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CD22 Regulates Time Course of Both B Cell Division and Antibody Response

Taishi Onodera,*† Jonathan C. Poe,§ Thomas F. Tedder,§ and Takeshi Tsubata2*†‡

Because pathogens induce infectious symptoms in a time-dependent manner, a rapid immune response is beneficial for defending hosts from pathogens, especially those inducing acute infectious diseases. However, it is largely unknown how the time course of immune responses is regulated. In this study, we demonstrate that B cells deficient in the inhibitory coreceptor CD22 undergo accelerated cell division after Ag stimulation, resulting in rapid generation of plasma cells and Ab production. This finding suggests that CD22 regulates the time course of B cell responses and suggests that CD22 is a good target to shorten the time required for Ab production, thereby augmenting host defense against acute infectious diseases as “universal vaccination.”


Bec ause many pathogens cause infectious symptoms in a time-dependent manner, the time course of acquired immune responses affects host defense against pathogens (1). This is underscored by “immunity” mediated by memory responses, in which quick acquired immune reactions contribute to efficient host defense against pathogens. Several lines of evidence suggest that differentiation of lymphocytes depends on cell division times rather than duration of stimulation (2–4). During T cell differentiation, division number profoundly affects production of cytokines such as IFN-γ and IL-4, which are crucial for differentiation to either Th1 or Th2 cells, respectively (3, 4). Although cell cycle dependence of B cell differentiation has not been extensively studied, Ig class switching has been shown to require three cell divisions (2). Thus, how rapidly cell division proceeds is crucial for regulating the time course of immune responses.

CD22 (also known as Siglec-2), a 140-kDa B cell membrane protein, is a negative regulator of signaling through BCR, which plays a crucial role in activation and differentiation of B cells (5). CD22 contains the ITIMs in the cytoplasmic region and is phosphorylated at ITIMs by the BCR-associated kinase Lyn upon BCR ligation (6, 7). The phosphorylated ITIMs then recruit and activate Src homology domain 2-containing phosphatase-1 (8), which negatively regulates BCR signaling by both dephosphorylating proximal BCR signaling molecules (9) and regulating the calcium pump, plasma membrane calcium-ATPase (10). Functionally, CD22 serves as a molecular switch that determines the fate of Ag-stimulated B cells, whether they undergo apoptosis or proliferation (11), and prevents autoimmune (12), probably through regulation of BCR signaling. However, Ab production to T cell-dependent Ags showed marginal increase, if any, in most CD22−/− mouse lines compared with wild-type mice (13–16), suggesting that CD22 regulates in vivo Ab responses only weakly.

In this study, we address the early phase of the immune response of CD22−/− B cells by immunizing the recipient mice that are adaptively transferred with CD22−/− B cells. We demonstrate that CD22−/− B cells undergo memory B cell-like rapid expansion, thereby forming plasmablastic burst, and rapid differentiation to plasma cells and Ab production by accelerated cell division. This finding suggests that BCR signaling strength regulates the time course of Ab responses through cell division control and suggests that CD22 is a good target to shorten the time course of B cell response, thereby augmenting host defense against acute infectious diseases.

Materials and Methods

Adoptive transfer and immunization

Spleen cells were prepared from donor mice (Ly5.2, IgM*), and erythrocytes were lysed by incubation with a solution consisting of 0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA (pH 7.2). Cells were stained with PE-conjugated anti-CD23 Ab (BD Pharmingen), followed by incubation with anti-PE microbeads (Miltenyi Biotec), and CD23−/− follicular B cells were purified using an autoMACS (Miltenyi Biotec). The purity of CD23+ cells was determined by flow cytometry using an LSR (BD Biosciences) (purity >97%). In some experiments, cells were labeled with 5 μM CFSE (Molecular Probes) as described previously (2). For adoptive transfer, Ly5-congenic C57BL/6 (B6.Ly5.1) mice were treated i.p. with 100 μg of chicken γ-globulin (CGG) in CFA. After 7 days, mice were injected i.v. with 4 × 10⁷ purified CD23− cells. One day later, recipients were immunized i.p. with 50 μg of 4-hydroxy-3-nitrophenyl acetyl (NP)-conjugated CGG (NP6-CGG) in alum.

ELISA and ELISPOT

Serum NP-specific Ab titers were measured by a standard ELISA using 96-well plates coated with 2 μg/ml NP-conjugated BSA (NP-BSA) (17). NP-specific Ab-forming cells (AFCs) were enumerated by an ELISPOT assay as described previously (17). In brief, MultiScreen-HA 96-well plates (Millipore) were coated with 50 μg/ml NP-BSA. Spleen or bone

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3 Abbreviations used in this paper: B6.Ly5.1, Ly5-congenic C57BL/6; AFC, Ab-forming cell; NP, 4-hydroxy-3-nitrophenyl acetyl; CGG, chicken γ-globulin; GC, germinal center; HEL, hen egg white lysozyme; NIP, 4-hydroxy-3-iodo-5-nitrophenyl acetyl; FNA, peanut agglutinin.
marrow cells were distributed to the plates and were incubated for 5 h at 37°C in a CO2 incubator. Plates were incubated with biotin-labeled anti-IgMa Ab and anti-IgG1a Ab followed by reaction with alkaline phosphatase-labeled streptavidin (BD Pharmingen).

**Flow cytometry and cell sorting**

Spleen cells were incubated with anti-low-affinity Fc receptor for IgG (FcγRII/III) Ab (2.4G2) and were stained with the following reagents: FITC- and biotin-labeled anti-Ly5.2 Ab, PE- and biotin-labeled anti-IgG1 Ab, PE-conjugated anti-CD38 Ab (BD Pharmingen), Cy5-labeled anti-B220 Ab, allophycocyanin-conjugated anti-CD80 Ab (eBioscience), Alexa Fluor 647-labeled anti-Fas Ab, 4-hydroxy-3-iodo-5-nitrophenyl acetyl (NIP)-conjugated allophycocyanin, biotin-labeled anti-IgM Ab (Southern Biotechnology Associates), and biotin-labeled peanut agglutinin (PNA) (Vector Laboratories). Biotin-labeled Abs were visualized by PerCP-conjugated streptavidin (BDPharmingen); 4,6-diamino-2-phenylindole (Sigma-Aldrich) was used for exclusion of dead cells. Cells were analyzed using an LSR with CELLQuest software (BD Biosciences).

**Immunohistochemical analysis**

Spleens were embedded in Tissue-Tek OCT compound (Sakura), snap-frozen in liquid nitrogen, and stored at −80°C. Cryostat sections (7-μm thick) were mounted onto slide glass, air dried, and fixed in acetone for 20 min at room temperature. The sections were incubated with blocking buffer PBS containing 0.5% BSA and 0.05% NaN3, containing anti-FcγRIII/I Ab (2.4G2) for 30 min and were stained at room temperature for 60 min with PE-conjugated anti-CD38 Ab, NIP-allophycocyanin, and either biotin-labeled anti-IgM Ab or biotin-labeled anti-IgG1 Ab, followed by...
IgG1 cells were isolated from CD22−/− GC B cells after Ag stimulation previously (19). PCR products were inserted into the pGEM-T vector (Promega), cloned, and sequenced using an Applied Biosystems 3130xl Genetic analyzer (Applied Biosystems). Framework and CDRs were determined as described and were immunized as indicated in the legend of Fig. 1. Spleen cells from recipients were analyzed by flow cytometry for expression of B220 (A−F), Ly5.2 (A−F), IgM (C), IgG1 (D), and binding to PNA (E and F) on days 3, 5, and 7 after immunization. Dead cells were excluded by staining with 4,6-diamino-2-phenylindole. Numbers of donor-derived total B cells (B220−Ly5.2−) (A and B), IgM+ B cells (B220−Ly5.2−IgM+), C, IgG1+ B cells (B220−Ly5.2−IgG1+), D, and GC B cells (B220−Ly5.2−PNA+) (E and F) in 2 × 10⁵ total spleen cells are shown. Dots represent data from individual recipients, and bars show the mean of each group (B–D and F). Filled circles, recipients transferred with CD22−/− QM B cells; open circles, recipients transferred with CD22+/− QM B cells. Statistical analysis was done by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Sequence analysis of the VH 17.2.25 gene

Genomic DNA was extracted from 6 × 10⁶ cells and was subjected to PCR that preferentially amplifies VH 17.2.25 as described previously (18). The PCR products were inserted into the pGEM-T vector (Promega), cloned, and sequenced using an Applied Biosystems 3130xl Genetic analyzer (Applied Biosystems). Framework and CDRs were determined as described previously (19).

Results

CD22−/− B cells rapidly expand and generate IgG+ B cells and GC B cells after Ag stimulation

To address how CD22 regulates B cell activation and differentiation at the early phase of immune responses, we crossed CD22−/− mice with QM mice (15, 20), in which almost all B cells are reactive to the hapten NP due to their expression of knocked-in VH 17.2.25 and λ L chain. Because CD22high follicular B cells are involved in the Ab response to protein Ags, including proteins conjugated with hapten such as NP (21, 22), CD22high spleen B cells were isolated from CD22+/+ or CD22−/− QM mice (Ly5.2) (Fig. 1A). A small number of these follicular QM B cells, mostly reactive to NP, were adaptively transferred to recipient B6.Ly5.1 mice (23). Mice were then immunized with NP-CGG, and the spleen section was histologically examined. Donor B cells (IgH+) were distinguished from recipient B cells (IgH−) by staining with allotype-specific anti-Ig Ab. On day 3 after immunization, a small number of NP-reactive CD22+/+ QM B cells were observed on the border between the follicle and the T cell zone (Fig. 1, B and E). QM B cells then formed small foci in the bridging channel and small GCs on day 5 (Fig. 1, C and F), in agreement with previous findings (24, 25). Astonishingly, CD22−/− donor B cells generated a large number of NP-reactive plasmablasts, mostly IgM+, in the bridging channels (Fig. 1, H and K) and formed GCs already on day 3 (Fig. 1, N and O). On day 5, a large number of CD22−/− plasmablasts, which mostly underwent class switching to IgG, filled both the bridging channels and the red pulp (Fig. 1, I and L). CD22−/− B cells also formed large GCs (Fig. 1, I and L). Naive CD22−/− donor B cells thus underwent rapid expansion and generated a burst of plasmablasts, characteristic of memory responses, in the early phase of primary immune responses.

To further analyze the response of CD22−/− B cells during primary immune responses, we transferred either CD22+/+ or CD22−/− QM B cells to recipient B6.Ly5.1 mice and examined, by flow cytometry, the expansion of donor B cells and generation of both IgG1+ B cells and GC B cells from donor B cells upon immunization (Fig. 2). Donor-derived CD22+/+ QM B cells slightly increased in number up to day 3 of immunization and showed substantial expansion from day 3 to day 5 (Fig. 2, A and B). The number of IgG1+ B cells and GC B cells generated from CD22−/− QM B cells also strongly increased from day 3 to day 5 (Fig. 2, D–F). In contrast, expansion of CD22−/− donor B cells showed a distinct time course and magnitude from those of CD22+/+ donor B cells. The most striking expansion of CD22−/− donor B cells was observed up to day 3 (Fig. 2, A and B), and the
expansion of CD22/−/− donor B cells in the first 3 days was much greater than that of CD22+/+ donor B cells for the first 5 days (Fig. 2, A and B), consistent with histology (Fig. 1). Essentially the same results were obtained with the number of IgG1+ B cells and GC B cells generated from CD22+/− donor B cells (Fig. 2, D–F). These results indicate that CD22−/− B cells rapidly expand and generate a large number of IgG1+ B cells and GC B cells during primary immune responses.

Rapid cell division of Ag-stimulated CD22−/− B cells

To address how CD22−/− donor B cells undergo rapid expansion in recipients during a primary immune response, we analyzed the division of B cells by labeling B cells from CD22+/+ and CD22−/− QM mice with CFSE and transferring CFSE-labeled B cells into the recipient mice. One day after immunization, cells showing diluted CFSE fluorescence were not observed either in CD22+/+ nor in CD22−/− donor B cells, indicating that B cells did not undergo cell division regardless of the presence or absence of CD22 during the first day of immunization (Fig. 3A). On day 2, CD22+/+ donor B cells underwent up to four cell divisions, whereas some CD22−/− donor B cells divided already six times. This result clearly indicated an accelerated cell division of CD22−/− B cells during primary immune responses.

Class switching to IgG was shown to depend on cell division of B cells (2). Therefore, we asked whether the early generation of a large number of IgG+ B cells from CD22−/− donor B cells upon immunization is due to an augmented cell division of B cells. When we analyzed the generation of IgG1+ B cells from the CFSE-labeled QM B cells in immunized recipients, CD22+/+ donor B cells underwent class switching to IgG1 at the third cell division in agreement with previous findings (2) (Fig. 3B). CD22−/− donor B cells also generated IgG1+ cells at the third cell division, indicating that class switching occurs in CD22−/− B cells after the same number of cell division as in CD22+/+ B cells (Fig. 3B). On day 3 after immunization of recipient mice, CD22+/− donor B cells generated three times as many IgG1+ B cells as CD22+/+ B cells. These donor-derived CD22−/− IgG1+ B cells contained more diluted CFSE fluorescence than CD22+/+ IgG1+ B cells, indicating more cell divisions in CD22+/− IgG1+ B cells. CD22−/− B cells thus generated higher numbers of IgG1+ B cells probably due to an augmented expansion rather than an enhanced class switch recombination.

Next, we examined generation of GC B cells from CD22−/− QM B cells. Both CD22+/− and CD22−/− donor QM B cells generated B cells with GC phenotype (B220+CD38low) (26) GC B

Table I. Sequence analysis of the VH 17.2.25 gene

<table>
<thead>
<tr>
<th></th>
<th>R°</th>
<th>S°</th>
<th>Percentage of Replacement</th>
<th>Mutations/kbp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD22+/+ QM B cells</td>
<td>33 clones</td>
<td>24</td>
<td>8</td>
<td>75</td>
</tr>
<tr>
<td>FR°</td>
<td>6</td>
<td>2</td>
<td>75</td>
<td>2.4</td>
</tr>
<tr>
<td>CDR</td>
<td>36 clones</td>
<td>22</td>
<td>7</td>
<td>76</td>
</tr>
<tr>
<td>FR°</td>
<td>17</td>
<td>4</td>
<td>81</td>
<td>5.9</td>
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</table>

*The DNA sequences of VH 17.2.25 were analyzed on CD22+/+ or CD22−/− QM B cells with GC phenotype (Ly5.2+/B220+CD38low), which were sorted by MoFlo on day 3 after immunization. The numbers of replacement (R) and silent (S) mutations and mutation frequencies in the framework (FR) and complementarity-determining regions are shown.

° Percentage of replacement mutations of all observed mutations.

Mutations/kbp was calculated as total number of mutations/segment length/number of clones.
cells at the 7th or 8th cell division on day 3 of immunization (Fig. 3B), suggesting that generation of GC B cells depends on cell division times. The number of CD22−/− CD38low B cells increased by five times compared with CD22+/− CD38low B cells, and CD22−/− CD38low B cells showed more diluted CFSE fluorescence than CD22+/− CD38low B cells, indicating that CD22−/− B cells generate a much larger number of CD38low B cells than CD22+/− B cells do by augmented cell division. To assess whether expanded CD22−/− CD38low B cells are functional GC B cells, we amplified the knocked-in Vh17.2.25 gene isolated Ly5.2 B220− CD38low spleen cells at day 3 of immunization. The frequency of somatic mutation at the CDR in CD22−/− QM B cells was higher than that in CD22+/− QM B cells (Table I), indicating CD38low cells generated from CD22−/− B cells efficiently undergo somatic hypermutation of Ig V genes characteristic for GC B cells. Taken together, CD22−/− B cells generate a higher number of IgG1 B cells and GC B cells at the early phase of immune responses, probably as a consequence of rapid cell division.

Rapid generation and loss of plasma cells and Ab production from Ag-stimulated CD22−/− B cells

We examined primary Ab production from CD22−/− QM B cells upon immunization with NP-CGG, by ELISPOT and ELISA, for anti-NP Ab carrying donor-derived Ig allotype. When we measured the number of donor-derived anti-NP AFCs in the spleen of recipient mice, the number of both IgM and IgG1 AFCs generated from CD22−/− QM B cells was much higher than that from CD22+/− QM B cells from day 3 to day 5 (Fig. 4, A and B). Thus, CD22−/− B cells rapidly generated plasma cells compared with CD22+/− B cells. Accordingly, specific Ab titers in sera were rapidly increased when recipient mice transferred with CD22−/− QM B cells were immunized with NP-CGG (Fig. 4, C and D). In these mice, donor-derived anti-NP IgM was already detectable on day 3, and the anti-NP IgM titer was markedly higher than in the recipients carrying CD22+/− B cells on day 5 (Fig. 4C). These mice also showed much higher serum titer of anti-NP IgG1 than mice transferred with CD22+/− QM B cells (Fig. 4D). Taken together, CD22−/− B cells generate plasma cells more rapidly and produce a larger amount of specific Abs than CD22+/− B cells in the early phase of primary immune responses.

When we examined Ab production in the later phase, the number of CD22−/− anti-NP AFC in the spleen was reduced on day 7, whereas the number of CD22+/− anti-NP AFCs was gradually increased up to day 7 (Fig. 4, A and B). Although plasma cells generated in spleen migrate to the bone marrow (27, 28), AFCs in the bone marrow at day 7 account for only a few percent of the

Table II. The total number of donor-derived AFCs in spleen and bone marrow of immunized recipient mice transferred with CD22+/− or CD22−/− QM B cells

<table>
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<tr>
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<th>Spleen (×10⁴)</th>
<th>Bone Marrow (×10⁴)</th>
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<tbody>
<tr>
<td>CD22+/− QM AFCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM*</td>
<td>3.5 (±1.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>IgG1*</td>
<td>3.3 (±1.7)</td>
<td>0.0</td>
</tr>
<tr>
<td>CD22−/− QM AFCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM*</td>
<td>25.7 (±15.7)</td>
<td>3.6 (±2.4)</td>
</tr>
<tr>
<td>IgG1*</td>
<td>19.1 (±1.5)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

⁷(Number of AFCs per 10⁶ spleen cells) × (total spleen cells)/10⁶. Data are shown as average (±SD).

⁸(Number of AFCs per 10⁶ bone marrow cells) × (total nucleated cells in the bone marrow)/10⁶. The number of total nucleated cells in the bone marrow was assumed to be 3×10⁹ as described previously (47). Data are shown as average (±SD).

⁹(The highest number of AFCs among those at days 3, 5, and 7) − (number of AFCs at day 7).

¹⁰NA. Not applicable.
reduction of CD22−/− AFCs in the spleen (Table II). Further, the serum levels of both donor-derived anti-NP IgM and IgG were gradually decreased after day 7, regardless of whether the donor B cells express CD22 (Fig. 4, C and D). This suggests that the decline of the number of CD22−/− anti-NP IgM and IgG AFCs in spleen after days 5 and 7, respectively, is due to loss of AFCs but not migration of AFCs from spleen. Since memory B cell compartment is formed as early as day 6 after immunization (29), we examined the donor-derived memory B cells at day 7. The frequencies of B cells with memory phenotype (Fas+CD80+ CD38highIgG+) (30, 31) derived from CD22+/+ and CD22−/− QM B cells were 2460 ± 254 and 514 ± 180 cells in 106 spleen B cells, respectively, suggesting that CD22+/+ B cells poorly generate memory B cells. Thus, in the late stage of primary immune response, CD22−/− AFCs are rapidly lost and memory B cells are not efficiently generated from CD22−/− B cells.

**Discussion**

In this study, we demonstrate that naive CD22−/− B cells rapidly generate IgG+ B cells, GC B cells, and plasma cells and rapidly produce Abs after immunization. Both CD22+/+ and CD22−/− B cells differentiate to IgG+ B cells and GC B cells after three and 7–8 cell divisions, respectively, suggesting that differentiation of B cells requires certain cell divisions regardless of presence or absence of CD22. In contrast, CD22−/− B cells undergo cell divisions more rapidly than CD22+/+ B cells. These results clearly indicate that CD22−/− B cells undergo rapid cell division after Ag stimulation, resulting in rapid B cell differentiation and Ab production. Thus, CD22 regulates time course of B cell response by functioning as a crucial regulator of B cell division after Ag stimulation. We demonstrate, in this study, that CD22−/− B cells form burst of plasma blasts at the early phase of immune response and generate rapid Ab production, both of which are characteristic for memory immune response (22, 32–34). Thus, lack of CD22 causes memory response-like rapid expansion and differentiation of B cells. Our previous evidence suggests that CD22 is expressed but regulates BCR signaling only weakly in differentiated B cells and generates B cell activation by a mechanism independent of CD22 signaling (35). We have shown that CD22 does not regulate BCR signaling through IgG-BCR, which is mostly expressed on memory B cells, so efficiently as signaling through IgM-BCR and IgD-BCR. Thus, lack of efficient CD22-mediated signal regulation may contribute to rapid expansion of memory B cells, leading to burst formation of plasma blasts and rapid Ab production in memory responses.

Recently, Horikawa et al. (36) and Waisman et al. (37) presented evidence against our finding that CD22 regulates IgM-BCR signaling more strongly than IgG-BCR signaling. They demonstrated that CD22 regulates IgM-BCR signaling to the extent similar to its regulation on IgM-BCR signaling when BCR is ligated by the Ag hen egg white lysozyme (HEL) or anti-IgM chain Ab. This is probably because ligation of IgM-BCR by these monovalent or divalent reagents induces weak activation of CD22-mediated signal regulation comparable to that induced by IgG-BCR ligation. Indeed, NP-conjugated protein Ags (38) but not HEL (39, 40) or anti-Ig Ab (our unpublished data) induce translocation of CD22 to lipid rafts, in which Lyn responsible for CD22 phosphorylation is enriched (41). In contrast, B cells expressing HEL-reactive IgG are shown to generate stronger immune response than IgM+ B cells (36, 42), suggesting that IgG-BCR induces augmented B cell activation by a mechanism independent of CD22 after HEL stimulation. Thus, IgG-BCR appears to induce strong B cell response through both CD22-dependent and -independent pathways, although the latter depends on Ags. Memory B cell-like rapid response of CD22−/− B cells is quite unexpected because earlier studies demonstrated that CD22−/− mice show either no increase or only marginal increase, if any, in Ab response in most of the mouse lines (13–16). In the absence of CD22, expansion of B cells and generation of AFCs rapidly decline as early as 7 days after immunization (Figs. 1, 2, and 4). Decline of AFCs in spleen appears to be caused by rapid loss of AFCs but not by their migration because Ab titer is declined after day 7 and also because AFCs do not migrate to the bone marrow (Fig. 4, C and D; Table II). Rapid loss of B cells and AFCs may be caused by enhanced cell death of CD22−/− B cells (14, 16, 43, 44). Further, decline of B cell expansion may be involved in poor formation of CD22−/− memory B cell compartment. Thus, enhanced response of CD22+/+ B cells is mostly restricted to the early phase of the immune response. Earlier studies failed to show enhanced response of CD22−/− B cells, probably because early B cell response was not examined. In our present study, analysis of the very early phase of B cell response using adaptive transfer of monoclonal B cells enabled us to demonstrate augmented response of CD22−/− B cells.

The finding we report here demonstrates that CD22 is a good target in inducing early Ab production in primary immune responses. Although primary responses do not efficiently produce high affinity Abs, unmutated Abs are also capable of protecting host against pathogens (45, 46). Therefore, CD22 antagonists may augment host defense against pathogens by inducing rapid Ab responses. In the presence of CD22 antagonists, B cell response may rapidly decline, but this problem may be overcome by a reagent that augments B cell survival. Because early Ab production is crucial for efficient prevention of acute infectious diseases by immunological memory, CD22 antagonists together with inducers of B cell survival may be useful to develop a new strategy for host defense as a “universal vaccine” that prevents acute infectious diseases regardless of pathogens.

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**Disclosures**

The authors have no financial conflict of interest.

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