lesions, about 1 to 3 mm in diameter. No skin lesions were found on the other two IRF-1$^{-/-}$ mice, but they too were less alert and less active than the BCG-infected wild-type mice, which showed no clinical signs of disease. Histologically, the skin lesions of the one IRF-1$^{-/-}$ mouse were granulomas with a morphological pattern compatible with miliary cutaneous tuberculosis (*26*), which contained large numbers of acid-fast bacilli (Fig. 5A). Many acid-fast bacteria were also found in abundant granulomatous lesions in the livers (Fig. 5B), lungs, and spleens (*27*) of all three IRF-1$^{-/-}$ mice. In contrast, acid-fast rods were not found in the livers (Fig. 5C) and other organs of wild-type mice, and these organs contained smaller, and slightly fewer, granulomatous lesions than were found in IRF-1$^{-/-}$ mice (*27*). These findings indicate that, in comparison with wild-type mice, BCG infection is much more extensive and severe, and *M. bovis* elimination is impaired, in IRF-1$^{-/-}$ mice. Although it is tempting to conclude that the lack of NO generation by IRF-1$^{-/-}$ macrophages is responsible for the more severe course of BCG infection, other reasons cannot be ruled out. However, it seems unlikely that the more severe BCG infection was due to the reduced CD8$^+$ T cell numbers in the IRF-1$^{-/-}$ mice (*14*), because mice with a null mutation in the β_2-microglobulin gene that lack functional CD8$^+$ T cells are no different than control mice in their response to BCG infection (*28*).

This study identifies an essential role for IRF-1 in iNOS gene induction in macrophages. This role is specific for IRF-1, because macrophages from mice with a null mutation in the IRF-2 gene did release substantial amounts of NO_2^- on stimulation with IFN-γ and LPS (*27*). The demonstration that IRF-1 is required for iNOS gene activation explains the earlier postulated need for intermediary protein synthesis in iNOS mRNA induction by cytokines (*29*). The essential role for IRF-1 may make it possible to develop new strategies for enhancing or blocking NO generation through modulation of IRF-1.

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30. Homozygous IRF-1$^{-/-}$ mice (*14*) and wild-type (129/SV/EV × C57BL/6 × DBA2) mice, aged between 2 and 12 months, were used in the experiments. (C57BL/6 × DBA2)F_1 mice (Jackson Laboratory, Bar Harbor, ME) were used as wild-type controls in some experiments. Mice were killed 3 to 4 days after intraperitoneal injection with 2 ml of 4% thioglycollate broth (Sigma). Peritoneal exudate cells, harvested in 10 ml of phosphate-buffered saline (PBS), were washed and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). The endotoxin level of the FBS lot used was 0.3 endotoxin units per milliliter, as determined by the Limulus amebocyte lysate assay. Adherent monolayers were obtained by plating 1×10^5 cells per well in 96-well microplates. Peritoneal cells were adhered for 1 hour at 37°C in 5% CO_2 and washed three times with complete medium to remove nonadherent cells. Adherent monolayers were treated with cytokines, with or without LPS, for 48 hours. NO_2^- concentration in the medium was measured by a microplate assay method (*2, 16*).
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Positive and Negative Thymocyte Selection Induced by Different Concentrations of a Single Peptide

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T lymphocyte maturation is dependent on interactions between the T cell receptor (TCR) expressed on the developing thymocyte and intrathymic major histocompatibility complex (MHC)–peptide ligands. The relation between the peptide-MHC complex that results in negative or positive selection has not been identified. Here, the requirements for the maturation of thymocytes expressing a defined transgenic TCR specific for a viral peptide are studied in fetal thymic organ culture. Low concentrations of the viral peptide antigen recognized by this transgenic TCR can mediate positive selection, whereas high concentrations result in thymocyte tolerance. These findings support the affinity-avidity model of thymocyte selection.

Only 5% of the immature T cells generated in the thymus develop to form part of the peripheral T cell repertoire (*1*). These cells are selected by interactions between the TCR and MHC class I or class II molecules present on cortical thymic epithelial cells (*2, 3*). Positive selection depends on the presence of CD4 and CD8 coreceptors (*4*),

the specificity of the heterodimeric receptor (5, 6), and for the CD8+ T cell lineage, peptides complexed with class I MHC (7–9). Negative selection eliminates or functionally inactivates thymocytes bearing receptors specific for self peptides complexed with self MHC molecules (10). Thus, MHC-associated self peptides mediate both positive and negative selection. One explanation for this conceptual paradox has led to the affinity-avidity model. This model postulates that a low-affinity interaction between the TCR and thymic ligand causes positive selection and survival of the thymocyte, whereas a higher affinity interaction results in tolerance.

To characterize a peptide capable of mediating positive selection in a well-defined system, we have studied the TCR transgenic mouse model (327 line) that expresses receptors specific for the lymphocytic choriomeningitis virus glycoprotein peptide (LCMV-gp) (p33-41) presented by H-2D^b (11). Mice expressing the 327 transgenic receptor were bred with mice containing a disrupted β_2-microglobulin gene (β_2M^-) (3) to generate TCR transgenic mice that were homozygous for the β_2M mutation (327,β_2M^-). Initial experiments using β_2M^- mice showed that the maturation of the CD8 lineage could be restored in fetal thymic organ culture by the addition of exogenous β_2M and a mixture of peptides (7). Therefore, we used a similar system and cultured 327,β_2M^- fetal thymic lobes with various concentrations of peptides and exogenous β_2M to determine the conditions that could restore maturation of the thymocytes expressing the 327 transgenic receptor.

Initial characterization of thymocytes from 327,β_2M^- mice was performed because previous evidence has suggested that low levels of the class I molecules may still be present on the cell surface in the absence of β_2M (12). Because the transgenic receptor is restricted to H-2D^b, we wanted to eliminate the possibility that residual expression of H-2D^b on the surface could mediate positive selection. Thymocytes from adult 327,β_2M^- (H-2^b), β_2M^- (H-2^b), 327,β_2M^+ (H-2^b), and 327,β_2M^+ (H-2^d) mice were stained with monoclonal antibodies specific for CD4 and CD8, CD3, the transgenic $V_\alpha2$ chain, and the transgenic β chain, $V_\beta8.1$. As shown in Fig. 1, positive selection of the transgenic receptor in 327,β_2M^+ (H-2^b) mice is characterized by skewing of the CD4,CD8 profiles to the CD8 single positive lineage (11), as well as by up-regulation of the transgenic receptor on CD4$^+$8$^+$ double positive and CD8$^+$ single positive thymocytes (13). In nonselecting 327,β_2M^+ (H-2^d) and 327,β_2M^- (H-2^b) mice, the TCR profiles showed the characteristic biphasic peak of immature and mature cells, and no skewing to the CD8$^+$ lineage (Fig. 1). The majority of CD3^{hi} cells also expressed high levels of $V_\beta8$ and had endogenously rearranged V_α chains. There was a variable but low number of CD8$^+$ thymocytes (0 to 4%) in β_2M^- (H-2^b) mice, and up to 5% of the thymocytes expressed either $V_\beta8$ or $V_\alpha2$. Because maturation of the transgenic TCR to the CD8$^+$ lineage did not occur in 327,β_2M^- (H-2^b) mice, this line was used to generate fetuses for thymic organ cultures.

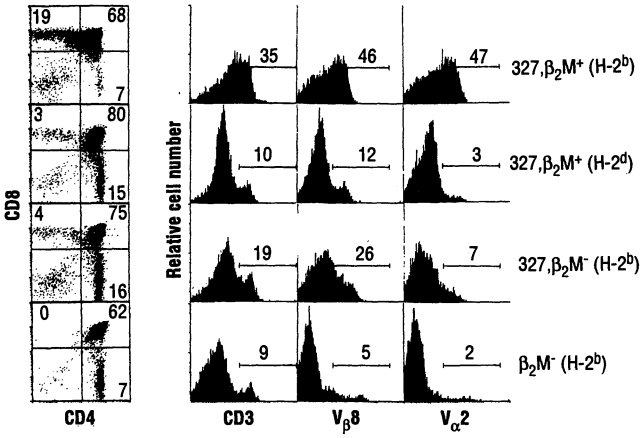

Fig. 1. Positive selection of the transgenic TCR does not occur in 327,β_2M^- mice. Thymocytes from 327,β_2M^+ (H-2^b), 327,β_2M^+ (H-2^d), 327,β_2M^- (H-2^b), or β_2M^- (H-2^b) mice were double stained with monoclonal antibodies specific for CD8 and CD4 or single stained with CD3, $V_\beta8$, or $V_\alpha2$ (24). The data are representative of thymocytes from at least three independent experiments (25).

To mimic signals for positive selection, we hypothesized that changes in avidity may be reflected by the "number" of transgenic TCRs engaged in a peptide-specific interaction. In terms of the affinity-avidity model, one would predict that high concentrations of a defined peptide would lead to a high "level" of TCR occupancy and negative selection, whereas lower concentrations of the peptide would lead to a lower "level" of TCR-specific interactions and result in positive selection. Previous studies have suggested that the peptide required for positive selection may be similar to the peptide antigen (14). Therefore, we decided to test whether different concentrations of the LCMV-gp peptide (p33-41) could promote positive or negative selection of TCR transgenic thymocytes in thymic organ culture.

Proliferation assays were done to obtain an estimate for the concentrations of peptide antigen to be used in thymic organ culture. Mature T cells from 327,β_2M^+ (H-2^b) transgenic mice responded to antigen-presenting cells pulsed with peptide antigen in concentrations ranging from 10^{-4} to 10^{-14} M. Maximal proliferation occurred at $\sim10^{-8}$ M and was reduced to minimal levels at 10^{-12} M (Fig. 2). Therefore, 10^{-6} M and 10^{-12} M peptide concentrations were chosen in an attempt to mimic the differences in signals required for negative and positive selection, respectively.

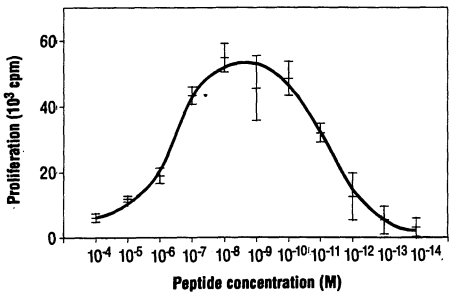

Fig. 2. Viral peptide concentrations required to generate a proliferative response from LCMV-specific T cells. Proliferation of spleen cells from 327 (H-2^b, β_2M^+) transgenic mice stimulated with macrophages (H-2^b, β_2M^+) pulsed with various concentrations of peptide (26).

Day 16 β_2M^- or 327,β_2M^- fetal thymic lobes were incubated in the presence of exogenous β_2M, at 10^{-6} M or 10^{-12} M peptide or in the absence of peptide. After 8 days of culture, thymocytes were analyzed with monoclonal antibodies specific for CD4 and CD8, and $V_\alpha2$ (Fig. 3). $V_\alpha2$-specific staining was used because it represented the characteristic increase in TCR density associated with positive selection (Fig. 1) (13). In the absence of exogenous peptide, the profiles of the 327,β_2M^- mice appeared similar to those of the β_2M^- mice, and there was no prominent $V_\alpha2^+$ or CD8$^+$ population. Control β_2M^- thymocytes cultured with 10^{-12} M LCMV-gp (p33-41) showed no alteration in the thymocyte profiles examined by flow cytometry. An average of 9.6 ± 1.3% (n = 5) CD8$^+$ cells were observed in thymocytes from 327,β_2M^- thymic lobes incubated with 10^{-12} M peptide. Control 327,β_2M^- thymic lobes cultured in the absence of peptide had an average of 3.5 ± 0.2% (n =

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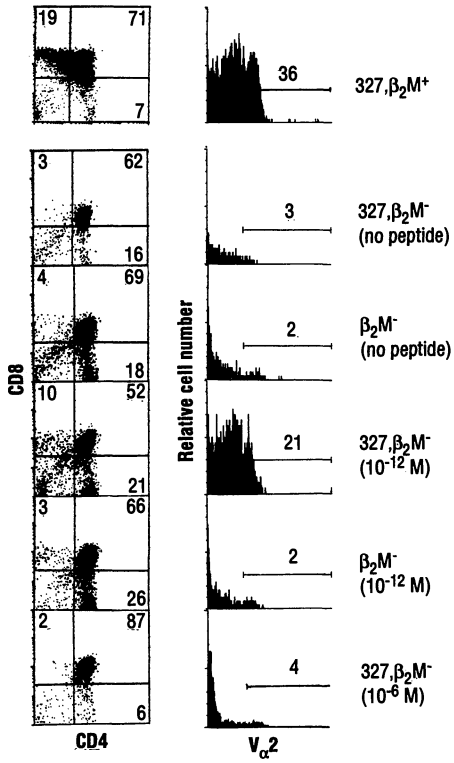

Fig. 3. Addition of viral peptide results in positive selection of transgenic T cells in fetal thymic organ culture. Representative profiles from a control adult 327,β_2M^+ thymus; 327,β_2M^- and β_2M^- fetal thymuses cultured without peptide; 327,β_2M^- and β_2M^- fetal thymuses cultured in the presence of 10^{-12} M peptide; and 327,β_2M^- fetal thymus plus 10^{-6} M peptide (*27*). The intensity of transgenic TCR staining of cultured fetal thymic lobes from 327,β_2M^+ mice was shown to be similar to the profiles obtained from 327,β_2M^+ adult thymus.

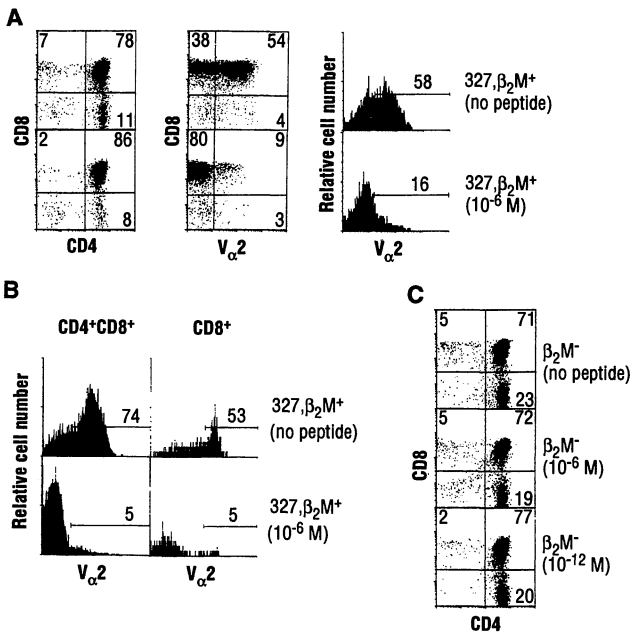

Fig. 4. Elimination of transgenic T cells in the presence of high concentrations of peptide. (**A**) Fetal thymic lobes from mice expressing the 327 transgenic receptor were cultured in the presence or absence of 10^{-6} M peptide. Two-color analyses using anti-CD4 and anti-CD8, or anti-CD8 and anti-$V_\alpha2$, and single-color profiles of $V_\alpha2$ are shown (*28*). (**B**) Three-color analysis of cultured fetal thymic lobes from 327,β_2M^+ mice were analyzed with antibodies specific for CD4, CD8, and $V_\alpha2$. The $V_\alpha2$ profiles from gated $CD4^+CD8^+$ and $CD8^+$ single positive cells are shown. (**C**) High concentrations of peptide did not prevent maturation of thymocytes in culture. β_2M^- lobes were cultured alone or in the presence of 10^{-6} or 10^{-12} M peptide, and two-color fluorescence profiles of CD4 and CD8 analysis are shown.

3) $CD8^+$ cells, and β_2M^- lobes incubated with peptide (10^{-12} M) contained 4.0 ± 0.4% (n = 5) $CD8^+$ cells. Therefore, a statistically relevant increase ($P < 0.005$) of $CD8^+$ thymocytes was observed in 327,β_2M^- fetal thymic lobes cultured with 10^{-12} M peptide. It is unlikely that all $CD8^+$ cells represent the early immature lineage for two reasons. First, we have observed that day 16 fetal thymocytes have a detectable immature $CD8^+$ population that becomes $CD4^+8^+$ after several days in culture. Second, if the $CD8^+$ cells detected in 327,β_2M^- thymic lobes cultured with 10^{-12} M peptide represent the immature population, then all thymic lobes, regardless of phenotype, should have similar numbers of these cells.

In addition, culture of 327,β_2M^- thymic lobes in the presence of 10^{-12} M peptide resulted in a substantial up-regulation of $V_\alpha2$ to levels similar to those observed on thymocytes from 327,β_2M^+ ($H\text{-}2^b$) mice undergoing positive selection (Fig. 3). This increase in the TCR density to high levels on the double positive cells has previously been well characterized and correlated with positive selection in both transgenic and nontransgenic systems (*13, 15*). Taken together, these data indicate that positive selection of thymocytes expressing the 327 transgenic receptor has occurred in the presence of 10^{-12} M peptide antigen.

The 8-day culture of 327,β_2M^- fetal thymic lobes in the presence of 10^{-6} M viral peptide resulted in a reduction in the number of thymocytes expressing the transgenic $V_\alpha2$ chain, as compared to thymic lobes incubated with 10^{-12} M peptide. A reduction in CD4 single positive mature cells from 18 to 6% was also observed (Fig. 3). To clearly demonstrate that negative selection had occurred, we cultured thymic lobes from positively selecting 327 mice for 5 days in the presence of 10^{-6} M peptide. A marked reduction in the number of $V_\alpha2^+$ cells occurred on $CD4^+8^+$ cells as compared with thymic lobes cultured in the absence of peptide (Fig. 4A). In addition, three-color analysis demonstrated that the $CD8^+$ cells did not express $V_\alpha2$ when cultured in the presence of 10^{-6} M peptide (Fig. 4B). To demonstrate that this was not due to toxic effects of the peptide, we cultured β_2M^- thymic lobes with various concentrations of peptide and analyzed the results by two-color CD4 and CD8 staining (Fig. 4C). The total cell number of the thymic lobes or maturation of the β_2M^- thymocytes was not altered in the presence of peptides. These results demonstrate that tolerance of double positive thymocytes expressing the transgenic TCR occurred in fetal thymic lobes cultured in the presence of high concentrations of peptide. These profiles are similar to previously published data where clonal deletion was demonstrated in the thymus of animals chronically infected with virus (*10, 13*) and where deletion of thymocytes occurred in the presence of this peptide in vitro (*16*).

Several reports have suggested that the "strength" of the interaction between the TCR on a developing thymocyte and its ligand (or ligands) will govern whether it undergoes positive or negative selection. This interaction may be strengthened or weakened by minor variations of the restriction element or by alterations in the level of coreceptor expression. For example, maturation of thymocytes expressing the 2C TCR transgene was investigated by breeding the transgenic mice with mouse strains expressing various mutations of the restriction element $H\text{-}2K^b$. A few amino acid substitutions in the $H\text{-}2K^b$ molecule led to either positive or negative selection of thymocytes bearing the transgenic TCR (*17*). Further studies have shown that the maturation of the 2C transgenic TCR can be switched from positive selection to negative selection by an increase in the amount of coreceptor CD8 molecule expressed on the surface (*18*). These and other experiments suggest that a range of avidities exist for positive and negative selection, where "stronger" interactions result in negative selection (*6, 19*).

Although negative selection of a defined TCR has been shown to occur in the presence of the peptide antigen, other experiments suggest that the interactions that lead to negative selection of thymocytes are less stringent than those required for peripheral T cell activation. Studies have

demonstrated that the concentration of antigen required for tolerance of double positive thymocytes is below the concentration required to detect a mature T cell response (*20*). It has also been shown that presentation of particular antigens in the thymus can delete a defined population of thymocytes, whereas the same antigens presented to mature T cells expressing the same TCR do not elicit a response (*21*). Although some of the differences may be attributed to the comparison of immature and mature T cells, these experiments also suggest that the "avidity" required for T cell activation is stronger than negative selection.

Collectively, these models suggest that differences in the affinity between the TCR and its ligand exist for positive selection, negative selection, and T cell activation. One way to explain the different developmental fates of the T cell has been to invoke the presence of two sets of peptides, one for low-affinity and one for high-affinity interactions, that mediate positive or negative selection, respectively (*22*). Our model has shown that no special peptides are involved in discriminating between positive or negative selection, since the viral peptide antigen that is recognized by mature T cells will mediate both positive and negative selection. Further experiments with unrelated H-2D^b–binding peptides will define how precise the interaction must be for positive selection. Our findings suggest that the avidity of the interactions between the thymocyte and stromal elements is reflected by the number of TCRs engaged with MHC molecules at a given time. This in turn may alter the strength of intracellular signals. It is likely that other molecules influence the stability of the TCR:peptide-MHC interactions and directly affect the signals generated through the TCR. Further studies with this model will help define the key molecules and interactions required for selection in the thymus.

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23. Thymocytes (1×10^6) were stained with phycoerythrin (PE)-conjugated antibody to CD4 (anti-CD4) (Becton Dickinson), fluorescein isothiocyanate (FITC)–conjugated anti-CD8 (Becton Dickinson), or supernatant from hybridomas KT3 (anti-CD3), KJ16 (anti-V_β8.1,8.2), or B20.1 (anti-V_α2) (*24*), followed by staining with FITC-conjugated goat antibody to rat immunoglobulin G (TAGO). Ten thousand events were collected in list mode storage with FACScan software (Becton Dickinson), and samples were gated on live cells by a combination of forward and side scatter and analyzed with LYSYS software (Becton Dickinson).
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25. Animals used in this study were handled in accordance with our institutional guidelines.
26. Spleen cells (1×10^5) from TCR transgenic mice were incubated with 2×10^4 macrophages that were prepulsed with various concentrations of p33-41 (Lys-Ala-Val-Tyr-Asn-Phe-Ala-Thr-Met) for 3 hours at 37°C. After 48 hours of cocultivation, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham) for 16 hours. Cells were harvested and counted on a Matrix 96 direct beta counter (Canberra Packard). Background proliferation for T cells incubated with macrophages in the absence of peptides was 300 cpm.
27. Day 16 fetal thymic lobes were cultured on 0.8-μm polycarbonate filters (Costar) in serum-free media [Iscove's modified Dulbecco's media, 2% Ultroser (Gibco), human β_2M (2.5 μg/ml; Sigma), glutamine, and antibiotics]. Peptides were added daily at a concentration of 10^{-6} or 10^{-12} M. After 8 days in culture, the lobes were harvested and stained with monoclonal antibodies as described (*23*).
28. The V_α2 used in two-color analysis was B20.1 conjugated to PE (Pharmingen). For three-color fluorescence, biotinylated B20.1 (Pharmingen) was detected with streptavidin-red 670 (Gibco/BRL).

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Requirement for BDNF in Activity-Dependent Survival of Cortical Neurons

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Cultured embryonic cortical neurons from rats were used to explore mechanisms of activity-dependent neuronal survival. Cell survival was increased by the activation of voltage-sensitive calcium channels (VSCCs) but not by activation of *N*-methyl-D-aspartate receptors. These effects correlated with the expression of brain-derived neurotrophic factor (BDNF) induced by these two classes of calcium channels. Antibodies to BDNF (which block intracellular signaling by BDNF, but not by nerve growth factor, NT3, or NT4/5) reduced the survival of cortical neurons and reversed the VSCC-mediated increase in survival. Thus, endogenous BDNF is a trophic factor for cortical neurons whose expression is VSCC-regulated and that functions in the VSCC-dependent survival of these neurons.

The patterning of connections in the developing nervous system is determined by the axonal and dendritic growth of individual neurons as well as by naturally occurring cell death. There is considerable evidence that both the morphology and survival of neurons can be influenced by neuronal activity (*1–4*). The survival of peripheral neurons in vitro is enhanced by the activation of VSCCs. In central neurons another major source of calcium entry is the *N*-methyl-D-aspartate (NMDA) subtype of

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