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Protein Energy Malnutrition Impairs Homeostatic Proliferation of Memory CD8 T Cells

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Nutrition is a critical but poorly understood determinant of immunity. There is abundant epidemiological evidence linking protein malnutrition to impaired vaccine efficacy and increased susceptibility to infections; yet, the role of dietary protein in immune memory homeostasis remains poorly understood. In this study, we show that protein-energy malnutrition induced in mice by low-protein (LP) feeding has a detrimental impact on CD8 memory. Relative to adequate protein (AP)-fed controls, LP feeding in lymphocytic choriomeningitis virus (LCMV)-immune mice resulted in a 2-fold decrease in LCMV-specific CD8 memory T cells. Adoptive transfer of memory cells, labeled with a division tracking dye, from AP mice into naïve LP or AP mice demonstrated that protein-energy malnutrition caused profound defects in homeostatic proliferation. Remarkably, this defect occurred despite the lymphopenic environment in LP hosts. Whereas Ag-specific memory cells in LP and AP hosts were phenotypically similar, memory cells in LP hosts were markedly less responsive to polyinosinic-polycytidylic acid-induced acute proliferative signals. Furthermore, upon recall, memory cells in LP hosts displayed reduced proliferation and protection from challenge with LCMV-clone 13, resulting in impaired viral clearance in the liver. The findings show a metabolic requirement of dietary protein in sustaining functional CD8 memory and suggest that interventions to optimize dietary protein intake may improve vaccine efficacy in malnourished individuals. The Journal of Immunology, 2012, 188: 77–84.

Generation of long-lived immune memory is the basis of vaccination and infection-generated immunity (1). Memory CD8 T cells arise after Ag clearance from a small subset of effectors derived from the clonal expansion and differentiation of naïve T cells responding to Ag (2, 3). Upon Ag re-encounter, Ag-specific memory CD8 T cells respond more rapidly and efficiently than naïve T cells, conferring the host with long-term protection (4). This dynamic memory pool is maintained at a relatively constant size for prolonged periods, even up to a lifetime, by homeostatic proliferation (5).

Homeostatic proliferation is a process in which influx of newly generated memory cells, by slow turnover, is balanced by death of pre-existing memory cells (6). The signals needed for memory homeostasis are well studied; it is independent of Ag and dependent on the concerted action of the γ-chain cytokines IL-7, for survival, and IL-15, for turnover (7, 8). Cell survival and turnover also depend on nutrient availability; very little, however, is known about how nutrition impacts memory CD8 T cell homeostasis. Because proliferation of memory cells imposes a metabolic demand on amino acid supply to support protein synthesis, dietary protein may be critical in sustaining T cell memory.

The present study was designed to determine whether homeostatic proliferation of memory CD8 T cells is impaired in protein-energy malnourishment (PEM). PEM is a major form of malnutrition and defined as an imbalance between intake of protein and energy and the optimal requirement to ensure the most favorable body growth and function (9). Malnutrition is a major health concern among millions in the developing world, and accumulating evidence suggests that malnutrition also afflicts HIV-infected patients, individuals with chronic disease, and aged populations in industrialized nations (10, 11). There is abundant epidemiological evidence suggesting that malnutrition impairs vaccine efficacy and increases susceptibility to infections; yet, the underlying mechanisms remain poorly understood (9, 12). A better understanding of the role of nutrition in memory homeostasis may open avenues for nutritional interventions to improve vaccine efficacy in malnourished individuals.

We assessed quantitative and qualitative aspects of CD8 T cell memory in lymphocytic choriomeningitis virus (LCMV)-immune mice (>40 d postinfection) fed either a low-protein (LP) diet to develop PEM or an adequate-protein (AP) control diet. After wk of dietary intervention, LP mice demonstrated a 2-fold reduction in Ag-specific memory CD8 cells compared with AP-fed controls, suggesting impaired proliferation due to PEM. Using adoptive transfer of CFSE-labeled memory cells from AP mice into naïve LP or AP mice, we confirmed that PEM caused profound defects in homeostatic proliferation. Interestingly, memory cells in AP or LP hosts were phenotypically similar with respect to the γ-chain cy-
Materials and Methods

Mice, virus, and infections

Four- to 6-wk-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Thy1.1+ P14-transgenic mice with CD8 T cells expressing the TCR specific for the Db/GP33-41 epitope of LCMV were obtained from The Jackson Laboratory and backcrossed to B6 mice in our colony. Mice were housed in cages and maintained on a 12-h light/12-h dark cycle at the Division of Animal Resources at Emory University. All experiments were initiated during the light cycle. Mice were infected with 2 × 10^5 PFU/100 μl LCMV Armstrong i.p. For secondary challenge experiments, 2 × 10^6 PFU LCMV-clone 13 was given i.v. Recombinant vaccinia virus (Vv)-GP33 expressing the GP33 epitope of LCMV was propagated and used as previously described (13). Mice were infected with 2 × 10^6 PFU VV-GP33. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

Diets

Semi-purified diets were custom-prepared (Harlan-Teklad, Madison, WI) to test the specific effects of protein energy malnutrition on memory CD8 T cell homeostasis (14). Protein levels were controlled at the desired experimental level by decreasing amounts of essential and nonessential amino acids. The LP diet contained 0.6% (5 g/kg) of protein, and the AP diet contained 18% (210 g/kg) by weight. LP diets contained more carbohydrate in the form of sucrose and cellulose compared with AP diets but were otherwise isocaloric, and both diets contained equal and identical quantities of energy, fat, and micronutrients. Animals had free access to water and food at all experimental time points.

Flow cytometry

Single-cell suspensions were prepared from spleen, and 10^6 cells were stained in PBS containing 2% FBS and 0.02% sodium azide (FACS buffer) for 30 min at 4°C followed by three washes in FACS buffer. Cells were stained with anti–CD8a-PerCP (53-6.7), Thy1.1-PE (OX-7), CD122-PE, CD4–allophycocyanin (MP6-XT22), IL-2–PE (JES6-5H4), Ki-67–FITC (B50) (all from BD Biosciences), CD127–PE-Cy7 (AKR34; ebioscience, San Diego, CA), IL-15Ra–FITC (BAF551; R&D Systems, Minneapolis, MN), and CD44-Pacific Blue (IM7; BioLegend, San Diego, CA). Intracellular staining for Bcl-2 and Ki-67 was performed directly ex vivo. Cytokine staining for IFN-γ, TNF-α, or IL-2 was done after a 5-h in vitro stimulation with 0.1 mg/ml gp33 peptide using the Cytofix/Cytoperm kit, according to the manufacturer’s instructions (BD Biosciences). MHC class I peptide tetramers were generated and used as described (15). Samples were acquired using either FACS Calibur or FACS Canto II instruments (BD Biosciences).

Data were analyzed using FlowJo software (Tree Star).

CFSE labeling and adoptive transfer

CFSE labeling was performed as described (16). Splenocytes were obtained from immune C57BL/6 mice or immune P14 chimeric mice. Fifty million CFSE-labeled splenocytes were adoptively transferred i.v. via tail vein injection into naive recipients that had been on the LP or AP diet for 2 wk. Longitudinal monitoring of T cell proliferation was performed by saphenous vein bleeding into 4% sodium citrate under isoﬂuorane anesthesia.

Poly(I:C) treatment

Mice were treated with 150 μg poly(I:C) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 ml PBS or PBS alone by i.p. injection, as previously described (17). Splenocytes and bone marrow cells were analyzed 2 d later for cycling memory CD8 T cells by Ki-67 staining.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed by the two-tailed Student t test using Prism software (GraphPad, La Jolla, CA). A p value ≤0.05 was used to determine significance.

Results

PEM decreases CD8 T cell memory

Increased incidence of infections in protein-malnourished individuals suggests that dietary protein may be a critical determinant of immune memory. But the role of nutrition in memory homeostasis is yet to be precisely defined. The objective of the current study was to determine whether PEM impairs CD8 memory homeostasis. Because PEM compromises the CD8 effector response to LCMV (14), which, in turn, could contribute to decreased CD8 memory, we designed the study to specifically examine the effect of PEM on established CD8 memory.

Mice were infected with the Armstrong strain of LCMV, which initiates a vigorous immune response resulting in clearance of virus and the subsequent generation of a robust memory pool (3). LCMV-immune mice (>40 d postinfection) were randomly assigned to receive either an AP diet (18% dietary protein) or an LP diet (0.6% dietary protein) (Fig. 1A). The diets were formulated to be isocaloric; therefore, the LP diet provided excess energy from carbohydrates (14). Because optimal amino acid supply is critical for cell proliferation and function, the effect of LP diet on CD8 T cells may be directly attributed to limited amino acid supply in the LP diet.

As previously described, after 4 wk on a LP diet, mice become protein-energy malnourished (14). This experimental setup allows us to quantitatively assess the effects of PEM on CD8 memory homeostasis without the confounding effects of malnutrition on memory precursors and/or viral control.

As demonstrated previously, weight loss occurs within 1 wk of initiating the LP diet and continues throughout the dietary period (14). After 4 wk, mice on the LP diet showed a significant weight loss of 20% of body weight, whereas body weight in AP mice remained relatively stable (data not shown). We first examined whether PEM quantitatively impaired CD8 T cell memory. To this end, lymphocytes were isolated from the spleen of AP and LP mice and examined for memory cells specific for the LCMV epitopes Db/GP276-286 and Db/GP33-41. Whereas no differences in percentage of CD44hi (33% in both groups) or total Ag-specific memory CD8 cells were observed between both dietary groups (Supplemental Fig. 1A), estimation of total numbers of Ag-specific T cells in the spleen revealed a 2-fold decrease in LP mice compared with AP mice (p < 0.05; Fig. 1B). The size of the total CD44-high memory pool was decreased by 2.8-fold in malnourished mice.

We next determined whether the functional capacity of Db/ GP276 memory cells was also impaired in LP mice. For this purpose, splenocytes were stimulated for 5 h with GP276-286 peptide, and cytokine production was determined. As measured by the mean fluorescence intensity (MFI), IFN-γ, TNF-α, and IL-2 production was not different between the two dietary groups, suggesting that cytokine production on a per-cell basis ex vivo was not impaired by PEM (Fig. 2A, 2B). However, the total number of cytokine-producing cells was significantly reduced in LP mice (Fig. 2C).

PEM decreases acute homeostatic proliferation of CD8 memory T cells

Decreased CD8 memory in LP hosts could be due to defects in homeostatic proliferation; because the receptors for the γ-chain cytokines are key signals for homeostatic proliferation (8, 18), we first determined whether expression of IL-7R and IL-15R was decreased on memory cells in LP mice. We found no difference either in percentage of receptor-positive cells or MFI of Ag-specific CD8s between both dietary groups (Supplemental Fig. 1; solid histogram denotes expression in GP276 tetramer+ CD8
T cells, and black lines denote expression levels in CD44-low naive CD8 T cells. These data suggested that memory cells in the LP environment were poised to receive proliferative signals from IL-15. To directly assess responsiveness of memory CD8 T cells to acute proliferative signals, we treated AP and LP LCMV-immune mice with poly(I:C). Poly(I:C), an RNA analog that induces IFN-γ, which in turn upregulates IL-15 production, leading to proliferation of memory CD8 T cells in an IL-15–dependent manner (17, 19). Three days after poly(I:C) treatment, lymphocytes were isolated from the spleen and bone marrow, a major site for homeostatic proliferation (17), and division of memory cells was assessed by staining lymphocytes with the cell proliferation marker Ki-67 (Fig. 3A). As shown in Fig. 3B, frequency of Ki-67–positive cells (of total CD44-high CD8 T cells) was lower in the spleen and bone marrow in LP mice, suggesting that PEM caused defects in acute homeostatic proliferation. Frequency of LCMV-specific cells (gated on GP276 tetramer+ cells) responding to poly (I:C) treatment was also markedly decreased (5% in LP mice compared with 21% in AP controls; Supplemental Fig. 2).

**FIGURE 1.** Effect of PEM on LCMV-specific memory CD8 T cells. A, Experimental outline: 6–8-wk-old B6 mice were infected i.p. with LCMV Armstrong and after 40 d postinfection, mice were assigned to receive either an AP (18% dietary protein) or an LP diet (0.6% dietary protein). Analysis of LCMV-specific memory CD8 T cells in the spleen was performed after 4 wk of dietary treatment. B, Db/GP276 and Db/GP33 tetramer staining of CD8 T cells from the spleen of AP- (top panel) and LP-fed (bottom panel) mice. Data are percent of tetramer-positive CD8 T cells. C, Absolute numbers of Ag-specific CD8 T cells in the spleen in AP- (black bars) and LP-fed (white bars) mice. One representative experiment of three is shown. Data are mean of four mice per group ± SEM. *p < 0.05.

**FIGURE 2.** Effect of PEM on cytokine production by LCMV-specific memory CD8 T cells. A, Intracellular cytokine staining for IFN-γ, TNF-α, and IL-2 after 5 h of GP276 peptide stimulation. Data are percent of CD8 T cells that produced cytokine. B, MFI of cytokine expression after GP276 stimulation. C, Total cytokine-producing cells in the spleen after GP276 in AP- (black bars) and LP-fed (white bars) mice. One representative experiment of three is shown. Data are mean of four mice per group ± SEM. *p < 0.05.
PEM decreases basal homeostatic proliferation of CD8 memory T cells

In addition to defects in acute homeostatic proliferation, lower numbers of LCMV-specific memory CD8 T cells in LP mice suggested that PEM may also impair basal homeostatic proliferation. To determine if this was the case, memory cells generated in an AP environment were labeled with the division tracking dye CFSE and adoptively transferred into naive recipients that had been maintained either on AP or LP diets for 2 wk (Fig. 4A). Dietary regimes were continued for 4 wk after cell transfer. At this time, lymphocytes were isolated from the spleen to compare division of CFSE-labeled cells between AP and LP mice. Inspection
FIGURE 5. Protective immunity of memory CD8 T cells in PEM. A. To assess protective immunity of memory CD8 T cells in PEM, memory cells from AP-fed P14 chimeric mice were labeled with CFSE and transferred to naive AP- or LP-fed recipients. Recipients were challenged with LCMV-clone 13, and recall responses were assessed 7 d after transfer. B shows a representative flow plot of percentage of Thy 1.1\(^{+}\)Db/GP33\(^{+}\) tetramer-specific CD8 T cells in AP- and LP-fed hosts. Bar graph shows total Db/GP33 tetramer-specific CD8 T cells per spleen. In C, intracellular cytokine staining for IFN-\(\gamma\) and IL-2 after 5 h of GP33 peptide stimulation is shown. Data are percent of CD8 T cells that produced both IFN-\(\gamma\) and IL-2. In D, viral titers in liver and lung are shown. *\(p < 0.05\).

PEM impairs recall responses of memory CD8 T cells
Because rapid recall response to secondary infection is a characteristic feature of memory cells (3), we next investigated whether PEM also compromised the ability of memory CD8 T cells to respond to a secondary infection. For this purpose, memory cells from Thy 1.1\(^{+}\) P14 chimeric-immune mice were adoptively transferred into naive recipients that had been maintained either on AP or LP diets for 2 wk. Mice were infected with the clone 13 strain of LCMV at 1 d posttransfer, and the secondary memory response was assessed at day 7 posttransfer (Fig. 5A).

At 1 d posttransfer, similar numbers of Thy1.1\(^{+}\) P14 cells were present in adoptive AP and LP hosts (data not shown). However, examination of secondary effector proliferation at day 7 revealed a 10-fold decrease in secondary effectors in LP hosts compared with AP hosts (Fig. 5B; AP mice, 30.3 \(\pm\) 8.2; LP mice, 3.1 \(\pm\) 0.8; \(p < 0.05\)). A representative flow cytometric analysis of cells is shown. We stained for several surface activation markers such as CD27, CD127, CD69, CD62L, programmed death-1, CD122, and...
CD127 and found no difference in expression of these molecules between AP and LP hosts (data not shown). Furthermore, Thy.1.1+ P14 cells in AP and LP hosts were functionally competent and could produce IFN-γ, TNF-α, and IL-2 upon GP33 peptide stimulation ex vivo (Fig. 5C). The observation that LP hosts were unable to support robust expansion of secondary effectors would suggest that LP hosts would be less effective at controlling virus compared with AP hosts. Indeed, assay of viral titers in the liver revealed significantly higher titers in LP mice compared with AP mice (LP mice, 5.0 ± 0.07 log10/mg of tissue; AP mice, 4.2 ± 0.4 log10/mg of tissue; p < 0.05; Fig. 5D). No difference in viral titers was observed in the lung. In a separate experiment, we assessed recall proliferation of malnourished memory cells in AP versus LP hosts (Fig. 6A). Representative flow plots of donor cells in AP and LP hosts are shown in Fig. 6B. The data show that recall proliferation of LP memory cells in AP hosts was superior to that in LP hosts (Fig. 6C), suggesting that malnutrition-induced defects in proliferation may be rescued by AP supplementation.

Impaired memory maintenance in malnourishment is rescued by dietary protein supplementation

Finally, we wanted to assess whether malnutrition-induced impairment in memory maintenance could be rescued by dietary intervention with an AP diet. For this purpose, Armstrong immune P14 chimeric mice were assigned to one of three dietary regimens as outlined in Fig. 7A: 1) AP for 8 wk; 2) AP for 4 wk followed by switch to LP for 4 wk; and 3) LP for 4 wk followed by a switch to AP for 4 wk (LP-rescue). After 8 wk of each respective dietary treatment, lymphocytes were isolated from the spleen of AP, LP, and LP-rescue mice and examined for Thy.1.1+ memory cells specific for the LCMV epitope Db/GP33-41 (Fig. 7B). Estimation of total numbers of Ag-specific T cells in the spleen revealed that AP supplementation of LP hosts resulted in a complete quantitative rescue of memory CD8 T (Fig. 7C). Together, these data underscore the importance of dietary amino acid availability in optimal CD8 memory homeostasis (Fig. 8).

Discussion

Although nutrition is recognized as being a critical determinant of immunity, an understanding of the mechanisms by which poor nutrition impairs immune function remains poorly characterized (20–22). The current study bridges this gap by presenting two main findings: first, that maintenance of memory CD8 T cells is impaired in malnourishment due to defects in homeostatic proliferation; and second, that memory cells in malnourished hosts have defects in recall responses, leading to impaired viral clearance upon reinfection. These findings have important implications for understanding the basis of increased infections in malnourished individuals in the world’s poorest countries. The data also suggest that malnourishment that frequently accompanies aging and chronic diseases may contribute to impaired immunity observed in these states. In all, the data show that a robust memory response generated under nutritionally optimal conditions is impaired by poor nutrition.

The complex and reciprocal relationship between nutrition and infections has long been recognized. Studies in humans and experimental animals show that infections place a significant metabolic demand on the host; for instance, increased mobilization of amino acids from peripheral tissues, largely skeletal muscle, supports gluconeogenesis in the liver to meet the heightened glucose needs of rapidly dividing immune cells (12, 23, 24). Cell-culture studies show that activated T cells increase amino acid uptake by ~6-fold compared with resting cells (25). Collectively, these data indicate that activated T cells have increased amino acid requirements to support gluconeogenesis in the liver and meet increased metabolic demands placed on the host during infections. The data also underscore the importance of dietary amino acid availability in optimal CD8 memory homeostasis (Fig. 8).
acid requirements to support at least two fundamental processes, increased bioenergetic demands and increased protein synthesis.

It therefore comes as no surprise that cell-mediated immune responses are compromised in protein-malnourished individuals. Salimonu et al. (26) found significantly lower T lymphocyte count in malnourished compared with well-nourished children at baseline and at 3, 10, and 21 d postimmunization with measles virus. Several studies have also shown decreased responses to tuberculin test in infants and children with PEM (21, 27). More recently, we demonstrated that PEM compromises the effector response to LCMV infection in mice (14). Decrease in effector responses led to a 3 log-fold increase in serum viral titers in LP mice at day 8, whereas virus was completely cleared in control mice. Thus, there is abundant evidence indicating that malnutrition impairs effector CD8 responses to infection. However, relatively little is known about whether malnutrition impairs memory maintenance.

In the current study, we demonstrate that PEM induced a 2-fold decrease in the memory CD8 compartment. Total lymphocyte numbers were also decreased, suggesting that mice on an LP diet were lymphopenic. Studies have shown that lymphopenia, resulting from severe infection, chemotherapy, irradiation, or malnourishment, induces proliferation of naive cells to memory cells that are phenotypically indistinguishable from true Ag-experienced memory cells (28). Lymphopenia-induced proliferation of naive cells to memory-phenotype cells would lead to an overestimation of the memory compartment in LP hosts. Thus, the true deficit induced by PEM may be underrepresented. However, it is also possible that thymic output was decreased in LP mice, and/or circulating levels of IL-7 and IL-15 in LP mice were not sufficient to induce proliferation of naive cells to memory-phenotype cells (29).

Impaired memory homeostasis in PEM could result from decreased proliferation, increased cell death, or a combination of both. Adoptive transfer experiments using CFSE-labeled cells showed that at 4 wk posttransfer, 77% of donor cells had an undiluted CFSE profile in LP hosts compared with 25% in AP hosts. This suggests that defects in cell proliferation contributed to decreased memory pool in PEM. Further, comparable levels of the prosurvival protein Bcl-2 and Bcl-XL and the proapoptotic protein Bim in memory cells from LP and AP mice argue against a proapoptotic memory phenotype in LP hosts (Fig. 3).

In addition to defects in basal homeostatic proliferation, memory cells in LP hosts showed impaired responses to poly(I:C), which induces acute proliferation of memory cells in an IFN-γ-, IL-15-dependent manner (19). This defect, which was also seen in the bone marrow, one of the major sites of homeostatic proliferation, could be due to at least three reasons. First, cytokine levels induced by poly(I:C) in LP hosts may not have been sufficient to drive increased proliferation. Indeed, amino acids such as glutamine are critical for production of IFN-γ in T cells (25). Second, impaired activation of APC such as dendritic cells in LP hosts could also be a contributing factor. Third, limited amino acid availability in LP hosts could interfere with efficient cell-cycle progression and turnover. In vitro studies by Angelini et al. (30) showing that APC control T cell proliferation by controlling availability of cysteine in the extracellular fluid underscore the importance of nutrient availability in T cell turnover.

Interestingly, despite demonstrating marked deficiencies in basal and acute proliferation, memory cells in LP hosts did not appear to be phenotypically different from fully functional memory cells. This observation raises the possibility that the effects of malnutrition may not be T cell intrinsic but rather may be environmental and suggest that impaired memory maintenance in malnourished individuals may be amenable to rescue by protein supplementation.

Indeed, replenishing protein in malnourished hosts rescued the proliferative defects due to malnourishment (Figs. 7, 8). Furthermore, recall proliferation was superior during adoptive transfer of LP memory cells into AP hosts compared with LP hosts (Fig. 6). Although this result points to the critical role of environmental/host factors in regulating memory homeostasis, it does not exclude the possibility that any T cell-intrinsic defect resulting from malnutrition was also corrected as a consequence of protein supplementation. It is likely, therefore, that a combination of factors, both environmental and T cell intrinsic, contribute to impaired memory homeostasis in malnourishment. Experimental targeting of pathways involved in amino acid transport and metabolism in T cells and subsequent response in well-nourished and malnourished mice would be required to thoroughly dissect the role of T cell-intrinsic versus host/environmental factors in malnutrition.

It is noteworthy that in malnourished children, an improvement in immune function, as measured by Ab titers, after protein supplementation has been reported (31). In contrast, however, numerous studies have failed to find significant benefit of protein supplementation in the severely malnourished (32, 33). It is possible that there may be a narrow window for successful intervention, and the ability to rescue immune function may depend on a number of factors including severity of malnourishment, type of infection, and age of the host. Further studies are needed to determine whether memory homeostasis can be rescued by amino acid supplementation in malnourished individuals. Indeed, rigorous studies are also needed to determine the impact of malnutrition on immune responses to vaccines with marginal efficacy, as this is of direct clinical and public health relevance.

In summary, the data show that dietary protein intake is critical for the maintenance of a functional CD8 memory pool. The findings suggest that interventions to optimize dietary protein intake may improve vaccine efficacy in malnourished individuals and decrease susceptibility to infections in chronically infected malnourished patients.

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Disclosures

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