Restoring T cell competence is a significant clinical challenge in patients whose thymic function is severely compromised due to age or cytoreductive conditioning. Here, we demonstrate in mice that mesenteric LNs (MLNs) support extrathymic T cell development in euthymic and athymic recipients of bone marrow transplantation (BMT). Furthermore, in aged murine BMT recipients, the contribution of the MLNs to the generation of T cells was maintained, while the contribution of the thymus was significantly impaired. Thymic impairment resulted in a proportional increase in extrathymic-derived T cell progenitors. Extrathymic development in athymic recipients generated conventional naive TCRαβ T cells with a broad Vβ repertoire and intact functional and proliferative potential. Moreover, in the absence of a functional thymus, immunity against known pathogens could be augmented using engineered precursor T cells with viral specificity. These findings demonstrate the potential of extrathymic T cell development for T cell reconstitution in patients with limited thymic function.

Introduction
The scarcity of T cells in recipients of BM transplantation (BMT) leads to a devastating susceptibility to pathogens and is associated with increased malignant relapse (1–4). Moreover, age-related thymic involution leads to inefficient thymopoiesis and T cell regeneration in adult BMT patients, who are often decades into the process of thymic involution (5). Thymic involution results in the dramatic diminution of thymic function over time, with a loss of thymic epithelial cells as early as 1 year after birth and the decline of thymopoiesis hastened by hormones during puberty (6–9). Recent evidence suggests that naive T cell export from the thymus is completely absent in aging humans, where the primary source of T cell reepopulation is homeostatic proliferation (10). Expansion of peripheral T cells after BMT can lead to a pool of memory phenotype T cells with limited T cell receptor diversity (11, 12), leading to decreased immune function as well as poorer prognosis for older BMT patients (13–15). Consequently, mechanisms to circumvent the thymus to generate functional T cells could drastically improve not only BMT outcomes, but outcomes for all immunodeficient patients.

We have previously demonstrated that adoptive transfer of T cell precursors (preTs) generated ex vivo on the OP9-DL1 system into BMT recipients significantly increased thymopoiesis, circulating T cell numbers, and protection against bacterial infection (16, 17). While this is primarily dependent on improved thymopoiesis, based on studies in thoracically thymectomized BMT recipients, we hypothesized that extrathymic T cell development could contribute to the regeneration of the postransplant T cell pool (16).

Extrathymic development of conventional TCRαβCD4+ and CD8αβ T cells in physiologic settings is controversial, though recent evidence indicates that extrathymic T cell development occurs in human tonsil (18). In oncostatin-M (OM) transgenic mice, which have no thymic function, LNs support T cell development of largely dysfunctional mature T cells with a premature activation phenotype and limited antiviral function (19–32). Following BMT, early T lineage cells including CD4+CD8− (double positive [DP]) cells have been identified in extrathymic sites, including LNs, spleen, and BM (19, 27, 29, 30, 32). While thymic function is important for restoring T cell immunity after transplant, the potential for extrathymic development to support the reconstitution of functional T cells is unknown. Further study could offer clinically feasible strategies to support T cell regeneration through stimulation of extrathymic T cell development.

We therefore sought to determine whether extrathymic T cell development produces functional T cells during regeneration following BMT and whether adoptive transfer of ex vivo–generated preTs enhance T cell function in athymic recipients.

Results
Gut-associated tissues support extrathymic T cell differentiation after BMT. To determine the role of extrathymic T cell development after BMT, we performed a kinetic analysis of DP cells from donor BM origin in the lymphoid organs of BMT recipients for 8 weeks (CD45.2−BALB/c→CD45.2−BALB/c). As expected, the number of DP cells in the thymus increased early after BMT, reaching a plateau after 2 to 3 weeks (Figure 1A). Although the spleen, peripheral LNs (PLNs), and BM had previously been reported to support early lineage-negative or DP T cell progenitors after BMT (30), we did not detect extrathymic T cell development in these sites (Figure 1, A and B, and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI60630DS1). Moreover, the intestinal environment appeared to be critical for extrathymic development, as mesenteric LNs (MLNs), Peyer's patches, intraepithelial sites, and lamina propria supported DP cells (Figure 1, A and B, and Supplemental Figure 1B). MLN DP cells expressed CD8αβ, CD3, TCRβ, Thy1, and CD69 at levels equivalent to thymic DP cells, with lower expression of CD24 (Figure 1C).
Taken together, these data demonstrate that gut-associated tissues can support extrathymic T cell progenitors following BMT.

Adoptively transferred preTs undergo T cell development in MLNs. Having observed extrathymic development of BM-derived T cell progenitors after BMT, we sought to determine whether preTs also underwent extrathymic development (16, 17). We generated preTs by coculturing lineage–c-kit+Sca-1+ hematopoietic progenitors on the OP9-DL1 stromal cell layer for up to 26 days in the presence of IL-7 and Flt3L. We have previously determined that preT incubated for this period primarily share a phenotype with double-negative (DN) CD4−CD8−DN2 (CD44+CD25+) or DN3 (CD44−CD25+) cells and engraft in the thymus, generating mature peripheral T cells after BMT (16). We used luciferase+ preTs for in vivo bioluminescence imaging (BLI) to analyze the kinetics and sites of preT engraftment after transplant (BMT+preT, luciferase+H2-Kb+ C57BL/6 [B6] preTs in CD45.2+ BALB/c → CD45.2+ BALB/c recipients). Consistent with our previous findings, thymic engraftment of preTs peaked 2 weeks after BMT (Figure 2, A and B). Additionally, we confirmed that DN2 preTs, but not DN1 (CD44+CD25−) or DN3, recapitulated the thymic engraftment phenotype of the whole cell milieu resulting from preT culture (referred to throughout simply as preT) (Figure 2, A and B). Strikingly, we found engraftment of preTs and DN2 preTs in the abdomen of recipients prior to the peak of thymic engraftment (Figure 2, A and B).

We then investigated whether preT engraftment in the abdomen corresponded to extrathymic T cell development. Consistent with our findings with BM-derived cells, we found that MLNs supported a wave of preT-derived (CD45.1+) DP cell development (Figure 2, C and D) with moderately lower CD24 expression, but comparable expression of CD8β, CD3, TCRβ, Thy1, and CD69 to thymic DP cells (Figure 2E). These findings confirm that extrathymic T cell development occurs in the first 3 weeks after BMT. Additionally, they indicate that preTs can be used both therapeutically and as a tool to study the development and function of extrathymic-derived T cells.

In order to assess their ultimate fate, we examined the lineage potential of preTs in BMT recipients. In euthymic BMT+preT recipients (CD45.1+CD45.2−B6→CD45.2+ WT B6 or B6 nude), preTs and DN2 preTs (CD45.1+ B6) gave rise to many hematopoietic lineages, including B, NK, and dendritic cells and macrophages, though they were primarily restricted to a T cell fate (Supplemental Figure 2, A–F). Even without the strong signals of the thymic environment where 70% of preTs became T cells (Supplemental Figure 2, A and B), greater than 30% of preT and DN2 preT progeny in athymic recipients gave rise to T lineage cells (Supplemental Figure 2, C and D). After transfer across MHC barriers into allogeneic recipients, preTs also gave rise to T cells (data not shown), indicating that extrathymic T cell development is not strain specific and preTs can be used as a universal therapy.
We previously demonstrated that preTs undergo nuclear factor of activated T cell (NFAT) activation in the thymus, consistent with thymic positive selection (33–35). We administered NFAT vector + preTs (CD45.1 + B6) into WT or nude BMT recipients (CD45.2+ BALB/c → CD45.2+ BALB/c) (Figure 3, A–D). NFAT activation was restricted to the abdominal region in all recipients and the thoracic region in WT recipients (Figure 3, A and B). Upon ex vivo analysis of the abdominal area, we found that NFAT activation occurred primarily in MLNs, where a large proportion of preTs gave rise to DP cells (Figure 3, C and D), indicating that extrathymic developing T cells in BMT recipients employ signaling mechanisms similar to those of thymocytes.

We next sought to determine if extrathymic T cell development was regulated similarly to thymopoiesis at the level of progenitor trafficking. We detected direct engraftment of preTs (CD45.1 + B6) in the spleen, MLNs, and PLNs of WT recipients (CD45.2+) one day post-transfer, as well as engraftment in WT thymus (Figure 3E). We confirmed our prior observation that DN2...
preTs express high levels of the trafficking molecules SELPLG (or PSGL-1) and CCR9 (Figure 3F), which are known to mediate entry into thymus and intestinal sites (36–38). Using competitive reconstitution assays (CD45.1+CD45.2+ B6 → CD45.1+CD45.2+ B6), we observed that preTs require PSGL-1 but not CCR9 (CD45.2+ KO B6 preT vs. CD45.1+ WT B6 preT) for thymic and MLN DP cell reconstitution after BMT (Figure 3G). We detected P-selectin and ICAM expression in the MLNs, but not VCAM, with similar expression patterns in the PLNs (Supplemental Figure 3, C and D). However, there was no change in expression after transplant, nor a reorganization in the stromal compartment, to account for amplified extrathymic T cell development after BMT (Supplemental Figure 3, A and B). Taken together, these studies indicate that preTs engraft directly in extrathymic sites after BMT and require the trafficking molecule PSGL-1 for reconstitution.

**The relative contribution of extrathympic T cell development increases in aging BMT recipients.** Given that we observed extrathympic T cell development after transplant into healthy young recipients, we next investigated its role in a clinically relevant model of impaired thymic function by using aging recipients. In 24-month-old recipients (CD45.1+CD45.2+ B6 → CD45.2+ B6), we found significantly fewer thymic DP cells than in 3-month-old controls at day 21 after BMT. Taken together, these studies indicate that preTs engraft directly in extrathymic sites after BMT and require the trafficking molecule PSGL-1 for reconstitution.
BMT (Figure 4, A and C). Importantly, although nontransplanted old mice did not have MLN DP cells (Supplemental Figure 4E), we found that the MLNs in 24-month-old mice maintained their capacity to support DP cells (Figure 4, A and C). MLNs contributed more than 30% of DP cells in aged BMT recipients (Figure 4B), indicating that extrathymic T cell development plays an increasingly important role in T cell reconstitution in patients with severe thymic impairment. We confirmed these findings in young and aged CBF1 (H2-b/d) syngeneic BMT recipients, which suggests these findings are not strain or MHC specific (Supplemental Figure 4). DP cells found in aging MLNs were phenotypically comparable to thymic DP cells (Figure 4D). These studies demonstrate for what we believe is the first time that extrathymic T cell differentiation plays a greater role in restoring the T cell pool after BMT when thymic function is compromised.

Given the maintenance of T cell generation in MLNs when there is considerable thymic atrophy, we then investigated whether LNs themselves are critical for extrathymic T lineage generation. We performed congenic transplants (CD45.1+ CD45.2+ B6→CD45.2+ WT B6) into thymectomized WT or LTα KO recipients, which lack LNs and Peyer’s patches (39, 40). Although total engraftment was unaffected, mice lacking LNs exhibited significantly reduced de

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Figure 4
Extrathymic T cell development increases in aging BMT recipients. (A) BM-derived CD4+CD8+ cells in the thymus and MLNs of 3-month-old (n = 10) and 24-month-old (n = 8) recipients at day 21 after BMT (CD45.1+CD45.2+ B6→CD45.2+ WT B6). Data combined from 2 experiments. (B) Percentage of total BM-derived CD4+CD8+ cells derived from MLNs in A. (C) Representative plots showing BM-derived cells from the thymus and MLNs at day 21 after BMT in A. (D) Representative histograms of CD4+CD8+ cells from BM origin in the thymus (gray shaded histogram) and MLNs (black line) of 24-month-old recipients. (E) BM-derived CD45+ and CD3+ cells in thymectomized WT (n = 10) and LTα KO recipients (n = 10) (CD45.1+CD45.2+ B6→CD45.2+ B6) on day 28 after BMT. Data combined from 2 experiments. **P < 0.01.
Extrathymic T cell development yields a broad repertoire of TCRαβ+ CD4+ and CD8αβ+ T cells after BMT. Analysis of mature T cells at day 42 after congeneric (CD45.1+CD45.2+ B6→CD45.2+ WT B6 or B6 nude) BMT. Analyzed by nonparametric Mann-Whitney test. White bars, host; gray bars, BM; black bars, preT. Quantification of mature T cells in WT (nontransplanted, n = 2; BMT only, n = 2; BMT+preT, n = 10) and nude (nontransplanted, n = 6; BMT only, n = 29; BMT+preT, n = 18) mice. Data combined from 1 to 7 experiments. (A) CD45+ cells, CD4+ and CD8αβ+ T cells in WT and nude recipients. (B) CD8αα+ and TCRγδ+ T cells in WT and nude recipients. (C) Percentage of Vβ usage measured by flow cytometry in CD4+ and CD8+ T cells from preT origin in nude BMT+preT recipients (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 6
Thymus-independent development yields naive T cells. (A and B) Naive (CD62L^+CD44^-), effector memory (CD62L^-CD44^+), central memory (CD62L^+CD44^+), and CD62L^-CD44^- T cells. WT: BMT only, n = 3; BMT+preT, n = 6. Data combined from 2 to 3 experiments. Nude: BMT only, n = 19; BMT+preT, n = 12. Data combined from 4 to 5 experiments. (C and D) CD45.1^+CD45.2^- B6 or CD45.1^-CD45.2^+ B6 nude BMT recipients ± CD45.1^+ preTs on day 90 after transplant. Black bars, preTs; gray bars: BMT+host. Nontransplanted, n = 5; BMT only, n = 11; BMT+preT, n = 13. Data combined from 1 to 3 experiments. (C) CD4^+ T cells and (D) CD8^+ T cells. *P < 0.05; **P < 0.01; ***P < 0.001.
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Figure 7
Extrathymic T cells generated after BMT are functional in vitro. Analysis of T cells after congenic (CD45.1+CD45.2− B6→CD45.2− WT B6 or B6 nude) BMT. Analyzed by Mann-Whitney test. White: host; gray: BM; black: preT. (A) Stimulation index after αCD3/CD28 activation in vitro of splenic and PLN T cells from WT and nude BMT+preT recipients at day 42. One representative experiment of 2; n = 3 for each condition, in triplicate. (B–D) Quantification of TNF-α− and IFN-γ−producing splenic T cells from WT (n = 2) (B) and nude BMT alone (n = 6) and nude BMT+preT (n = 10) (C and D) recipients at day 42 following PMA/ionomycin stimulation. Data combined from 2 to 3 experiments. (C) CD4+ T cells. (D) CD8+ T cells. *P < 0.05; **P < 0.001.

In order to assess the functional capacity of extrathymic-derived T cells in vivo, we selected the lymphocytic choriomeningitis virus (LCMV) model, which induces potent CD8+ T cell responses. At the peak of LCMV-specific T cell responses on day 8 after challenge, we found an increased number of IFN-γ−producing CD4+ T cells in LCMV-infected nude BMT+preT recipients (Figure 8B), though less than WT controls (Figure 8A). We also found an increased number of TNF-α− and IFN-γ−producing CD8+ T cells in LCMV-infected nude BMT+preT recipients (Figure 8D), albeit 1 to 2 logs lower.

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The ability of extrathymic sites to support the generation of functional T cells of the TCR-αβ lineage has been a subject of debate owing to conflicting data in disparate model systems. Conventional CD4+ and CD8αβ+ T cells have routinely only been found to be of thymic origin, though in select systems, such as the overexpression of OM, LNs can support conventional T cell development, resulting in functionally inferior T cells (20–23). In the context of BMT, the potential for extrathymic T cell development appears to be significantly enhanced, with several groups describing early T cell precursor cells in BM, spleen, and LNs of BMT recipients (19, 27, 29, 30, 32). However, the ability of these extrathymic progenitors to generate functional T cells was poorly defined. In this study, we demonstrate that extrathymic T cell development after BMT generates a broad pool of functional T cells that, importantly, can contribute toward restoring immune competence in patients with limited thymic function.

LNs express many of the critical factors required for T cell development, including Notch ligands, IL-7, and peripheral tissue antigens required for T lineage cell commitment and selection (26, 41, 42). However, the intestinal environment was critical to extrathymic T cell development than in WT controls (Figure 8C), indicating enhanced antiviral responses conferred by preT-derived T cells in athymic mice.

Finally, we assessed viral burden after LCMV infection in WT and nude mice. As expected, WT mice effectively clear the virus due to thymic T cell development (Figure 8, E and G). Nontransplanted athymic mice, however, are persistently infected with LCMV (Figure 8F). Importantly, nude recipients of BMT have significantly lower viral burden (Figure 8F), suggesting the extrathymic generation of a functional T cell receptor repertoire from the polyclonal BM progenitor pool. Despite a trend in individual experiments toward lower viral burden, we did not detect a significant enhancement of antiviral immunity in mice that received preTs compared with nontransplanted nude controls (Figure 8F). However, athymic BMT recipients that received p14 transgenic preTs, which express the LCMV-specific T cell receptor p14, had several logs lower viral burden than nude recipients of WT preTs, indicating a significant improvement in pathogen clearance (Figure 8H). These findings indicate that extrathymic T cell development after BMT generates fully functional T cells from BM and preT origin. Importantly, genetically engineered preTs can further enhance antiviral immunity in athymic recipients.

**Discussion**

The ability of extrathymic sites to support the generation of functional T cells of the TCR-αβ lineage has been a subject of debate owing to conflicting data in disparate model systems. Conventional CD4+ and CD8αβ+ T cells have routinely only been found to be of thymic origin, though in select systems, such as the overexpression of OM, LNs can support conventional T cell development, resulting in functionally inferior T cells (20–23). In the context of BMT, the potential for extrathymic T cell development appears to be significantly enhanced, with several groups describing early T cell precursor cells in BM, spleen, and LNs of BMT recipients (19, 27, 29, 30, 32). However, the ability of these extrathymic progenitors to generate functional T cells was poorly defined. In this study, we demonstrate that extrathymic T cell development after BMT generates a broad pool of functional T cells that, importantly, can contribute toward restoring immune competence in patients with limited thymic function.

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development, as anatomically distant PLNs failed to support DP cell development in multiple mouse strains. The unique signals provided by the intestinal environment that allow MLNs to support T cell development, along with the intra-nodal conditions that actually support that development, are an exciting area for future investigation. While MLNs in aging euthymic and athymic mice do not support appreciable numbers of DP cells, extrathymic T cell development is documented in athymic mice (19). However, this process is inhibited by the presence of mature T cells in the LNs (19). The postransplant setting, with its extreme lymphopenia and depletion of T cells from the periphery, may condition the LNs for extrathymic T cell development until such time as mature T cells reconstitute the organ (19, 20). It will be interesting to investigate the roles of lymphopenia, tissue damage, and the abundance of circulating progenitor cells in transiently amplified extrathymic T cell development after transplant. Limited thymic function in aged BMT patients leads to slow and often incomplete T cell recovery, causing susceptibility to opportunistic pathogens and tumor relapse. In fact, aging takes an even greater toll on the thymus in humans than in mice (10), and it has been suggested that athymic mice would potentially serve as more appropriate BMT recipients than aged mice in modeling transplant into older patients (12). As such, it is therefore likely that extrathymic T cell differentiation plays an even more prominent role in T cell reconstitution in BMT patients than we have been able to demonstrate in our models. Here we show that Foxn1-deficient, genetically athymic mice can generate significant numbers of conventional TCRαβ T cells following BMT. Neither CD4+ nor CD8+ T cells displayed the premature activation or memory phenotype reported in previous studies of extrathymic-derived T cells (22). While the number of TCRαβ T cells in athymic BMT recipients is approximately 2 logs lower than in euthymic recipients, the successful generation of functional extrathymic T cells constitutes an attractive target for therapeutic optimization. The extrathymic microenvironmental cues provided to either polyclonal BM or preTs are adequate to produce a qualitatively functional extrathymic-derived T cell pool, as extrathymic-derived T cells in our system proliferate after TCR ligation and mount antiviral cytokine responses. We also demonstrate that, in the absence of a functional thymus, rapid immune competence can be conferred up on previously unseen pathogen suggesting the generation of a diverse receptor repertoire. The inability of polyclonal preTs to enhance antiviral immunity compared with BM alone indicates that the increased size of the T cell pool does not result in increased LCMV-specific T cell frequency. In addition, several factors may have masked any potential effects of the WT preTs, including administration of the standard infection dose despite 2-log lower T cell numbers and assessment of PFU at the standard time point despite persistent infection. Importantly, using genetically engineered preTs with viral specificity, we could substantially enhance viral clearance in both euthymic and athymic recipients. These findings indicate that patients with limited thymic function due to genetics, aging, or prolonged cytoreductive therapies may yet have the requisite machinery to generate a diverse repertoire of functional T cells. Moreover, these extrathymic pathways of T cell development can be harnessed to provide rapid specific antimicrobial and antitumor immune competence.

Methods

Mice. B6 (CD45.2+ B6), B6.SJL-Plp1−/− Pepl3+BoyJ (CD45.1+ B6), BALB/c (CD45.2+), and CB6F1 mice were obtained from the Jackson Laboratory. B6 and BALB/c background Foxn1-deficient nude mice (CD45.2) were obtained from Taconic Farms. Cer9 KO, Select (PSGL-1) KO, P1 CD45.1+ CD45.2+ B6, luciferase+ B6 maintained at Memorial Sloan-Kettering Cancer Center in accordance with Institutional Animal Care and Use Committee standards. BMT. Transplants were performed as previously described (33, 43). Briefly, mice received split-dose lethal total body irradiation (B6 background mice: 1100 cGy; BALB/c background mice: 850 cGy; CB6F1 background mice: 1300 cGy). BM, prepared by aseptic preparation of femoral BM using MACS lineage depletion kit (Miltenyi Biotec), was administered i.v. at a dose of 10⁶ lineage-depleted BM cells per mouse. Where indicated, preTs (4–8 × 10⁵ cells) or sorted preTs (1–2 × 10⁶ cells) were coadministered with BM.

Culture. Femoral BM was harvested aseptically and processed using a MACS lineage depletion kit to deplete mature hematopoietic cells expressing CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and c-Kit. Lineage Scamice; c-kit cells were isolated on MoFlo (Beckman Coulter) or Aria (BD Biosciences) sorters, then plated on OP9-DL1. Cells were maintained in MEM-α+ 20% FCS + 1% penicillin/streptomycin (P/S) with 10 ng/ml murine IL-7 and 10 ng/ml murine Flt3 ligand. Cultures were passaged every 4 days and used for analysis or transplant between days 14 and 28 of coculture.

Antibodies and flow cytometry. All antibodies were obtained from BD Biosciences — PharMingen other than CD62L PE—Texas Red (Caltag Laboratories), CD44 Alexa Fluor 700 (BioLegend), CD25 PE—Texas Red (Caltag Laboratories), and CCR9 PE (R&D Systems). For cell-surface markers, cells were stained for 15 minutes at 4°C in PBS with 0.5% BSA (PBS/BSA), washed, and resuspended in DAPI in PBS/BSA. For intracellular cytokine staining, cells were stimulated for 5 hours with 50 ng/ml PMA and 500 ng/ml ionomycin. Cell-surface staining was as above, followed by intracellular staining with the eBioscience kit per the manufacturer’s instructions. All flow cytometry was performed on an LSR II (BD Biosciences) and analyzed with FlowJo (TreeStar Software).

BLI. PreTs generated from mice expressing firefly luciferase were adoptively transferred into BMT recipients as described above. Engraftment of preTs was monitored several times per week by in vivo bioluminescence. Mice received D-luciferin (6 mg/mouse; Caliper Life Sciences) i.p., were anesthetized by isoflurane, and imaged 10 minutes after injection. Mice were placed in the light-tight chamber of an IVIS-200 imaging system (Caliper Life Sciences), and 5 minute exposures were acquired. Bioluminescence data were analyzed using Living Image 2.5 (Caliper Life Sciences).

Proliferation assay. Spleens and PLNs were harvested at day 42 following BMT and aseptically processed to single-cell suspension; then 5 × 10⁶ cells each were plated in a volume of 200 μl of RPMI + 10% FCS + 1% P/S + 1% β-mercaptoethanol in a 96-well U-bottom plated coated with antiCD3 (10 μg/ml) and antiCD28 (1 μg/ml). Each sample was plated in triplicate. Cells were stimulated for 72 hours at 37°C in a humidified atmosphere with 5% CO₂ content. [3H]Thymidine was added 72 hours into stimulation, and cells were harvested 16 hours later.

LCMV challenge. At day 28 after BMT, mice were challenged i.p. with 2 × 10⁶ LCMV-Armstrong PFUs. Splenocytes were harvested 8 days after challenge and plated with either CD4 epitope gp61 or CD8 epitope gp33 peptides. After 5 hours, cells were harvested and processed for intracellular cytokine (ICC) as above. PFU assays were performed as previously described (44). Briefly, 7.5 × 10⁶ Vero cells were plated per well in a 6-well plate on day –1 of assay. On day 8 after infection, spleens were harvested. Spleens were sonicated in 1 ml of RPMI and medium was aspirated from Vero cells; then 0.2 ml of sonicate was plated in serial dilution (10⁻¹ through 10⁻¹⁰) and covered with a 1:1 complete Medium 199:1% agarose mixture following 60 minutes of adsorption on day 0 of assay. Plates were kept at 37 degrees in a CO₂ incubation and after 4 days, additional 1:1 complete 199 medium (1% agarose containing neutral red dye) was added to wells. The following day, plaques were read.


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