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Plasmodium Suppresses Expansion of T Cell Responses to Heterologous Infections

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Plasmodium remains a major pathogen causing malaria and impairing defense against other infections. Defining how Plasmodium increases susceptibility to heterologous pathogens may lead to interventions that mitigate the severity of coinfections. Previous studies proposed that reduced T cell responses during coinfections are due to diminished recruitment of naïve T cells through infection-induced decreases in chemokine CCL21. We found that, although Listeria infections reduced expression of CCL21 in murine spleens, lymphocytic choriomeningitis virus (LCMV)-specific T cell responses were not impaired during Listeria + LCMV coinfection, arguing against a major role for this chemokine in coinfection-induced T cell suppression. In our experiments, Plasmodium yoelii infection led to a reduced CD8+ T cell response to a subsequent Listeria infection. We propose an alternative mechanism whereby P. yoelii suppresses Listeria-specific T cell responses. We found that Listeria-specific T cells expanded more slowly and resulted in lower numbers in response to coinfection with P. yoelii. Mathematical modeling and experimentation revealed greater apoptosis of Listeria-specific effector T cells as the main mechanism, because P. yoelii infections did not suppress the recruitment or proliferation rates of Listeria-specific T cells. Our results suggest that P. yoelii infections suppress immunity to Listeria by causing increased apoptosis in Listeria-specific T cells, resulting in a slower expansion rate of T cell responses. The Journal of Immunology, 2015, 194: 000–000.

Cointfections with different pathogens (e.g., HIV, Mycobacterium tuberculosis, helminths, hepatitis viruses, and Plasmodium) affect about one third of the human population (1). In some cases, chronic viral infections are beneficial to host immune responses to bacterial and viral coinfections (2, 3). However, most epidemiological data indicate that bystander infections suppress host immunity to heterologous infections (1). Consistent with these observations, previous work showed that lymphocytic choriomeningitis virus (LCMV) and Listeria infections result in decreased levels of the T cell–trafficking chemokine CCL21 (4-6), which correlated with the ability of LCMV to impair heterologous T cell responses to vaccinia virus (VV), virus-like particles, or vesicular stomatitis virus infections (4). Thus, one mechanism by which pathogens may suppress immunity to heterologous infections is through impaired recruitment and activation of naïve T cells during a coinfection (4).

Plasmodium infections caused ~200 million cases of malaria that resulted in >600,000 deaths in 2012 (7). There is good evidence that Plasmodium infections can negatively impact immunity to bacterial (8–12) and viral (13, 14) infections, as well as responses to some vaccines (15–17). One of the most well-known examples is EBV, which contributes to the high rate of endemic Burkitt’s lymphoma in equatorial Africa (18). During EBV + Plasmodium coinfection, impaired control of EBV-infected B cells correlates with Plasmodium-induced suppression of EBV-specific CD8+ T cells (19); however, the precise mechanism by which this suppression occurs is not known. The best understood mechanism of suppressed immunity by Plasmodium is the release of heme during malaria, which culminates in the release of immature granulocytes and defective oxidative burst by neutrophils following infection with non-typhoid Salmonella (20).

To further address how Plasmodium suppresses host immunity to heterologous infections, we used the rodent model of malaria. For these studies, mice were infected with P. yoelii 17XNL (Py), followed by bacterial (Listeria monocytogenes) or viral (LCMV or VV) coinfections. We found lower numbers of Listeria-specific effector CD8+ T cells during coinfections as a result of the slower expansion kinetics of the effector T cell response. By combining in vivo experiments with in silico mathematical modeling, we propose that increased apoptosis, and not reduced recruitment or proliferation rates of naïve T cells, is responsible for the slower expansion kinetics of Ag-specific CD8+ T cells and, thus, suppression of host immunity to Listeria during Py infections.

Materials and Methods

Mice and infections

Female C57BL/6Ncr mice (6–10 wk of age) were purchased from the National Cancer Institute (Frederick, MD). Thy1.1+ OT-I TCR-transgenic CD8+ T cells were maintained at the University of Tennessee. Mice were housed at the University of Tennessee animal care facility under the appropriate biosafety level. For Plasmodium infections, mice were infected with 105 Py parasitized RBCs (pRBCs). Mice that were infected with...
Plasmodium spp. were infected at the indicated times with 5 × 10^6 CFU acta-deficient L. monocytogenes expressing OVA (Lm-OVA), 5 × 10^6 CFU acta-deficient L. monocytogenes (strain DPL1942; no OVA expression), 2 × 10^6 PFU LCMV Armstrong strain (Arm), or 5 × 10^6 PFU VV expressing OVA257-264 (generously provided by Jonathan Yewdell, National Institutes of Health). Mice coinfected with wild-type L. monocytogenes followed by LCMV were infected with 5 × 10^5 CFU L. monocytogenes (strain 10403S) and infected at the indicated times with 2 × 10^5 PFU LCMV Armstrong strain (Arm), which were infected with LCMV followed by L monocytogenes were infected with 2 × 10^5 PFU LCMV Arm or 2 × 10^5 PFU LCMV clone 13 (cl-13), followed by 5 × 10^6 CFU Lm-OVA at the indicated times. LCMV Arm infections were performed i.p. All other infections were done i.v. The Institutional Animal Care and Use Committee approved all animal experiments.

**Quantification of bacterial burden**

Spleens were removed on the indicated day, placed in 0.2% Igepal (Sigma-Aldrich, St. Louis, MO), and homogenized. Serial dilutions of tissue homogenate were plated on trypticase soy agar plates with 50 μg/ml streptomycin. Plates were incubated overnight at 37˚C.

**Quantification of Ag-specific T cells**

Spleens and inguinal lymph nodes were manually disrupted to generate single-cell suspensions in HyClone RPMI 1640 media (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% PBS (Atlanta Biologicals, Lawrenceville, GA), 1.1 mg/ml HEPES (Thermo Fisher Scientific), 0.2 mg/ml l-glutamine (Research Products International, Mt. Prospect, IL), penicillin/streptomycin (0.05 U/ml and 0.05 mg/ml; Invitrogen, Grand Island, NY), 0.05 mg/ml gentamicin sulfate (Invitrogen), and 0.05 μM 2-ME (Thermo Fisher Scientific). Livers were perfused with cold PBS through the hepatic portal vein and made into single-cell suspensions. Lungs were perfused through the left ventricle with cold PBS (pH 7.4) and treated with DNase/collagenase for 1 h prior to generation of a single-cell suspension. Lymphocytes from liver and lung single-cell suspensions were isolated using a 35% Percoll/BSA gradient. Single-cell suspensions were treated with ammonium chloride potassium to lyse RBCs. Blood was collected in heparinized Natelson collection tubes and treated with ammonium chloride potassium to obtain PBMCs. Tissues were harvested as indicated at the number of days postinfection.

Ag-specific T cells were detected using intracellular cytokine staining for IFN-γ or MHC I tetramers. For intracellular cytokine staining, cells were incubated for 5 h at 37˚C with brefeldin A (BioLegend, San Diego, CA) with or without CD8+ T cell epitope; OVA257-264, NP 396-404, GP33-41, and GP276-286 (concentration of each peptide was 200 nM) and CD4+ T cell epitope; and LLO190-201 and GP61-80 (concentration of each peptide was 250 nM) were added to HyClone RPMI 1640 media. After incubation, cells were stained with Fe block (anti-CD16/32, clone 2.4G2) and indicated cell surface Abs resuspended in FACS buffer (1× PBS, 1% BSA, 0.02% sodium azide); fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Diego, CA); stained for intracellular IFN-γ, TNF, IL-2, or granzye B; and washed with BD Perm/Wash (BD Biosciences). OVA-specific CD8+ T cells also were detected by tetramer staining. Cells stained negative for CD11c, MHC II, IFN-γ, TNF, or granzyme B; and washed with BD Perm/Wash (BD Biosciences, San Diego, CA); stained for intracellular IFN-γ (clone XMG1.1), MHC II, and CD45.2 (clone 104, anti-CD127 PE (clone A7R34), anti-KLRG1 allophycocyanin (clone 2F1/KLRG1), anti-IFN-γ FITC (clone XMG1.1), anti-TNF allophycocyanin (clone MP6-XT22), and anti–IL-2 PE (clone JES6-5H4). Anti-CD27 PE (clone LG7F9) was purchased from eBioscience (San Diego, CA), and anti–granzye B–PE was purchased from CalTag Laboratories.

**Parasitemia**

Percentage parasitemia was calculated by performing thin blood smears. Blood was obtained by performing tail snips. Slides were fixed with methanol and then stained with Giemsa stain (Thermo Fisher Scientific) diluted 1:20 in PBS for 30 min.

**BrdU assay**

Mice were injected i.v. with 2 mg BrdU (Sigma-Aldrich) solution 3 h prior to spleen removal. BrdU was put into solution with 1× sterile PBS. The BrdU was injected i.v. (BD Pharmingen, San Diego, CA) and administered according to the manufacturer’s recommendation for labeling BrdU+ cells.

**Caspase-3/7 assay**

Spleens were disrupted manually into single-cell suspension. The Caspase-3 and -7 Assay Kit (Invitrogen, Grand Island, NY) was used to detect apoptosis. A 30X FLICA working solution was added to cells and incubated for 1 h at 37˚C and 5% CO2. Cells were washed with 1× wash buffer and stained with a tetramer, followed by cell surface fluorescent Abs. Caspase-3 and -7 were detected using flow cytometry.

**Adaptive transfer of OT-I TCR-transgenic CD8+ T cells**

Spleens and lymph nodes from Thy1.1+ OT-I TCR-transgenic mice were removed and disrupted into a single-cell suspension. Thy1.1+ OT-I TCR-transgenic T cells were enriched using a CD8α+ T Cell Isolation Kit II (Miltenyi Biotec, Cologne, Germany). OT-I TCR-transgenic T cells were quantified and injected i.v. at the indicated amount. For some experiments, OT-I TCR-transgenic T cells were labeled with 0.8 μM CFSE prior to adoptive transfer.

**CCL21 ELISA**

Spleens were removed from naive control mice or from mice on day 7 postinfection with ∼5 × 10^7 wild-type L. monocytogenes (10403S), 10^4 Py rRBCs, 2 × 10^5 PFU LCMV Arm, or 2 × 10^6 PFU LCMV cl-13. Spleens were placed in 1 ml PBS supplemented with 1% BSA (New England Bioslabs, Ipswich, MA) and 10 μl/ml protease inhibitor mixture P8340 (Sigma-Aldrich) and then homogenized. CCL21 was quantified using Mouse CCL21/CXCL13 DuoSet (R&D Systems, Minneapolis, MN), according to the manufacturer’s recommendations.

**In vitro survival of Thy1.1+ cells**

Mice were infected with Py and L. monocytogenes as indicated, and Thy1.1+ OT-I TCR-transgenic T cells were sorted from the spleens of mice using mouse and rat CD90.1 MicroBeads (Miltenyi Biotec) 7 d post–L. monocytogenes infection. Thy1.1+ cells were resuspended at 1 × 10^6 cells/ml in supplemented RPMI 1640 and incubated in tissue culture plates at 37˚C, 5% CO2. At the indicated times, cell density was determined by resuspending the cells and counting them using trypan blue exclusion. Cell numbers were determined at each time point from the same sample.

**Statistical analysis**

All statistical analyses were performed using Prism 6.0c (GraphPad). Mathematical models were fitted to experimental data by log transforming the model predictions and data to ensure normality of the residuals; residuals of the best fit were checked for normality using the Shapiro–Wilk normality test. We used nonlinear least squares and function FindMinimum in Mathematica 5.2 (Wolfram) for model fitting to data. Ninety-five percent confidence intervals (CIs) for model parameters were estimated by bootstrapping residuals with 1000 simulations (21). Statistically significant comparison of the models was done using the F-test for nested models (22). The quality of the model fits to data with repeated measurements (Lack of Fit test) was determined as described previously (22). In short, we decomposed the total residual sum of squares, Sr, into replication sum Sr and lack of fit sum Sr. Replication sum is calculated as the total sum of squares of deviations of the replication values above their averages and has vr, df. Then lack of fit is given by Sr = Sr − Sr with vr = n − p − vr, df, where n is the total number of data points and p is the number of model parameters (22). The ratio Sr−vr/vr was compared with the F distribution with (vr−n, p+vr) df to determine whether there is a significant lack of fit (22). Low vr values indicate the presence of lack of fit and, thus, of a poor model fit of the data. To determine the difference in the rates of decay of OT-I T cells in vitro culture, we log-transformed the measured cell numbers and fitted
two models to these data: one model assuming that the initial number of OT-1 cells and the rate of decay are different between cells from \( L.\) monocytogenes–infected and Py+ L. monocytogenes–coinfected mice and another model in which the decay rates were fixed to the same value. The difference in the quality of models’ fits to the data (due to differences in decay slopes) was determined using F-tests for nested models (22).

Mathematical model

To investigate potential mechanisms leading to suppression of CD8\(^+\) T cell responses to heterologous infections, we extend a mathematical model that was used previously to quantify the kinetics of LCMV-specific CD8\(^+\) and CD4\(^+\) T cell responses (23–25). In the model, Ag-specific naive CD8\(^+\) T cells are recruited in the response at a rate \( \lambda \) (see Fig. 4). Recruited T cells become activated and start dividing after an initial delay of 7 hours. Activated cells divide until time \( T_{\text{off}} \), and the initial maximal rate of T cell proliferation is given by \( \rho \). To account for the observed saturation in the rate of increase in the number of Ag-specific T cells at the peak of the immune response, we also let the rate of T cell proliferation decline with the T cell density due to competition between T cells of the same specific for survival factors (e.g., Ag, cytokines); the per capita rate of T cell proliferation is then \( \rho e^{-A} \), where \( A \) is the number of activated Ag-specific T cells. The parameter \( \mu \) was necessary to explain our kinetics data because a model assuming constant exponential growth of T cell numbers did not describe the data accurately, based on the F-test for nested models (data not shown). Of note, the reduction in the rate of T cell proliferation with time since infection was confirmed in additional experiments involving BrdU labeling (Fig. 7). Importantly, the model in which saturation in T cell numbers near the peak is due to cell density–dependent apoptosis rate, did not describe the data well in comparison with the model with density-dependent cellular proliferation as judged by Akaike information criterion (results not shown). After reaching the peak, Ag-specific T cells die at a constant rate \( \alpha \). To explain our experimental data with best quality, we let activated cells die during the expansion phases at the same rate \( \alpha \), so the maximum net rate of the growth of the T cell population during the expansion phase is \( \rho - \alpha \). Thus, our basic mathematical model assumes that activated T cells undergo apoptosis at a constant rate \( \alpha \) during both expansion and contraction phases. Cell numbers increase during the expansion phase as a result of rapid proliferation, which ceases after the peak of the immune response, leading to a decline in cell numbers. With these assumptions, the model is written as a set of delay differential equations:

\[
\frac{dN(t)}{dt} = -\lambda N(t),
\]

\[
\frac{dA(t)}{dt} = \lambda N(t - T) + \left( \rho e^{-A(t)} - \alpha \right) A(t), \quad \text{if } t < T_{\text{off}}, -\alpha A(t), \text{otherwise}.
\]

In our fits, we fixed \( T = 24 \) because it was determined that CD8\(^+\) T cells start proliferating only \( \geq 24 \) h following Ag recognition (26–28). In all shown fits, the rate of naive T cell recruitment into the response was fixed to \( \lambda = 5/d \), because allowing for different recruitment rates did not improve the quality of the model fit to data (see text for details).

Results

Plasmodium impairs the maturation of DCs and activation of Listeria-specific T cells

Plasmodium-infected RBCs impair the ability of DCs to respond to subsequent TLR stimulation (29, 30), and \( P.\) chabaudi and \( P.\) yoelli infections in mice impair the ability of DCs to stimulate naive T cells in vitro (31, 32). These observations support the possibility that \( P.\) Plasmodium infections impair host immunity during coinfections through suppression of T cell responses against the heterologous infection. Consistent with these observations, Py infections impaired the maturation of DCs following coinfection with actA-deficient Lm-OVA. CD11c\(^+\) DCs from Lm-OVA–infected mice upregulated the costimulatory molecules CD40, CD80, and CD86; however, these molecules were significantly lower in Py+ L. monocytogenes–coinfected mice (Fig. 1A–C). In particular, there was a profound defect in the upregulation of CD86 on DCs in Py+ L. monocytogenes–coinfected mice. Furthermore, Py infections alter the hierarchy of various DC subsets over the course of infection (Supplemental Fig. 1).

To determine whether reduced expression of T cell costimulatory molecules on DCs impaired the activation of naive T cells in vivo, OT-1 CD8\(^+\) T cells were transferred to C57BL/6 mice that were infected 1 d later with Py. One week later, naive control or Py-infected mice were infected with Lm-OVA, and the geometric mean fluorescence

**FIGURE 1.** \( P.\) yoelli impairs the ability of DCs to upregulate costimulatory molecules following a \( L.\) monocytogenes coinfection and the activation of \( L.\) monocytogenes–specific T cells. (A) Experimental design. (B) Representative graphs showing the expression of CD40, CD80, and CD86 on CD11c\(^+\) MHCII\(^+\) DCs. (C) gMFI of CD40, CD80, and CD86. Data (mean + SEM) from three to six mice are cumulative results from two independent experiments. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), **** \( p < 0.0001 \), one-way ANOVA, followed by the Tukey multiple-comparison test. (D) A total of \( 1 \times 10^5 \) Thy1.1\(^+\) OT-1 T cells was transferred to Thy1.2\(^+\) C57BL/6 mice 1 d before infection with Py pRBCs. One week later, naive control and Py-infected mice were infected with Lm-OVA. gMFI of CD25 on OT-1 TCR-transgenic CD8\(^+\) T cells on the indicated day. Data (mean + SEM) from six mice are cumulative results from two independent experiments and were analyzed using the unpaired two-tailed \( t \) test. n.s., not significant.
intensity (gMFI) of CD25, which is used as a marker of recent T cell activation by DCs (33), was measured on OT-I CD8+ T cells at days 1–3 post–Lm-OVA infection. Consistent with the reduced expression of T cell costimulatory molecules on DCs, the expression level of CD25 was significantly lower each day on OT-I CD8+ T cells in Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice (Fig. 1D). These data indicate that Py infections impair both the ability of DCs to upregulate T cell costimulatory molecules and the activation of naive T cells responding to the L. monocytogenes infection.

Plasmodium suppresses T cell responses to bacterial and viral coinfections

To address whether Plasmodium infections impair host immunity to Listeria coinfections, C57BL/6 mice were infected with Py and then infected 7 d later with actA-deficient Lm-OVA. Bacterial burden was quantified 4 d after Lm-OVA infection (Fig. 2A). Although most control mice cleared the Lm-OVA infection by 4 d postinfection, Py-infected mice exhibited a profound defect in the clearance of Lm-OVA (Fig. 2B). It is likely that the Py infection impaired anti-Listeria innate immunity, but this deficiency in

FIGURE 2. P. yoelii suppresses T cell responses to a bacterial coinfection. (A) Experimental design. (B) Bacterial burden in the spleen. Each symbol represents an individual mouse from two independent experiments. Data points below the limit of detection (LOD) represent mice in which we could not detect a response. (C) Experimental design. (D) Kinetics of Py parasitemia. Data (mean + SEM) are from five mice and are representative of multiple experiments. Representative contour plots showing the fraction of CD8+ (E) or CD4+ (G) cells that are IFN-γ+. Number of OVA-specific CD8+ T cells (F) and LLO190-specific CD4+ T cells (H) per spleen at the indicated time points. Data (mean + SEM) from 9–21 mice (F) and 3–12 mice (H) are cumulative results from at least two independent experiments. (I) Experimental design. (J) Representative contour plots showing the fraction of CD8+ cells that are Kb-OVA+. (K) Number of Kb-OVA–specific CD8+ T cells/spleen. Data (mean + SEM) from five or six mice are cumulative results from two independent experiments. All data were analyzed by the unpaired two-tailed t test. n.s., not significant.
clearance could also arise as a result of impaired \textit{L. monocytogenes}–specific T cell responses.

To address whether \textit{Plasmodium} infections impaired T cell responses to \textit{Listeria} coinfections, C57BL/6 mice were infected with Py and then were infected 7, 14, 28, or 63 d later with Lm-OVA; 7 d later, the \textit{L. monocytogenes}–specific CD8$^{+}$ T cell response was measured in the spleen (Fig. 2C). Of note, Py causes an infection that reaches peak parasitemia ~3 wk postinfection and is cleared ~4 wk postinfection (Fig. 2D). Mice coinfected with Lm-OVA on day 7 post-Py infection exhibited a 20-fold decrease in \textit{L. monocytogenes}–specific CD8$^{+}$ T cells (Fig. 2E, 2F), and most mice had no detectable \textit{L. monocytogenes}–specific CD4$^{+}$ T cells at day 7 post–\textit{L. monocytogenes} infection (Fig. 2G, 2H). We recovered significantly smaller numbers of \textit{L. monocytogenes}–specific T cells in mice coinfected with \textit{L. monocytogenes} at 14 d post-Py infection, but coinfections at later time points led to normal \textit{L. monocytogenes}–specific T cell responses (Fig. 2F, 2H). Interestingly, the degree of \textit{L. monocytogenes}–specific T cell suppression was lower when mice were infected on day 14 post-Py infection compared with on day 7, despite higher parasite burden between days 14 and 21 compared with days 7–14 (Fig. 2D).

These observations suggest that Py infections also may impact the quality of \textit{Listeria}-specific T cells. Identification of \textit{Listeria}-specific CD8$^{+}$ T cells through production of IFN-$\gamma$ following TCR stimulation or MHC I tetramer staining revealed similar numbers of OVA-specific CD8$^{+}$ T cells, regardless of quantification methodology, in \textit{L. monocytogenes}–infected and Py+\textit{L. monocytogenes}–coinfected mice, respectively (Supplemental Fig. 2A). These data indicate that Py infections do not impair production of IFN-$\gamma$ in \textit{L. monocytogenes}–specific CD8$^{+}$ T cells. Furthermore, \textit{Listeria}–specific CD8$^{+}$ T cells in Py+\textit{L. monocytogenes}–coinfected mice exhibited no defect in the production of TNF, IL–2, or granzyme B (Supplemental Fig. 2B) or the fraction of cells that were CD27$^{+}$, CD62L$^{+}$, CD127$^{+}$KLRG$^{+}$, or CD127$^{+}$KLRG1$^{−}$ (Supplemental Fig. 2B). Collectively, these data indicate that Py infections do not alter the functional characteristics of \textit{Listeria}-specific CD8$^{+}$ T cells.

During a \textit{Plasmodium} infection, the spleen becomes enlarged, in part as a result of the inflammation associated with Py infection and because RBCs are retained and cleared in the spleen. Therefore, it is possible that the decreased \textit{L. monocytogenes}–specific T cell response in the spleen could be attributed to alterations in spleen homeostasis and T cell trafficking. To address this possibility, mice were infected with Py and were coinfected 7 d later with Lm-OVA; \textit{L. monocytogenes}–specific T cells were quantified in multiple tissues 7 d post–\textit{L. monocytogenes} infection (Fig. 2I). There were significant decreases in \textit{L. monocytogenes}–specific CD8$^{+}$ T cells observed in all tissues with the exception of the inguinal lymph nodes, which had few detectable \textit{L. monocytogenes}–specific CD8$^{+}$ T cells in both groups of mice (Fig. 2I, 2K). These results indicate that Py caused a generalized decrease in \textit{L. monocytogenes}–specific CD8$^{+}$ T cells.

To determine whether \textit{Plasmodium} infections impair T cell responses to viral coinfections, mice were infected with Py and then uninfected control mice and Py-infected mice were infected with LCMV Arm (Fig. 3A). Consistent with the bacteria coinfection data, Py induced a substantial decrease in both LCMV-specific CD8$^{+}$ T cells (Fig. 3B, 3C) and LCMV-specific CD4$^{+}$ T cells (Fig. 3D, 3E). Similar results were observed when Py-infected mice were coinfected with VV expressing OVA (Fig. 3F–H). Collectively, these results demonstrate that \textit{Plasmodium} infections impair host T cell responses to heterologous bacterial and viral coinfections. These results are also consistent with the previously observed suppression of CD8$^{+}$ T cell responses to several viral coinfections following an initial infection of mice with LCMV (4).

**FIGURE 3.** \textit{P. yoelii} inhibits T cell responses to viral coinfections. (A) Experimental design. Representative contour plots showing the fraction of CD8$^{+}$ (B) or CD4$^{+}$ (D) cells that are IFN-$\gamma$–. Number of NP396-, GP33-, and GP276-specific CD8$^{+}$ T cells (C) and GP61-specific CD4$^{+}$ T cells (E) per spleen. Data (mean + SEM) from six mice are cumulative results from two independent experiments. (F) Experimental design. (G) Representative contour plots showing the fraction of CD8$^{+}$ cells that are IFN-$\gamma$–. (H) Number of OVA-specific CD8$^{+}$ T cells/spleen. Data (mean + SEM) from five or six mice are cumulative results from two independent experiments. All data were analyzed by the unpaired two-tailed $t$ test.

**Viral, but not bacterial, infections suppress T cell responses to coinfections**

Both LCMV and \textit{L. monocytogenes} infections result in decreased levels of the T cell trafficking chemokine CCL21, which correlate
with impaired T cell responses to subsequent viral infections (4). It is not known how long LCMV impairs host T cell responses to heterologous infections or whether L. monocytogenes suppresses heterologous T cell responses by decreasing expression of CCL21. To address these questions, mice were infected with LCMV Arm, which causes an acute infection that is cleared within ~1 wk, or LCMV cl-13, which causes a chronic infection that can persist for life in some tissues (34). Various days later, LCMV-infected and control mice were coinfected with Lm-OVA (Fig. 4A). Both LCMV Arm–infected mice and LCMV cl-13–infected mice exhibited substantial suppression of L. monocytogenes–specific CD8+ T cells (>100-fold decrease in most mice) when coinfected with Lm-OVA 7 d post-LCMV infection (Fig. 4B). L. monocytogenes–specific CD4+ T cell responses exhibited greater suppression by LCMV Arm and LCMV cl-13 at this time point, with many mice not exhibiting detectable L. monocytogenes–specific CD4+ T cells (Fig. 4C). Despite this substantial impairment, there was little to no suppression of L. monocytogenes–specific T cells in LCMV Arm–infected mice coinfected with Lm-OVA just 1 wk later (day 14 post-LCMV infection), as well as at subsequent time points (Fig. 4B, 4C). In contrast, mice infected with LCMV cl-13 exhibited impaired L. monocytogenes–specific T cell responses even up to 2 mo post-LCMV cl-13 infection (Fig. 4B, 4C). These results show that the duration of virus-induced immunosuppression is not universal, but in the case of a chronic infection, it can last many weeks.

To determine whether L. monocytogenes infections also suppress T cell responses to subsequent viral coinfections, mice were infected with virulent L. monocytogenes. Of note, this was a potent infection from which 33% of the L. monocytogenes–infected mice died prior to day 7 (data not shown). Control mice and L. monocytogenes–infected mice were coinfected with LCMV Arm on day 7 or 14 post-L. monocytogenes infection (Fig. 4D). Surprisingly, L. monocytogenes–infected mice showed no impairment in LCMV-specific CD8+ T cells (Fig. 4E) and only a 2-fold decrease in LCMV-specific CD4+ T cells when coinfection occurred at 7 d post-L. monocytogenes infection; no impairment was observed when coinfection occurred at 14 d after primary L. monocytogenes infection (Fig. 4F). These data demonstrate that infection-induced suppression of T cell responses to coinfections is not generalizable to all microorganisms.

Previous experiments correlated LCMV-induced suppression of heterologous virus–specific T cell responses with decreased levels of the T cell–trafficking chemokine CCL21 (31). Consistent with that report, mice infected with L. monocytogenes, Py, LCMV Arm, or LCMV cl-13 showed reduced levels of CCL21 (Fig. 4G). However, L. monocytogenes+LCMV coinfections caused little to no impairment in LCMV-specific T cells (Fig. 4D–F). Taken together, these results indicate that impairment of T cell responses during coinfections may not be due to reduced amounts of CCL21, as previously suggested (4).

Plasmodium alters the expansion kinetics of the Listeria-specific T cell response

To address the mechanism by which Py infections suppress T cell responses to coinfections, we first characterized the kinetics of the L. monocytogenes–specific T cell response. Mice were infected with Py and then both uninfected control mice and Py-infected mice were infected with Lm-OVA. L. monocytogenes–specific CD8+ T cells were quantified daily between days 3 and 10 post-Lm-OVA infection (Fig. 5A). L. monocytogenes–specific CD8+ T cells were detected in the spleen as early as day 3 in L. monocytogenes–infected mice but not until day 4 in Py+L. monocytogenes–coinfected mice (Fig. 5B), which was consistent with the impaired clearance of L. monocytogenes at day 4 post-L. monocytogenes infection (Fig. 2B). There were 15–70-fold differences in L. monocytogenes–specific CD8+ T cell numbers between the two groups of mice through the peak (day 6 post-L. monocytogenes infection) of the L. monocytogenes–specific CD8+ T cell response in L. monocytogenes–infected mice (Fig. 5B). However, the peak of the L. monocytogenes–specific CD8+ T cell response in Py+L. monocytogenes–coinfected mice was not observed until day 9 post-L. monocytogenes infection (Fig. 5B), indicating a suppressed response. In addition, the peak L. monocytogenes–specific CD8+ T cell response was significantly (p < 0.0001) lower in Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice (Fig. 5C). Thus, Py altered the expansion kinetics of the L. monocytogenes–specific CD8+ T cell response.

The differential magnitude of L. monocytogenes–specific CD8+ T cell responses between L. monocytogenes–infected and Py+L. monocytogenes–coinfected mice could arise from three processes: a reduced recruitment rate of naive L. monocytogenes–specific T cells into the response in coinfected mice, a reduced proliferation rate of L. monocytogenes–specific T cells in coinfected mice, or an increased apoptosis rate of L. monocytogenes–specific T cells in coinfected mice. We extended a previously used mathematical model to discriminate among these mechanisms (Fig. 4D, see Materials and Methods) (23–25). The model was fitted to the kinetics of OVA-specific CD8+ T cell numbers in both L. monocytogenes–infected mice and Py+L. monocytogenes–coinfected mice to investigate which model parameters (e.g., naive T cell recruitment, proliferation, apoptosis) were influenced by the Py infection. Although the T cell kinetics data may imply that L. monocytogenes–specific CD8+ T cells are recruited more slowly into the response (Fig. 5B), the model predicted that the rates of naive T cell recruitment (λ) were very high and similar between L. monocytogenes–infected mice and Py+L. monocytogenes–coinfected mice (Fig. 5E, 5F), with half of L. monocytogenes–specific T cells being recruited into the response within log(2)/λ = 3 h postinfection. This is consistent with previous studies on rapid and efficient CD8+ T cell recruitment into immune responses during L. monocytogenes infection (35). In fact, allowing for different recruitment rates of naive CD8+ T cells into the response in L. monocytogenes–infected or Py+L. monocytogenes–coinfected mice did not improve the quality of the model fit to the experimental data (F2,101 = 1.68, p = 0.19). The modeling predicted that the main effect of Py infection on the kinetics of the L. monocytogenes–specific CD8+ T cell response was the reduced rate of expansion before reaching the peak. The estimated doubling time of the number of L. monocytogenes–specific CD8+ T cells occurred in ln(2) day−1 = 5.7 h (95% CI, 4.6–6.0) in L. monocytogenes–infected mice and ln(2) day−1 = 11.1 h (95% CI, 10.5–11.6) in Py+L. monocytogenes–coinfected mice (Fig. 4E, 4F). Because L. monocytogenes–specific CD8+ T cells declined more rapidly after reaching the peak in Py+L. monocytogenes–coinfected mice (Fig. 5E), we hypothesized that the difference in expansion kinetics was due to increased apoptosis of L. monocytogenes–specific T cells. The model in which L. monocytogenes–specific CD8+ T cells proliferate at the same maximal rate p in L. monocytogenes–infected and Py+L. monocytogenes–coinfected mice, but die more rapidly during the coinfection, explained the data better and with a minimal number of parameters compared with the model with different proliferation and apoptosis rates (F1,102 = 0.34, p = 0.56). The mathematical model in which L. monocytogenes–specific CD8+ T cells die more rapidly in Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice also described the data very well, as judged by the Lack of Fit test (F9,94 = 1.63, 1991.
FIGURE 4. Viral, but not bacterial, infections suppress T cells responding to coinfections, despite decreased CCL21. (A) Experimental design. Number of OVA-specific CD8+ T cells (B) and LLO190-specific CD4+ T cells (C) per spleen at the indicated time points. Each symbol in the day-14 plot is from one mouse. Data (mean + SEM) from five or six mice are cumulative results from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, followed by the Tukey multiple-comparison test. (D) Experimental design. Number of NP396-, GP33-, and GP276-specific CD8+ T cells (E) and GP61-specific CD4+ T cells (F) per spleen at the indicated time points. Data (mean + SEM) from four to six mice are cumulative results from two independent experiments. Data were analyzed by the unpaired two-tailed t test. (G) Levels of CCL21 in various groups of mice. Data are cumulative results from 2-independent experiments; each symbol represents an individual mouse. ****p < 0.0001, one-way ANOVA, followed by the Dunnett multiple-comparison test. LOD, limit of detection; n.s., not significant.

Plasmodium does not impair the recruitment of Listeria-specific T cells

To experimentally test the predictions from the mathematical modeling, we first examined T cell recruitment and activation through analysis of CD25 upregulation. The small number of naive precursor T cells present in a mouse complicates analysis of early activation events on endogenous T cells. However, adoptive transfer of large numbers of TCR-transgenic T cells, such as OT-I TCR-transgenic CD8+ T cells that recognize OVA, can be used to characterize early events in T cell activation. Prior to using this approach, it was important to determine whether OT-I T cells exhibited the same defects in Py+L. monocytogenes–infected mice as did endogenous OVA-specific CD8+ T cells. This was addressed by adoptively transferring a small number of OT-I T cells (~250) into C57BL/6 mice, half of which were infected with Py on the following day. One week later, both the uninfected control and Py-infected mice were infected with Lm-OVA; OT-I T cell numbers were quantified daily between days 5 and 10 post–Lm-OVA infection (Supplemental Fig. 3A). Consistent with the endogenous response, OT-I T cells exhibited both a substantial decrease early after L. monocytogenes infection, a delayed peak in expansion, and a lower peak in the Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice (Supplemental Fig. 3B–D). Importantly, our mathematical model assuming that only 10% of OT-I cells survive the transfer [N(0) = 25] (36) and are rapidly recruited into the response (λ = 5 d−1) accurately described these independent data and predicted nearly identical estimates for the rate parameters ρ (proliferation rate) and α (apoptosis rate) within CI (Supplemental Fig. 3F), as for the endogenous response (Fig. 5F). The predicted rates of recruitment of OT-I cells into the response were not significantly different in L. monocytogenes–infected mice and Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice (F2,46 = 0.12, p = 0.68). Similar results were observed assuming 40% of OT-I T cells [N(0) =100] survive the transfer (data not shown). Again, the mathematical model that assumed similar proliferation of OT-I T cells in both groups of mice while increased apoptosis of OT-I T cells in Py+L. monocytogenes–coinfected mice compared with L. monocytogenes–infected mice explained these data the best and with the minimal number of parameters (F1,47 = 2.6, p = 0.1; Supplemental Fig. 3E, 3F). Collectively, our data and analysis demonstrate that OT-I T cells function similarly to endogenous OVA-specific CD8+ T cells, which means that the adoptive-transfer system can be used to address the early activation events of T cells in mice coinfected with Py.

To determine whether Py infections alter the recruitment of L. monocytogenes–specific CD8+ T cells, ~1.5 × 10^6 OT-I T cells were adoptively transferred into C57BL/6 mice. The following day, half of the mice were infected with Py, and both uninfected control and Py-infected mice were infected with Lm-OVA 1 wk later. OT-I T cells were quantified on days 1–3 post–L. monocytogenes infection
Collectively, these results indicate that Py infection did not impair recruitment of naive T cells during heterologous infections, despite decreased levels of CCL21 in the spleen.

*Plasmodium infections do not impair proliferation but induce increased apoptosis in Listeria-specific T cells*

To test the hypothesis that Py infections did not alter the proliferation of *L. monocytogenes*-specific CD8\(^+\) T cells, but, in contrast, increased their apoptosis, the rate of *L. monocytogenes*-specific CD8\(^+\) T cell proliferation and the level of apoptosis were measured throughout the expansion and early contraction phases in both *L. monocytogenes*-infected and Py+*L. monocytogenes*-coinfected mice (Fig. 7A). To analyze proliferation of *L. monocytogenes*-specific CD8\(^+\) T cells, mice were pulsed with BrdU 3 h prior to removing spleens for T cell analysis. At the earliest time points (days 4 and 5 post-*L. monocytogenes* infection), there was robust proliferation of *L. monocytogenes*-specific CD8\(^+\) T cells in *L. monocytogenes*-infected and Py+*L. monocytogenes*-coinfected mice (Fig. 7B, 7C). We (38) and other investigators (37) developed mathematical models to estimate the rate of lymphocyte proliferation using BrdU data. For randomly dividing T cells, the predicted fraction of BrdU\(^+\) cells in the population during labeling is given by the equation \( f = 1 - e^{-\lambda t} \), where \( p \) is the rate of T cell division, and \( t \) is the time of the labeling (38). In our data, we observed 50–70% BrdU\(^+\) cells in \( t = 3\)-h labeling period; by taking the average \( f = 60\% \), we estimate that, during the early time points, the rate of cell division is \( p = 0.15/h = 3.6/d \), which is in reasonable agreement with our independent estimate using the mathematical model \( p = 3.38/d \) (Fig. 4E).

Initial robust proliferation of *L. monocytogenes*-specific CD8\(^+\) T cells in *L. monocytogenes*-infected mice was followed by
a rapid decline at day 6 and onward (Fig. 7C), which correlated with the \textit{L. monocytogenes}–specific CD8$^+$ T cells reaching peak expansion followed by contraction (Fig. 4B). There was no difference in \textit{L. monocytogenes}–specific CD8$^+$ T cell proliferation at day 5 post-\textit{L. monocytogenes} infection between the two groups (Fig. 7B, 7C). Consistent with the delayed peak of \textit{L. monocytogenes}–specific CD8$^+$ T cells in Py$^+$ \textit{L. monocytogenes}–coinfected mice compared with \textit{L. monocytogenes}–infected mice (Fig. 4B), there were higher rates of proliferation in Py$^+$ \textit{L. monocytogenes}–coinfected mice compared with \textit{L. monocytogenes}–infected mice on days 6–8 (Fig. 7C). These data also indicate that the rate of proliferation of \textit{L. monocytogenes}–specific CD8$^+$ T cells may decline through the response, because fewer BrdU$^+$ cells are found at later times postinfection in Py$^+$\textit{L. monocytogenes}–coinfected mice (Fig. 7C). This is consistent with the predictions of our mathematical model in which the rate of T cell proliferation is reduced as the immune response reaches the peak due to a postulated self-suppression rate $m$ (Fig. 4D and Materials and Methods). The reduction in the rate of T cell proliferation with time since infection predicted by the mathematical model was in good agreement with the proliferation rate measured using BrdU in \textit{L. monocytogenes}–infected mice (data not shown). However, in Py$^+$\textit{L. monocytogenes}–coinfected animals, there was a larger decline in the rate of proliferation, as measured by BrdU, compared with the predictions of the mathematical model (data not shown). This discrepancy suggests that reduced maximum
numbers of *L. monocytogenes*–specific CD8+ T cells during Py+L. *monocytogenes* coinfection also may be driven by the reduced rate of T cell proliferation near the peak of the immune response. Yet, our results strongly indicate that the lower numbers of *L. monocytogenes*–specific CD8+ T cells in Py+L. *monocytogenes*–coinfected mice are not due to an inability of these cells to proliferate rapidly early during the infection.

Measuring apoptosis ex vivo is challenging because it is expected that apoptotic cells are quickly removed by professional phagocytes (39). Nevertheless, *L. monocytogenes*–specific CD8+ T cells undergoing apoptosis were measured through detection of active caspase-3/7. A significantly higher fraction of active caspase-3/7+ cells was observed in Py+L. *monocytogenes*–coinfected mice compared with L. *monocytogenes*–infected mice on day 6 (p = 0.0041) and day 7 (p = 0.0002) post-L. *monocytogenes* infection (Fig. 7D, 7E). There was a consistent decline in the fraction of apoptotic cells (caspase-3/7+) over the course of both Py+L. *monocytogenes* and Py+L. *monocytogenes* infections (Fig. 7E). This was not expected, because most cell death should be occurring during the contraction phase when the number of *L. monocytogenes*–specific T cells declines. This observation of a declining fraction of apoptotic cells was not consistent with the basic assumption of the mathematical model of a constant apoptosis rate during an immune response. Importantly, after pooling day-12–15 data on caspase-3/7 staining, we still found a statistically significant difference between the average fraction of caspase-3/7+ cells in *L. monocytogenes*–infected mice and Py+L. *monocytogenes*–coinfected mice (9.5 ± 1.5% versus 14 ± 1.3%, respectively; p = 0.025, unpaired two-tailed t test). Additionally, OT-I T cells from Py+L. *monocytogenes*–coinfected mice exhibited reduced survival in vitro compared with OT-I T cells from *L. monocytogenes*–infected mice (Supplemental Fig. 4). Interestingly, we observed a 3–4-fold difference in the in vitro apoptosis rate of OT-I T cells isolated from *L. monocytogenes*–infected mice compared with Py+L. *monocytogenes*–coinfected mice, similar to what we also predicted for the in vivo infection (Fig. 5F). Taken together, our data indicate that apoptosis is likely to play the major role in regulating the magnitude and kinetics of CD8+ T cell responses during coinfections.

**Discussion**

The mechanisms responsible for *Plasmodium*–induced suppression of host immune responses to heterologous infections are poorly defined. Previous work indicated that suppression of CD8+ T cell responses to coinfection was due to decreases in the chemokine CCL21, which guides migration of naïve T cell APCs, such as DCs (4). The observed reductions in the magnitude of CD8+ T cell responses specific to VV, viral-like particles, and vesicular stomatitis virus in the presence of an LCMV coinfection were interpreted to be the result of failed recruitment of naïve CD8+ T cells during the priming phase (4). Our experimental data indicate that this cannot be the main mechanism of suppression, because profound depletion of CCL21 following L. *monocytogenes* infection did not impact the magnitude of the LCMV-specific T cell responses (Fig. 4D–G). In contrast, we observed that, during Py+L. *monocytogenes* coinfection, the L. *monocytogenes*–specific CD8+ T cell population expanded at a significantly slower rate than did the CD8+ T cell response during L. *monocytogenes* infection (Fig. 5E, 5F). Our mathematical modeling analysis and experimental data point to an increased apoptosis rate for proliferating effector T cells as the main mechanism for the slower expansion. Two alternative mechanisms—decreased recruitment rate of naïve T cells and reduced proliferation rate of T cells—also could contribute to the observed reduced expansion rate of the L. *monocytogenes*–specific CD8+ T cell response. However, we found no strong evidence for reduced recruitment of naïve T cells into the response or significantly reduced proliferation rates for L. *monocytogenes*–specific T cells in Py+L. *monocytogenes*–coinfected mice compared with L. *monocytogenes*–infected mice in either our mathematical modeling analysis or in vivo experiments.
Our data also show that Py infections affect the maturation status of DCs during coinfections; however, it is not clear how this occurs. These data are consistent with prior reports that demonstrated that both *P. falciparum* and *P. yoelii* pRBCs prevent DCs from upregulating costimulatory molecules, such as CD40, CD80, and CD86, when exposed to subsequent LPS stimulation (29, 30). Of note, the effect of *Plasmodium* on the functional characteristics of DCs is dependent on both the species and strain of *Plasmodium* (40). We demonstrate that Py infections impair the ability of DCs to upregulate T cell costimulatory molecules following *L. monocytogenes* infection, which correlates with decreased T cell activation (Fig. 1D). Although impaired T cell activation in Py+*L. monocytogenes*–coinfected mice might be directly linked to this observation, our data do not exclude the possibility that impaired T cell activation is independent of impaired DC maturation.

The mechanism(s) by which impaired T cell activation results in increased apoptosis is unclear. The transcription factor IRF4 is induced following TCR stimulation based on the strength of stimulation, which results in repression of the proapoptotic Bcl-2 family member Bim (41, 42). Decreased expression of CD25 on *L. monocytogenes*–specific T cells in Py+*L. monocytogenes*–coinfected mice indicates reduced T cell stimulation. However, we saw no differences in the expression levels of IRF4 or Bim in OT-I T cells between *L. monocytogenes*–infected mice and Py+*L. monocytogenes*–coinfected mice at days 2–3 or 5–8 post-*L. monocytogenes* infection (data not shown). Differences in the expression of the transcription factor Id2 is another potential explanation for the different levels of apoptosis in *L. monocytogenes*–specific T cells in *L. monocytogenes*–infected mice versus Py+*L. monocytogenes*–coinfected mice. Id2 provides survival signals during the generation of effector CD8+ T cells through the induction of pro-survival genes (e.g., Bcl-2 and Spi-6) and repression of genes that inhibit T cell survival (e.g., Bim and CTLA-4) (43–45). Alternatively, inhibitors of apoptosis proteins were recently implicated in the survival of LCMV-specific effector T cells during the expansion phase (46). Curiously, inhibitors of apoptosis proteins function via direct binding to, and inhibition of, caspases (47), which is consistent with the differential activity of caspase-3/7 in *L. monocytogenes*–specific T cells in *L. monocytogenes*–infected mice and Py+*L. monocytogenes*–coinfected mice. It is interesting to note that the role of apoptosis in regulating the magnitude of Ag-specific CD8+ T cell responses was proposed in several previous studies (48, 49). In particular, CD8+ T cells lacking expression of the type I IFN receptor (IFNAR) proliferated vigorously in vivo following LCMV infection but failed to accumulate (48), which is consistent with our data. Additional work is underway to identify the molecular mechanisms responsible for increased apoptosis in T cells responding to heterologous infections in Py-infected mice.

Previous studies addressed the impact of *Plasmodium* blood stage infections on the development of *Plasmodium* liver stage–specific CD8+ T cells. These studies reported conflicting results; one demonstrated that blood stage infections impair liver stage–specific CD8+ T cell responses (50), whereas the other did not observe any effect of a blood stage infection on liver stage–specific CD8+ T cell responses (50). In both of these studies the liver stage–specific CD8+ T cell responses were primed before the initiation of the blood stage infection. In contrast, we addressed the impact that a current or prior blood stage infection had on the induction of T cell responses to coinfections. Our data clearly demonstrate that, when mice have an active blood stage infection, particularly early during malaria, the T cell response to heterologous infection is impaired.

Although our analysis provides novel mechanisms for how immune responses could be suppressed by ongoing chronic infections, two features of such suppressed responses remain unclear. First, *L. monocytogenes*–specific CD8+ T cells in Py+*L. monocytogenes*–coinfected mice reach their peak abundance much later than do T cells during *L. monocytogenes* infection. It was argued that the timing of the peak CD8+ T cell response is programmed early in infection, possibly by the amount of Ag available to naive T cells, as well as by the inflammatory milieu present during priming (49, 51–55). However, our data indicate that the program of expansion of CD8+ T cells can be changed and prolonged in the presence of a bystander infection, raising questions about the mechanisms that regulate the Toff time of the immune response. Second, we observed a lower peak of the *L. monocytogenes*–specific CD8+ T cell response in Py+*L. monocytogenes*–coinfected mice, and our modeling predicted a faster rate of apoptosis for effector T cells, including a faster decline in T cell numbers, after the peak (Fig. 5E, 5F).

Because it was proposed that the number of memory CD8+ T cells formed following an acute viral infection is proportional to the number of effector T cells present at the peak of the response (56), we should expect fewer memory CD8+ T cells to be formed in Py+*L. monocytogenes*–coinfected mice. This outcome would be consistent with recent studies showing that the formation of memory T cells during a chronic bystander infection was impaired (57). Studies of whether this is the case and the potential mechanism(s) that regulate the formation of memory T cells during *Plasmodium* coinfections are underway in our laboratory. In conclusion, we identified a novel mechanism by which *P. yoelii* infections impair immunity to heterologous infections. Our results suggest that a better understanding of the pathways involved in the apoptosis of effector T cells during an immune response may lead to novel therapeutic strategies to improve the control of bacterial, and possibly viral, infections in individuals chronically infected with *Plasmodium* spp.

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References


