger signaling levels mediated by IL-1β rather than the antigenic properties of the hapten used.

Cytokine profile in tolerance vs sensitization in mouse lymph nodes

These results strongly suggested that the inflammasome determines, either at the skin or draining lymph node level, whether suppressive or effector T cells are produced. We reasoned that the cytokine environment surrounding T cells specific for the DNTB/DNFB epitope may vary accordingly. We therefore determined the cytokine profile in draining lymph nodes of DNFB treated ears from ASC deficient mice and their control littersmates. Mice sensitized with DNFB on day 1 and 2 were sacrificed at day 3 and the draining lymph nodes were dissociated and cultivated for 5 days. Under these conditions, we could not detect significant amounts of secreted IL-1β (data not shown). It is important to note here that IL-1β is secreted in minute amounts and that IL-6, one of its bona fide downstream target, is significantly increased after the injection of femto-moles of IL-1β, hence making IL-6 a surrogate marker for active IL1β secretion (25). Interestingly, we found that wild-type lymph nodes secreted significantly higher amounts of IL-6 and IL-12/23 than those from ASC-deficient mice, suggesting a cytokine environment likely to favor a Th1 and Th17 response and hamper regulatory T cell (Treg) development (26, 27) (Fig. 4 and see below). These results are consistent with a previous report showing that IL-12 can turn DNTB into a strong sensitizer (14); IL-10 secretion, which antagonizes LC migration and IL-12 priming of T cells (28), was slightly higher in ASC deficient mice, but the difference was not statistically significant and the very low levels observed may undermine the relevance of our finding. Hence, our results suggest but do not prove that the inflammasome may modulate the secretion levels of IL-10, which may in turn regulate T cell fate.

Mechanisms of tolerance transfer

The mechanism of DNTB induced tolerance is still controversial. However, early experiments, including adoptive transfer studies, have suggested that regulatory T cells (previously called suppressive T cells) may be implicated (11, 12, 14). We therefore studied the impact of regulatory cells on tolerance to DNFB. We first analyzed the repartition of CD4+ CD25+ FoxP3+ T cells in lymph nodes draining the skin of DNFB treated ASC-deficient mice and their control littersmates. We found that the overall number of CD4+ CD25+ FoxP3+ cells was the same, suggesting either that Treg are not implicated in tolerance or that the DNFB-specific
Mechanisms of tolerance transfer and inflammasome dependency. A, The repartition of CD4+ CD25+ FoxP3+ Tregs is the same in lymph node cells extracted from DNFB treated ASC heterozygote and deficient mice, as demonstrated by FACS staining. B, Adoptive transfer of DNTB exposed donor mice fails to induce tolerance to DNFB in the recipient mouse, if CD4+CD25+ cells are removed by magnetic associated cell sorting, before their transfer. (n = 4 mice/group).

FIGURE 5. Cytokine profile in tolerant vs sensitized mouse lymph nodes. Lymph node cells from DNFB treated ASC+/− mice (days −5 and −4 before lymph nodes extraction) secrete significant amounts of IL-6 and IL-12, as determined by ELISA. In contrast, cells from their ASC−/− littermates secreted only small amounts of these cytokines. IL-10 levels were slightly increased in ASC−/− cells, yet statistical significance was not reached. (n = 4 mice/group). ND: Nondetectable.

Discussion

The central role of innate immunity in the activation of the adaptive immune response is no longer a matter of debate, in particular regarding TLR signaling. We herein provide evidence that danger signaling through the inflammasome can also affect the fate of the adaptive immune response. Others and we had already shown that efficient sensitization does not occur in the absence of the inflammasome and IL-1β (9, 10, 22), yet these studies did not evaluate the impact of this signaling pathway, or its absence, on the type of adaptive response elicited. We now provide evidence that the inflammasome may act as a major switch between tolerance and sensitization, laying emphasis on the sequential role of innate immunity and adaptive immunity in CHS (Fig. 6). Indeed, we found that DNTB fails to activate the inflammasome in vitro, unlike other members of this family. Furthermore, DNTB becomes a sensitizer if concomitant inflammasome signaling is provided. More importantly, blocking inflammasome signaling turns a bona fide sensitizer, DNFB, into a tolerizer. Taken together, these findings suggest that the inflammasome controls the development of tolerance or sensitization to (irritant) chemicals. Hence, NLR and TLR not only share structural resemblance, but also a similar function. Because both signaling pathways ultimately result in NF-κB activation, it is reasonable to think that a certain amount of redundancy is present. We therefore propose that stimulation of TLR, IL-1R, IL-18R, and IL-12R may all favor the development of CHS to DNFB or other Ags, as reported for IL-12 (14). Consequently, NLR activation may be an interesting target as an adjuvant for vaccines and tumor vaccination, and it should be noted here that DNBC has been used to promote inflammation and favor immune rejection in metastatic melanoma patients (31).

Our data further suggest that the Treg-mediated development of tolerance may represent a default pathway in noninflammatory conditions, as we observed that ASC deficiency favors tolerance to hapten presents in the skin. The potential physiologic benefit to this may be to avoid unnecessary/harmful T cell responses against Ags that are not accompanied by significant cell stress or damage, and therefore are unlikely to originate from a pathogen (introducing the notion of an environmental self). This hypothesis may have
FIGURE 6. Putative model of the function of the inflammasome in T cell priming. DNFB-induced inflammasome activity results in the secretion of active IL-1β, which drives the expression of IL-6 and IL-12, thus creating an environment favoring Th1 development and thwarting Treg counter-effects. Hence, DNFB provided at the same time a danger signal and a suitable Ag, whereas DNTB provides only an Ag. In the absence of danger signals, tolerance is the default pathway, relying on the low levels of IL-6 to stimulate Tregs. Modulation of the danger-signaling pathway can be achieved at the inflammasome level (negative: ASC deficiency, zYVAD; positive: SDS) or at the cytokine level (negative: IL-1Ra, anti-IL-12; positive: IL-1β and IL-12).

important implications in the fields of virology and oncology. As an example, human papillomaviruses elicit little response from the immune system, although they can provide highly antigenic epitopes (32). Danger signaling mediated by imiquimod-induced TLR7-triggering results in a rapid destruction of the lesions by T cells (33), suggesting that the virus “invisibility” depends on the absence of inflammation. Cancer development and immune-escape may rely on similar mechanisms, as most tumors develop without eliciting inflammation. Interestingly, activation of the innate immune system through TLRs (33) or NLRs (31) may also result in an efficient antitumor response, further underlining the potential of these molecules in the field of immunotherapy (34).

We also provide evidence that activation of the inflammasome results in the secretion of cytokines likely to favor the development of a Th1 and Th17 response counterbalancing Treg activity and tolerance. Indeed, IL-12 is known to drive T cells toward a Th1 phenotype, while IL-6 favors Th17 over Treg development (26, 27). Th17 secrete IL-17, which further increases the activity of formerly arisen Treg cells (26, 27). Finally, decreasing IL-10 levels, a cytokine that can counterbalance the effect of IL-12 may further tip the balance in favor of the Th1 (28). These results are in accord with a recent study showing that IL-1β breaks tolerance by favoring the expansion of CD25+ effector cells (35).

Finally, our results may shed some light on the controversy regarding the sensitizing potential of DNTB. Indeed, unlike several reports, including the present work, some authors did not observe tolerance to DNTB but a weaker sensitization, concomitant with dendritic cell accumulation in regional lymph nodes (17, 18). We propose that the frontier between tolerance and sensitization is narrow and easily crossed. Indeed, in conditions where DNTB is found to induce tolerance, we found that adding danger signals turns DNTB into a sensitizer. It is likely that the nature of the vehicle, the concentration of the molecule, and skin-preparation such as shaving will tip the balance toward sensitization. In conditions where DNTB is a sensitizer, it induces similar levels of proliferation by draining lymph node cells such as DNBC, but fails to induce LC migration (36), suggesting the possible recruitment of dermal DC by DNTB (37). We propose that DNTB fails to activate the inflammasome and IL-1β activation, which is crucial for LC migration, but does activate other molecular pathways that may, depending on the context, be sufficient to trigger an efficient immune response and result in sensitization. We are currently generating LC-specific IL-1β and ASC-deficient mice to study this hypothesis. Interestingly, we have conducted experiments that indicate that the presence of an intact inflammasome is essential for DNFB and DNBC-induced LC migration (data not shown). Additional experiments are necessary, including a characterization of the role of the inflammasome on dermal DC, as the exact role of both cell types in CHS remains controversial (38–41). Finally, the CHS outcome may also depend on the viral and bacterial environment of the host, as TLR and NLR activate similar targets such as NF-κB and IL-12.

Although NLR discovery is recent, several therapeutic tools targeting their signaling pathways or downstream targets are already available. For example, down-modulation of the inflammasome has been achieved successfully in patients suffering from autoinflammatory disorders, either by blocking caspase-1 or IL-1β signaling (42–44). Additional tools will soon be added to this list, including anti-IL-1β, anti-IL-6, and anti-IL12/23 Abs. As for NLR activation, Freund’s adjuvant has been used extensively in vaccination studies (3, 8). We believe that gathering further insights into the role of each member of this large family will increase the number of patients benefiting from this therapeutic advance and will lead to the development of even more potent and specific drugs.

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Disclosures

The authors have no financial conflict of interest.

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