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IL-2 complexes have substantial effects on the cellular immune system, and this approach is being explored for therapeutic application in infection and cancer. However, the impact of such treatments on subsequent encounter with pathogens has not been investigated. In this study, we report that naive mice treated with a short course of IL-2 complexes show enhanced protection from newly encountered bacterial and viral infections. IL-2 complex treatment expands both the NK and CD8 memory cell pool, including a recently described population of preexisting memory-phenotype T cells responsive to previously unencountered foreign Ags. Surprisingly, prolonged IL-2 complex treatment decreased CD8 T cell function and protective immunity. These data reveal the impact of cytokine complex treatment on the primary response to infection. The Journal of Immunology, 2010, 185: 6584–6590.

Cytokine therapy, especially involving IL-2 treatment, has long been proposed as a way of manipulating the cellular immune response to enhance immunity to tumors and pathogens (1, 2). Limitations due to high-dose IL-2 toxicity may be circumvented by more recent approaches in which anti–IL-2 Ab/IL-2 complexes have been shown to be much more efficient at inducing changes in the cellular immune system (3). This has led to promising results using IL-2 complex treatment in mouse models of cancer (4–6), in reduction of persistent viral load (7), and as an adjuvant for enhancing immune responses (4, 8, 9). These studies all explore the effect of cytokine complex treatment on previously or simultaneously encountered Ags; however, the effect of such therapy on subsequent exposure to a novel pathogen has not yet been explored. Because substantial dysregulation of the immune system follows such treatments, it is not clear whether this is a benefit or a detriment to new immune responses.

IL-2 complex treatment has a dramatic effect on memory-phenotype (MP) (defined as CD44hiCD122hi) CD8+ T cells, leading to massive expansion of their numbers (3). Animals not exposed to an Ag previously would not be expected to contain Ag-specific memory T cells, yet our recent studies suggest that homeostatic processes produce memory-like T cells with specificities for unencountered Ags (10). Although we have previously shown that “homeostatic” memory cells, produced during the response to lymphopenia, can elicit effective protective immunity (11), it is not clear whether expansion of such cells influences the “primary” immune response to an infection with a novel pathogen. Experiments using OT-I TCR transgenic T cells showed some short-term protective benefit after IL-2 complex treatment, but deficiencies in cytokine production were noted, indicating these memory cells may not be fully functional (12). In addition to its effects on MP CD8+ T cells, IL-2 complex therapy also leads to transient expansion of the NK cell pool (3, 6), which may also contribute to innate immunity against newly encountered pathogens.

We sought to assess the protective potential of IL-2 complexes to elicit increased resistance against infection in naive mice. After short-term IL-2 complex immunization, naive mice are significantly protected against high-dose acute infections. We show that such IL-2 complex treatment effectively expands MP CD8+ T cells, increases their expression of granzyme B, and increases their capacity for IFN-γ production upon stimulation. Enhanced protection against Listeria monocytogenes infection involved contributions by both CD8 and NK cells. Furthermore, we found that CD8+ T cells primed after IL-2 complex treatment underwent more robust expansion upon a secondary infection. However, more extensive IL-2 complex therapy, despite producing even greater expansion of MP CD8+ T cells, led to a loss of protective immunity. Overall, these results indicate appropriate IL-2 complex cytokine therapy may enhance immune resistance to previously unencountered pathogens, suggesting potential as a novel vaccine tool.

Materials and Methods

Mice

Six- to twelve-week-old female C57BL/6 and B6.SJL mice were purchased from the National Cancer Institute (Bethesda, MD) or The Jackson Laboratory (Bar Harbor, ME). All mouse protocols were approved by the Institutional Animal Use and Care Committees at the University of Minnesota (Minneapolis, MN).

IL-2 complex treatment

Mice were injected i.v. with 1.0 μg carrier-free recombinant mouse IL-2 (eBioscience, San Diego, CA) combined with 10 μg anti–IL-2 Ab (S4B6; BioXCell, West Lebanon, NH). Mice given three injections of complex were injected on days 0, 2, and 4. Mice given five injections of complex received treatment on days 0, 1, 2, 3, and 4. Control mice were injected on the same schedule with 10 μg rat IgG2a (BioXCell) or left untreated.

In vitro stimulations

Splenocytes were incubated with PMA and ionomycin (Sigma-Aldrich, St. Louis, MO) or OVA peptide (SIINFEKL) and with brefeldin (BD Biosciences, San Jose, CA) for 6 h. Surface staining followed by intracellular staining for IFN-γ, TNF, and IL-2 was then performed. Cytokine stim-
ulations with IL-2, IL-12, and IL-18 were performed as described previously (10).

Flow cytometry and magnetic bead isolation
R8-B8R and R8-OVA tetramers were produced as described previously (13). Endogenous, tetramer-specific CD8+ T cells were enriched by magnetic bead separation (10, 14). Cells were analyzed on an LSR II (BD Biosciences), and data were evaluated using FlowJo software (Tree Star, Ashland, OR).

Bacterial and viral infections
Recombinant L. monocytogenes strains LM-OVA or attenuated LM-OVA (both expressing secreted OVA protein) were provided by Hao Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) and have been described elsewhere (15). Attenuated L. monocytogenes strain LM-B8R (expressing the B8R CD8 epitope from vaccinia virus) was provided by Dr. Ross Kedl (National Jewish Medical Research Center, University of Colorado, Denver, CO). Bacteria were grown in tryptic soy broth with 50 μg/ml streptomycin to an OD600 of ∼0.1. For LM-OVA challenges, 2 × 10^7 CFU were injected i.v. For primary infection with attenuated LM-OVA or LM-B8R, 3 × 10^6 CFU were injected i.v. The actual number of bacteria injected was confirmed by dilution and growth on tryptic soy broth plates containing streptomycin. For vaccinia virus infections, 2 × 10^6 PFU of Vaccinia virus strain Western Reserve (VV-WR) was diluted in PBS and injected i.v.

Determination of CFU
On day 3 postinfection, the spleen and liver were removed and placed in an 0.2% IGEPLA solution (Sigma-Aldrich). Organs were homogenized, and serial dilutions were plated onto tryptic soy broth plates containing 50 μg/ml streptomycin. Bacterial colonies were counted after plate incubation for ∼24 h at 37°C. The limit of detection (∼100 organisms) is indicated by a dashed line on graphs in the figures. Bacterial colonies were counted in Figs. 1, 3, 4, and 6.

Plaque assay
On day 3 postinfection with VV-WR, ovaries were harvested in PBS and frozen as a single-cell suspension. After two freeze–thaw cycles, the ovary homogenate was incubated at 37°C for 45 min with 0.25 mg/ml trypsin (Sigma). 143B cells (American Type Culture Collection) were grown to confluence. Dilutions of the ovary homogenate were added in duplicate to the cellular monolayer and left for 2 d. Staining with 1% crystal violet was then performed. Plaques were counted, and total viral load per ovaries was calculated.

Depletion of NK cells and CD8+ T cells
Mice were treated with 600 μg anti-NK1.1 (PK136; ATCC) or 1 mg anti-CD8ε (2.43; BioXCell) on day −1, day 1, and day 3 relative to IL-2 complex treatment (day 0, day 2, and day 4). Depletion of ≥97% was confirmed by staining of the spleen and liver.

Statistics
A two-tailed, unpaired Student t test was performed on the indicated data samples using Prism software (GraphPad, San Diego, CA). The p values are displayed in Figs. 1, 3, 4, and 6.

Results
Short-term IL-2 complex treatment protects naive mice from bacterial and viral infection
IL-2 complex treatment has been reported to expand selectively preexisting MP CD8 T cells and NK cells (3). However, the significance of such treatment on subsequent infection with a pathogen has not been explored. We sought to determine if IL-2 complex-treated naive mice would exhibit increased protection when challenged with a high-dose bacterial or viral infection. We treated mice with three injections of IL-2 combined with anti–IL-2 Ab S4B6 (as in Ref. 3) on days 0, 2, and 4. Four days after the last injection, mice were infected with a high dose of LM-OVA (Fig. 1A). After 3 d, high bacterial loads were recovered in the spleens and livers of control-treated mice, whereas L. monocytogenes-immune mice had reduced this bacterial burden by 3–4 logs (Fig. 1B, 1C). Remarkably, naive mice that received IL-2 complex reduced the bacterial load by 2–3 logs in both organs, despite the fact that these animals had not been primed with specific Ag. In a second cohort of mice, 5 of 9 control-treated mice died during the first week after infection, whereas all the IL-2 complex-treated mice (9 of 9) survived the infective dose (data not shown). As a second model of infection, IL-2 complex-treated naive mice were infected with 2 × 10^6 PFU of VV-WR. Three days postinfection, the ovaries (the major reservoir of infection) were harvested and assessed for viral load. Compared with controls, the IL-2 complex-treated animals showed a significant decrease in vaccinia virus load (Fig. 1D). Therefore, in two separate infection models (bacterial and viral), with either lymphoid (L. monocytogenes) infection or peripheral (vaccinia virus) sites of infection, IL-2 complex afforded significantly better elimination of pathogens in naive hosts.

IL-2 complex treatment increases CD8 T cells and NK cells in both lymphoid and nonlymphoid organs
We next sought to more closely examine the phenotype of CD8 T cells expanded with IL-2 complex treatment. This resulted in increased numbers of CD8 T cells and NK cells (NK1.1+, CD3+) in all organs analyzed (spleen, lymph nodes, bone marrow, lung, and liver) (Fig. 2A). CD4 T cells and NKT cells (CD3ε−, NK1.1+) were also slightly increased in number (data not shown). In addition, we found that the CD8 T cell population was overwhelmingly of
central-memory phenotype (CD44$^{hi}$, CD62L$^{hi}$) as well as expressing high levels of CD43 and CD27 (Fig. 2B and data not shown). Surprisingly, these cells also expressed high levels of granzyme B (Fig. 2B), which is typically associated with effector and effector-memory T cell populations, not central-memory T cells. However, this phenotype has been described previously for IL-2 complex-treated Ag-experienced memory cells in the context of a chronic infection (7). The enhanced number of cells and corresponding phenotypic changes appeared to last for approximately 2 wk (NK cells) to 3 wk (CD8 T cells) after the start of treatment (Supplemental Fig. 1). Therefore, after three injections of IL-2 complex over the course of a week, we found transiently increased numbers of MP CD8 T cells and NK cells in multiple organs, some of which were positive for a critical effector molecule (granzyme B).

Ag-specific MP CD8 T cells are increased in number with IL-2 complex treatment

Our previous studies indicated that in unimmunized animals, CD8 T cells specific for foreign Ags can be detected in the MP pool (10). It was therefore of interest to know whether IL-2 complex treatment led to an increase in numbers of MP CD8 T cells with specificities relevant to the pathogens studied. To examine endogenous, Ag-specific CD8 T cells, we used MHC class I tetramer staining followed by magnetic bead enrichment (10, 14). Using this technique, along with multiparameter flow cytometry, we found increases in the number of both K$^b$-B8R$^+$ and K$^b$-OVA$^+$ specific CD8$^+$ T cells (Fig. 3A). These are dominant epitopes in the CD8 T cell response to vaccinia virus and LM-OVA, respectively, and are likely to contribute to protection of the host (Fig. 1). By co-staining with the CD44 marker, we found that virtually all of the increase in cell number was due to an increase in the CD44$^{hi}$ compartment, which ranged from 10- to 12-fold (Fig. 3B, 3C). Importantly, the Ag-specific CD44$^{lo}$ compartment was conserved but did not significantly change in number. This agrees with previous adoptive transfer data showing that naive CD8 T cells do not divide in response to IL-2 complex treatment, which is likely due to their low expression of CD122 (3). However, our data contrast with that of another report that indicated both naive and memory OT-1 CD8 T cells were expanded by IL-2 complex (12). We conclude that in the endogenous, polyclonal repertoire, IL-2 complex treatment increases the overall number of tetramer-positive CD8 T cells by specifically amplifying MP (CD44$^{hi}$CD122$^{hi}$) cells.

IL-2 complex protection is mediated by both CD8 T cells and NK cells and lasts for several weeks

We next investigated the mechanism behind IL-2 complex-mediated protection during high-dose infection with LM-OVA. CD8 T cells are known to be critical mediators of L. monocytogenes clearance (16). IL-2 complex treatment increased both the overall number of MP CD8 T cells and the number of K$^b$-OVA$^+$-specific CD8 T cells. However, given the large increase in numb-
ers of NK cells post–IL-2 complex treatment, it was possible that these cells were also important mediators of protection. To address this, mice were depleted of NK1.1+ cells or CD8+ cells or both during the week of IL-2 complex treatment. After the week of injections and confirmation of >90% depletion of target cells (data not shown), mice were challenged with LM-OVA. Three days postinfection, IL-2 complex-treated mice displayed strong clearance (~3 logs) over control-treated mice (Fig. 4A, 4B). Depletion of either CD8+ cells or NK1.1+ cells alone decreased clearance of *L. monocytogenes* in both the spleen and liver. CD8+ T cells can display NK1.1+ with activation (17). However, the frequency of MP CD8+ T cells expressing NK1.1 in IL-2 complex-treated mice was not elevated compared with that in control animals (data not shown), indicating that NK1.1 depletion in these experiments is not depleting a significant number of CD8+ T cells. CD8+ cells were more critical in the spleen, whereas NK1.1+ cells appeared to contribute more to clearance in the liver. However, when both populations were removed, the number of bacteria found in the spleen was no longer significantly different from that in control-treated mice. Bacterial loads remained significantly lower in the liver even after removal of CD8+ and NK1.1+ cells, indicating other cell populations are actively contributing to clearance in this organ.

We also examined how long the protective effect was maintained after 1 wk (three injections) of IL-2 complex. Challenging mice with a high dose of LM-OVA on day 8 after the start of treatment continued to convey impressive clearance of bacteria within 3 d (Fig. 4C). When a greater interval of time was allowed between IL-2 complex treatment and challenge with *L. monocytogenes*, we found that protection lasted for at least 3 wk after the start of IL-2 complex treatment but waned after that time period. These data are supported by the observation that elevated numbers of CD8+ T cells and NK cells are maintained for 2–3 wk after treatment (Supplemental Fig. 1). These data demonstrate that short-term treatment with IL-2 complex provides measurable protection against infection for several weeks.

**Ag-specific memory cells are long-lasting and exhibit increased secondary expansion**

Our data show that IL-2 complex treatment can induce acute protection against bacterial or viral pathogens. We next sought to determine if after that encounter with infection, a long-lasting and effective memory compartment was created. Mice were injected with IL-2 complexes over the course of 1 wk and then infected with LM-OVA as shown in Fig. 1. Unexpectedly, despite the increase in Ag-specific CD8 T cells after IL-2 complex treatment (Fig. 3A), the number of K b-OVA–specific memory cells in the spleen at 6 wk postinfection was similar in mice that did or did not receive IL-2 complex treatment (Fig. 5A). This might suggest the IL-2 complex treatment results in responding cells with decreased potential to become memory cells after Ag encounter. Notably, studies in which IL-2 or IL-15 complexes were given during the contraction phase of an acute response indicated that cytokine therapy led to increased frequencies of KLRG-1hiCD127lo short-lived effector cells (SLECs) (8). To explore this in our system, we analyzed the phenotype of Ag-specific memory cells after LM-OVA infection. As shown in Fig. 5B, the K b-OVA–specific memory cells had a similar phenotype regardless of prior IL-2 complex treatment, except for a slight increase in the frequency of CD62Lhi and CD27hi cells in IL-2 complex-treated animals (Fig. 5B). Importantly, the frequency of KLRG-1hi cells was similar regardless of IL-2 complex treatment (Fig. 5B), suggesting no change in SLEC frequency. We also found that IL-2 complex treatment did not alter the patterns of KLRG-1 (and CD127) expression on MP CD8+ T cells prior to infection, nor on effector cells at day 5 after LM-OVA infection (data not shown).

We next asked whether these cells would respond if a second encounter with pathogen occurred. After LM-OVA rechallenge, vigorous expansion of K b-OVA–specific CD8+ T cells occurred with a slightly higher fold-expansion (1.7-fold) occurring in mice that previously received IL-2 complex treatment (Fig. 5C). These data indicate that strong IL-2 signals delivered by complex treatment do not impair (or strongly enhance) the development of long-term memory cells that effectively respond to a reencounter with Ag.

**Increasing the number of cytokine complex injections has a detrimental impact on protective immunity**

Lastly, we asked whether increasing the number of IL-2 complex injections would even further enhance protective immunity. Increasing the number of IL-2 complex injections to five total (given on days 0, 1, 2, 3, and 4) did enhance the number of CD8+ T cells and the frequency of cells that were MP (Supplemental Fig. 2A, 2B).
Infection. We treated mice with either three or five injections of IL-2 complex (Supplemental Fig. 3). Because this change in expansion might arise because of disparities in the frequency of responsive cells and/or changes in the bacterial loads sustained by the animals, we performed adoptive transfer of CD44hi cells from three-injection and five-injection mice into new hosts and infection with an attenuated strain of *L. monocytogenes* (LM-OVA ActA), which is equivalently cleared by all the mice. In this model, Ag-specific CD44hi cells from animals treated with three or five doses of IL-2 complex underwent similar expansion (Fig. 6D).

The loss of protection by animals given multiple doses of IL-2 complex might correlate with expression of inhibitory molecules, but neither PD-1 nor LAG-3 [both of which are implicated in decreased functionality of “exhausted” CD8 T cells (18)] were induced in CD8 T cells treated with five injections of IL-2 complex (Supplemental Fig. 4). Reduced protection might also correlate with loss of effector responses. Although granzyme B expression was actually greater in CD8 T cells from mice treated with five injections of IL-2 complex (Supplemental Fig. 4), we observed a defect in cytokine-induced production of IFN-γ in this population. During early phases of an immune response, MP CD8 T cells produce IFN-γ in response to IL-12 and IL-18, a function that can be mimicked in vitro (19). When comparing three-injection and control-treated mice in this system, we found a large increase in CD8 T cells from IL-2 complex-treated mice that were high producers of IFN-γ (Fig. 6E). In contrast, mice treated with five injections of complex exhibited decreased overall production of IFN-γ and were particularly deficient in the highest IFN-γ-producing cells. Interestingly, a similar deficit was also noted in the NK cell compartment (Supplemental Fig. 5). We conclude that expansion of protective MP CD8 T cells and NK cells can be achieved through IL-2 complex injections but that overstimulation can occur, leading to functional deficiencies in both lymphocyte populations.

### Discussion

In this study, we demonstrate that treatment of naïve mice with IL-2 complexes can increase resistance to newly acquired acute infections. Short-term treatment over the course of a week enhanced clearance of *L. monocytogenes* and protected mice from a lethal infection. A similar improvement in clearance was also seen after VV infection, a situation in which CD8 T cells and NK cells can mediate protective immunity (20). This indicates the potential usefulness of cytokine complexes against a wide range of infections. Additionally, the increased presence of MP CD8 T cells and NK cells in multiple organs (both lymphoid and nonlymphoid) also raises the possibility that IL-2 complex treatment could be beneficial during infections with peripheral reservoirs of infection. Others have shown cytokine complexes can improve responses to established tumor or chronic infection (4–7). In this study, we show that cytokine complex treatment can be used to positively manipulate the immune system prior to encounter with infection.

How does IL-2 complex treatment enhance protective immunity? We found that both CD8 T cells and NK cells were important for maximal clearance in the *L. monocytogenes* model. IL-2 complex treatment increased total numbers of CD8 T cells, including the numbers of precursors specific for the Ags studied by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from
Whether IL-2 complex treatment amplifies CD8 T cells of all specificities equally is of interest and is currently being explored. The amplified pool of CD8 T cells overwhelmingly displayed a central-memory phenotype, and earlier studies indicated that IL-2 complex treatment selectively induces proliferation of MP (rather than naive) CD8 T cells (3). However, other cytokine therapies (e.g., IL-15/IL-15Rα complexes) can promote proliferation of naive (CD44lo) CD8 T cells (21, 22), and IL-2 complexes have been shown to induce proliferation of some naive CD8 T cell clones (12). Hence, it is currently unclear whether the expanded Ag-specific MP CD8 T cell pool we detected after IL-2 complex therapy is derived from preexisting MP cells, naive cells, or both populations. In apparent contrast with their central-memory phenotype, the CD8 T cells induced by IL-2 complex treatment showed elevated expression of granzyme B, an important effector molecule. These findings mirror those from another report examining the effects of IL-2 complex therapy on Ag-primed memory CD8 T cells (7). We observe improved protection lasted for 2–3 wk after termination of cytokine therapy, which correlated with the reestablishment of normal numbers and phenotype of CD8 T cells and NK cells. Therefore, protection via this therapy is relatively short-lived but has the benefit of not irrevocably changing the immune system. In future studies, it will be interesting to determine how quickly enhanced protection is acquired after the start of IL-2 complex treatment, as well as the response by CD8 T cells and NK cells to infection while concurrently responding to IL-2 complexes.

Given the elevation in Ag-specific CD8 T cell numbers after IL-2 complex treatment, it was surprising that this did not translate into increased numbers of Ag-specific memory cells after primary infection or a substantial increase in the recall response (Fig. 5). This does not appear to reflect alterations in the production of SLECs versus memory precursor cells at the memory stage (Fig. 5), but might instead reflect the more efficient and rapid elimination of the pathogen used for priming by animals treated with IL-2 complexes. Further studies will be required to investigate this issue.

Unexpectedly, we found that the improved outcome observed after treatment with three injections of IL-2 complex was negated by the delivery of two additional doses (five injections over 1 wk). We noted that increased IL-2 complex treatment generated MP CD8 T cells and NK cells with a reduced capacity to produce IFN-γ, potentially suggesting some form of exhaustion. However, Ag-reactive CD8 T cells retained their expression of granzyme B and their capacity to expand after adoptive transfer and priming. Earlier studies using TCR transgenic models suggested that CD8 T cells expanded by IL-2 complex treatment had inferior functional capacity (12), but our data suggest that the functional state of the cells expanded by IL-2 complex depends on the dosing regimen. Whether additional cell types are compromised by sustained IL-2 complex therapy is not clear.

Given that suitable IL-2 complex treatment enhanced protective immunity in unvaccinated animals, it is interesting to speculate on potential use of cytokine therapy as a novel vaccine. Traditional approaches to vaccination have been unable to combat effectively many serious infections, particularly in situations where a strong (Kb-OVA and Kb-B8R). Whether IL-2 complex treatment amplifies CD8 T cells of all specificities equally is of interest and is currently being explored. The amplified pool of CD8 T cells overwhelmingly displayed a central-memory phenotype, and earlier studies indicated that IL-2 complex treatment selectively induces proliferation of MP (rather than naive) CD8 T cells (3). However, other cytokine therapies (e.g., IL-15/IL-15Rα complexes) can promote proliferation of naive (CD44lo) CD8 T cells (21, 22), and IL-2 complexes have been shown to induce proliferation of some naive CD8 T cell clones (12). Hence, it is currently unclear whether the expanded Ag-specific MP CD8 T cell pool we detected after IL-2 complex therapy is derived from preexisting MP cells, naive cells, or both populations. In apparent contrast with their central-memory phenotype, the CD8 T cells induced by IL-2 complex treatment showed elevated expression of granzyme B, an important effector molecule. These findings mirror those from another report examining the effects of IL-2 complex therapy on Ag-primed memory CD8 T cells (7). We observe improved protection lasted for 2–3 wk after termination of cytokine therapy, which correlated with the reestablishment of normal numbers and phenotype of CD8 T cells and NK cells. Therefore, protection via this therapy is relatively short-lived but has the benefit of not irrevocably changing the immune system. In future studies, it will be interesting to determine how quickly enhanced protection is acquired after the start of IL-2 complex treatment, as well as the response by CD8 T cells and NK cells to infection while concurrently responding to IL-2 complexes.

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Given that suitable IL-2 complex treatment enhanced protective immunity in unvaccinated animals, it is interesting to speculate on potential use of cytokine therapy as a novel vaccine. Traditional approaches to vaccination have been unable to combat effectively many serious infections, particularly in situations where a strong
cellular immune response needs to be generated (23, 24). Additionally, newly emerging or rapidly mutating pathogens make unavailable the time needed for determining correct antigenic targets for cellular immune responses. In this study, we show an alternative approach, using cytokine complexes to expand globally both innate and adaptive immune cells. This treatment proved to be an effective vaccine in two models of infections and removed the need to predetermine targets of the response.

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Disclosures
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