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Both Dendritic Cells and Macrophages Can Stimulate Naive CD8 T Cells In Vivo to Proliferate, Develop Effector Function, and Differentiate into Memory Cells

Lu-Ann M. Pozzi, Joseph W. Maciaszek, and Kenneth L. Rock

The generation of T cell immunity requires the acquisition and presentation of Ag on bone marrow-derived APCs. Dendritic cells (DC) are believed to be the most potent bone marrow-derived APCs, and the only ones that can stimulate naive T cells to productively respond to Ags. Because macrophages (MΦ) are bone marrow-derived APCs that are also found in tissues and lymphoid organs, can acquire and present Ag, and can express costimulatory molecules, we have investigated their potential to stimulate primary T cell responses in vivo. We find that both injected MΦ and DCs can migrate from peripheral tissues or blood into lymphoid organs. Moreover, injection of peptide-pulsed MΦ or DCs into mice stimulates CD8 T cells to proliferate, express effector functions including cytokine production and cytolyis, and differentiate into long-lived memory cells. MΦ and DCs stimulate T cells directly without requiring cross-presentation of Ag on host APCs. Therefore, more than one type of bone marrow-derived APC has the potential to prime T cell immunity. In contrast, another bone marrow-derived cell, the T lymphocyte, although capable of presenting Ag and homing to the T cell areas of lymphoid organs, is unable to stimulate primary responses. Because MΦ can be very abundant cells, especially at sites of infection and inflammation, they have the potential to play an important role in immune surveillance and the initiation of T cell immunity. The Journal of Immunology, 2005, 175: 2071–2081.

The generation of a CD8 T cell response requires the presentation of antigenic peptides (1–3) on a bone marrow-derived professional APC (4–6). APCs resident in the tissues or circulating in the blood gather Ag from the local environment and then migrate to secondary lymphoid organs (7). APCs that reside in the lymph nodes and spleen may also acquire Ag from lymph fluid or blood, respectively. These cells then generate peptides from the Ags they have acquired and display them on their MHC class I molecules (8–12). Once in the lymphoid tissue, the APCs present these peptide-MHC class I complexes and costimulatory molecules to naive T cells and initiate an immune response (11, 12). Cells of nonhematopoetic origin are unable to prime naive T cell responses (4, 5, 13), presumably because they lack the necessary costimulatory molecules and are unable to traffic to lymphoid organs (14–16).

Dendritic cells (DC) are bone marrow-derived APCs that reside in most tissues in the body. They are initially in an immature state in which they are highly phagocytic and express low levels of MHC class II and the costimulatory ligands CD80 and CD86 (7, 17). Once DCs acquire Ag and receive activation signals, e.g., from CD40-CD40L interactions, Toll-like receptors, and/or exposure to inflammatory cytokines, they mature and express high levels of MHC class II molecules, CD80, CD86, and cytokines such as IL-12 (7, 17). They also migrate to the T cell zones of the secondary lymphoid organs where they interact with naive T cells. It is presently thought that DCs are the only cells that can stimulate naive T cells in vivo (7, 18, 19).

Macrophages (MΦ) are another bone marrow-derived APC that can present Ag and express costimulatory molecules. MΦ are also found in most tissues, and their numbers rapidly increase at sites of infection or inflammation. Early studies suggested that MΦ could stimulate primary immune responses. For example, immunization with MΦ led to the priming of naive T cell responses (20). However, in these experiments, MΦ may not have been directly priming T cells because it is now known that DCs can acquire and cross-present Ag from other cells. Other studies using carrageenin and silica or toxic liposomes to deplete animals of phagocytic cells were initially interpreted to show that MΦ play an important role in the initiation of naive T cell responses (21–24). However, these treatments deplete the animals of all phagocytic cells including immature DCs. More recent studies have suggested that DCs are the key cells needed to initiate T cell responses, at least in certain situations (11, 12, 25, 26). The current view is that the presentation of Ag by MΦ to T cells is important for the effector phase of immune responses (i.e., T-dependent activation of MΦ) rather than in the afferent phase of responses (stimulating naive T cells).

Understanding what APCs stimulate immune responses is important for understanding how different kinds of immune responses are generated and for the rational design of vaccines and immunotherapy. When particulate Ag is injected s.c. in vivo, it is acquired and cross-presented by a bone marrow-derived APC and stimulates CD8 T cell response (10, 27–29). When the APCs responsible for acquiring the particulate Ag and transporting it to lymph node were characterized, only ~50% of the cells expressed...
the DC marker CD11c (29). The other Ag-containing phagocytic cells lacked CD11c and were presumably MΦ (29). We have demonstrated previously that MΦ are as good as DCs at cross-presenting Ag in vitro (27, 30, 31). Therefore, we investigated the capacity of MΦ to prime immune responses in vivo. Using an adoptive transfer model, we find that MΦ do prime naive CD8 T cells to proliferate and mature into both effector and memory cells. In addition, this priming occurs through the direct presentation of peptides by the MΦ to the T cells.

Materials and Methods

Animals

C57BL/6, B6/SJL F1, Rag−/−, BALB/c, β2-microglobulin-deficient (B6.129P2−/−B6.2m−/−), and mice congenic for Ly 5.1/CD45.1 (B6.129P2−/−Ptpcr−/−Pep3′Boy) and Thy 1.1/CD90.1 (C57BL/6−/−Igth1 Gpi1′) were initially obtained from The Jackson Laboratory. TCR transgenic (Tg) mice (OT-I, specific for the OVA peptide SIINFEKL bound to Kb) (32) were obtained from Steve Jameson (University of Minnesota, Minneapolis, MN); breeding pairs of P-14 TCR Tg mice specific for lymphocytic choriomeningitis virus (LCMV) gp33 peptide (KAVYNFATCGIFA) bound to Kb were from B6.SJL−/−Igh a (CD45.2, CD90.2) background or bred onto C57BL/6 (CD45.2, CD90.2) background or bred onto C57BL/6-Igh a thy1a Gpi1a (C57BL/6-Ptprca Pep3b/Boy) and C57BL/6-Igh a (CD45.1, CD90.1) congenic Ly 5.1/CD45.1 mice were bred onto C57BL/6-CD45.1 or C57BL/6-CD90.1 mice. Some of these strains were characterized previously that MΦ are as good as DCs at cross-presenting Ag in vitro (27, 30, 31). Therefore, we investigated the capacity of MΦ to prime immune responses in vivo. Using an adoptive transfer model, we find that MΦ do prime naive CD8 T cells to proliferate and mature into both effector and memory cells. In addition, this priming occurs through the direct presentation of peptides by the MΦ to the T cells.

Adoptive transfer experiments

Adoptive transfer experiments were done as described previously (39). Briefly, T cells were isolated from the lymph nodes of TCR Tg mice on a wild-type or Rag−/− background by purification on nylon wool or Ab plus complement (Pel-Freeze) depletion using an anti-class II (M5/114) and an anti-heat stable antigen (J11D) mAb (ATCC) and labeled with 1 μM CFSE (Molecular Probes) for 20 min at 37°C. The cells were then washed once with HBSS without Mg2+ and Ca2+ (Invitrogen Life Technologies), supplemented with 5% FCS, followed by serum-free HBSS. On day 1, 2.5 × 104–3 × 105 Tg T cells were adoptively transferred into host animals, which resulted in Tg T cells constituting ~1% of CD8 T cells. On day 0, DCs or MΦ pulsed with the appropriate peptide were injected either s.c. or i.v., depending on the experiment. Nonpeptide-pulsed APCs were used as a negative control. Draining lymph nodes and/or spleens were removed at various time points, stained with PerCp-conjugated anti-CD8 (BD Pharmingen), and either allophycocyanin-conjugated anti-CD45.1 (BD Pharmingen) or Cy5-conjugated anti-CD90.1 (eBioscience), and then analyzed by flow cytometry.

Intracellular cytokine staining

Lymph node and/or spleen cells were stimulated ex vivo with either media alone or peptide (SIINFEKL, 1 μg/ml; or KAVYNFATCGIFA, 5 μg/ml) in the presence of Brefeldin A for 5 h. The samples were then washed and stained for CD8 and a congenic marker (CD45.1 or CD90.1) for 30 min. The samples were then fixed with Perm/Fix (BD Pharmingen) for 20 min in the cold and washed with Perm/Wash (BD Pharmingen) twice. PE-anti-IFN-γ (clone XMG; BD Pharmingen) was added in the presence of Perm/Wash (BD Pharmingen) for 45 min. The samples were washed twice and fixed. All samples were assayed by flow cytometry.

In vivo cytosis

The in vivo cytosis assays were done as described previously (39). Briefly, at various times points after priming, peptide-pulsed and nonpeptide-pulsed CD45.1 CSFE-labeled splenocyte targets were injected i.v. Twenty to 24 h later, lymph nodes or spleens were removed and stained with anti-CD45.1 conjugated to allophycocyanin. Samples were analyzed by flow cytometry. Percentage of killing was determined according to the following formula: 100 − (((percentage of peptide pulsed in primed)/percentage of unpulsed in primed)/percentage of unpulsed in unpimed) × 100).

Viral challenge experiments

C57BL/6 mice were immunized with DCs or MΦ pulsed with 10 μg/ml gp33 peptide. Forty days later, the animals were challenged with 5 × 104 PFU of LCMV (Armstrong strain) i.p. Three days after infection, spleens were removed and homogenized. The homogenates were spun to remove debris and frozen at −80°C. Ten-fold serial dilutions of the homogenates were added to Vero cell monolayers plated (1–60–70% confluent) with an agar overlay and incubated for 24 h, at which time neutral red was added in more overlay mixture. Twenty-four to 48 h later, plaques were visualized using a light box and counted. Viral titers were calculated according to the following formula: PFU per millilitre = plaques counted × dilution × volume added.

Statistical analysis

The proliferation data was represented as percentage of Tg T cells proliferating and subjected to statistical analysis. Significant effects of condition and strains were evaluated using ANOVA for mixed models (SAS 9.1) by maximum likelihood cross tabulation (SPSS 12; SPSS Inc., Chicago, IL). Differences in proportion of nonresponders were evaluated using Fisher’s exact test. Viral plaques assay data was subjected to the student’s t test using Excel software (Microsoft).

Results

Phenotype and migratory properties of cultured primary DCs and MΦ

To study the ability of different types of APC to prime naive T cells, we grew MΦ and DCs from mouse bone marrow. First, we characterized their phenotype (Fig. 1). Approximately 70% of the cells cultured in GM-CSF and IL-4 differentiated into DCs, as defined by expression of the pan-DC marker CD11c (Fig. 1E);
Although these cells are not homogeneous, we will henceforth refer to them as DCs. In contrast, the bone marrow cultured in M-CSF, which stimulated the growth of MΦ, totally lacked the expression of the CD11c DC marker (Fig. 1E). Although a subpopulation (~30%) of MΦ had a lower level of MHC class I molecules than the DCs, the majority of these two cell types expressed equal amounts of this molecule (Fig. 1A). Similarly, the majority of both cells expressed equivalent levels of CD80 and CD86, although a small subpopulation of DCs (~20%) had higher levels of both markers (Fig. 1, C and D). Approximately 82% of DCs expressed MHC class II molecules at moderate levels, and ~18% expressed class II at high levels (Fig. 1B). The small subpopulation of DCs expressing the high levels of CD80, CD86, and MHC class II are ones that have undergone maturation. The population of MΦ was also biphasic for MHC class II, with ~40% expressing moderate levels and 60% expressing low to undetectable levels of class II molecules (Fig. 1B). When the MΦ were compared with DCs, the majority express similar levels of MHC class I and costimulatory molecules. We also examined the expression peptide-MHC class I complexes on these cells. MΦ and DCs were incubated with SIINFEKL peptide, stained with 25D1 (38), and analyzed by flow cytometry. Approximately 3-fold lower numbers of K\(^\alpha\)-SIINFEKL complexes initially formed on the MΦ as compared with DCs (data not shown).

In the experiments described below, we compare the stimulation of T cells in vivo after injection of DCs and MΦ into mice. Therefore, we also examined the ability of the DCs and MΦ to migrate into lymphoid tissue 24 h after injection into s.c. sites or from the blood. Migrating DCs and MΦ were present in the draining lymph nodes (Fig. 2A). However, when equivalent numbers of these APCs were injected, ~8-fold more of DCs migrated to the draining lymph node as compared with MΦ (Fig. 2A). In contrast, when these same APCs were injected i.v., equal numbers of both APCs were detected in the spleen (Fig. 2B). We were unable to reliably detect by flow cytometry the migration of either APC to the inguinal lymph nodes when delivered i.v. (although T cell stimulation assays described below indicate that small numbers of both cell types may migrate into lymph nodes). Therefore, both MΦ and DCs can migrate to secondary lymphoid organs, but the relative efficiency of this process for MΦ vs DCs depends on the site of injection.

**FIGURE 1.** Characterization of APC populations. Bone marrow-derived MΦ (solid line) and DCs (dashed line) were cultured and stained as described in Material and Methods. A, MHC class I; B, MHC class II; C, CD80; D, CD86; E, CD11c; and F, irrelevant control (PE-anti-IA\(^\alpha\)).

**FIGURE 2.** DCs migrate more efficiently out of the s.c. space to the lymph nodes than MΦ, but with equal efficiency out of the blood into the spleen when injected i.v. A, A total of 10\(^6\) CFSE-labeled MΦ or DCs was injected s.c. Twenty-four hours later, the draining lymph nodes were analyzed by flow cytometry. Lymph nodes from an animal that did not receive any APC were used as negative controls. Each bar represents an individual mouse’s lymph node. The mean number of cells that migrated for MΦs was 102.2, and the mean number of cells that migrated for DCs was 798.7. Similar results were obtained in five experiments. B, A total of 10 \(\times\) 10\(^6\) CFSE-labeled MΦ or DCs was injected i.v. Twenty-four hours later, spleens were removed, and a single-cell suspension was prepared as in A. A spleen from an animal that did not receive any APC was used as a negative control. Samples were then analyzed by flow cytometry. The mean number of cells that migrated for MΦs was 2474.5, and the mean number of cells that migrated for DCs was 2298.6. Similar results were obtained in five experiments.

Both DCs and MΦ can stimulate primary T cell responses in vivo

To test the ability of DCs vs MΦ to stimulate T cells, we used an adoptive transfer system similar to that described by Jenkins and colleagues (11). We used T cells purified from P-14 LCMV TCR Tg mice (specific for the gp33 peptide KAVYNFATC presented on D\(^\alpha\)) because they uniformly have a naive phenotype as assessed by expression of CD44, CD62, and CD62L (data not shown). C57BL/6 mice received injections i.v. with CFSE-labeled P-14 TCR Tg T cells, and 24 h later they received injections s.c. with KAVYNFATC-pulsed or unpulsed bone marrow-derived DCs or MΦ. After 4 days, the draining lymph nodes (popliteal) were removed from these mice, and the transferred T cells (CD8\(^+\), CD4\(^+\), and CD45.1\(^+\)) were analyzed by flow cytometry. In animals immunized with either MΦ or DCs coated with peptide, the Tg T cells
proliferated, as indicated by the progressive loss of CSFE label (Fig. 3A). In contrast, Ag-specific T cells did not proliferate in animals primed with APCs without KAVYNFATC (gp33).

Because the prevailing view has been that DCs are the principal cells that stimulate naïve T cells (7), it was surprising to find that MΦ stimulate such strong primary T cell responses. Consequently, we evaluated whether contaminating DCs might actually account for the ability of cultured MΦ to prime T cell responses. We first determined that the minimum number of injected DCs that stimulated T cell responses was 50,000 (Fig. 3B). Therefore, in order for DCs to be responsible for stimulating the responses we observe with MΦ, they would be need to contaminate these preparations at a level >10%. However, as described above, the cultured MΦ contained undetectable levels of cells expressing the CD11c DC marker, which is expected because DCs are not stimulated by the M-CSF used to grow MΦ. To further assess this point, we also tested MΦ and DCs lines, which are cloned and lack any contaminating cell type. We found that both the C2.3 (Fig. 3C) and A3.1 (data not shown) MΦ cell lines as well as the DC2.4-cloned DC line (Fig. 3C) stimulated primary T cell responses in vivo. Although there was some mouse to mouse variability in the magnitude of the responses observed, the overall responses they stimulated were similar. Therefore, we conclude that peptide-pulsed MΦ can stimulate primary T cell responses.

Comparison of DC and MΦ priming of T cell responses

Although the overall level of proliferation and accumulation of terminally divided Ag-specific T cells was similar when animals were stimulated with either MΦ or DCs at higher doses of peptide s.c., the kinetics of the responses were different. Tg T cells in the MΦ-primed animals consistently reached their peak frequency 3 days after priming, whereas they peaked on day 4 in animals primed with DCs (data not shown).

The degree of T cell proliferation as well as the number of T cells present in the terminal division peak was dependent on the dose of peptide used to coat the APCs (Fig. 3A). Figure 3A also demonstrates that the amount of peptide needed to induce proliferation of the Ag-specific T cells was ~10-fold higher for MΦ than it was for DCs when they were injected at equal numbers s.c. (1 μg/ml vs 0.1 μg/ml, respectively). At the high (nonlimiting) doses of peptide, DCs and MΦ stimulated equal proliferation and percentage accumulation of terminally divided T cells (77% for DCs vs 81% for MΦ; Fig. 3A). Similar results were observed in five experiments with a statistically significant difference in responses stimulated by MΦ and DCs compared at equal peptide concentration (p < 0.0001), but there was no significant difference when MΦ pulsed with 10-fold more peptide were compared with DCs (p = 1.0). A similar 10-fold difference in potency was also observed when MΦ and DCs coated with SIINFEKL were used to stimulate OVA-specific OT-I Tg T cells (data not shown).

Because our earlier data (Fig. 2A) indicated that after s.c. injection DCs migrated to lymph nodes up to 8-fold better than MΦ, we attempted to increase the number of migrating MΦ by giving mice injections with larger numbers of these APC. When mice were given injections with four times the number of gp33-pulsed MΦ as DCs, the two APCs stimulated an equal expansion of the Ag-specific T cells to the same concentration of peptide (Fig. 4). Similarly, because our earlier data indicated that after i.v. injection DCs and MΦ migrate similarly to secondary lymphoid tissue, we equalized the number of migrating cells by i.v. injection. Under these conditions, the peptide-pulsed MΦ and DCs stimulate equal numbers of P-14 Tg T cells to proliferate in the spleen at all time points examined (days 3–6) (Fig. 5). Similar results were obtained in three experiments, and again there was no statistically significant difference between the responses stimulated by the two APC types at all peptide concentrations (p > 0.1). Although we were unable to visualize by flow cytometry the migration of i.v.-injected

![FIGURE 3.](http://www.jimmunol.org/) Ability of s.c.-injected DCs and MΦs to stimulate naïve T cells to proliferate. A. CD45.1+ congenic CFSE-labeled P-14 T cells were adoptively transferred i.v. into C57BL/6 mice. After 24 h, the mice were immunized s.c. with 0.5 × 10^6 of DCs (top panels) or MΦs (bottom panels) pulsed with or without the indicated concentration of gp33 peptide. Four days later, the draining lymph nodes were analyzed, stained with Abs against CD8 and CD45.1, and analyzed by flow cytometry. The samples were gated on live, CD8+ and CD45.1+ cells and analyzed for CFSE content. The x-axis depicts the level of CFSE content of the Tg T cells, whereas the y-axis represents relative cell number. The groups in this experiment were repeated at least five times in independent experiments with similar results. B. CD45.1+ congenic CFSE-labeled P-14 T cells were adoptively transferred into C57BL/6 mice. The next day, mice were immunized i.v. with various numbers of DCs pulsed with 10 μg/ml gp33 peptide. Three days later, the draining lymph node was removed and stained. The different lines represent independent mice (n = 1 for no Ag and 10^2; n = 2 for 1.8 × 10^2 and 1.5 × 10^2; and n = 3 for 5 × 10^2) from the same experiment. This experiment was repeated three times with similar results. C. CD45.1+ congenic P-14 T cells isolated from lymph nodes were adaptively transferred into C57BL/6 mice. The next day, mice were immunized s.c. with the indicated cell lines pulsed with the indicated concentrations of gp33 peptide. Three days later, the draining lymph node was removed and analyzed. The different lines represent independent mice from the same experiment (n = 2 for gp33-pulsed MΦ cell line; n = 3 for gp33-pulsed DC cell line; and n = 1 for the no Ag control). This experiment was repeated three times with similar results.
APCs in the lymph nodes, we observed similar levels of P-14 T cell proliferation in the lymph nodes of mice immunized with DCs or MΦ (data not shown); because this T cell proliferation was observed at very early time points, it may represent T cells stimulated in the nodes rather than ones that were stimulated elsewhere and subsequently trafficked into the nodes. In any case, this set of experiments indicates that when MΦ are injected i.v., they are able to prime naive P-14 Tg T cells as well as DCs.

Peptide-pulsed T cells are unable to stimulate responses

To determine whether any MHC class I-positive cell can prime naive Tg T cells, we investigated whether T cells could also function as APCs in this model. T cells were chosen because they express class I molecules at high levels, can present pulsed peptides on MHC class I molecules, and migrate into the T cell areas of the secondary lymphoid organs. As a source of T cell APCs in these experiments, CD8+ T cells were isolated from the lymph nodes of OT-I mice bred on to a Rag-deficient background (providing a homogeneous pure population of T cells of irrelevant specificity that lacked B cell contamination). When injected s.c., this pure population of gp33-pulsed CD8+ T cells was unable to stimulate the naive P-14 T cells to proliferate (Fig. 6A). In contrast, in the same experiment, MΦ and DCs stimulated P-14 T cells, demonstrating that the CSFE-labeled P-14 T cells were capable of proliferating (data not shown). As shown in Fig. 6B, when the OT-I T cells APCs were injected i.v., they induced only a small amount of proliferation of the P-14 T cells, and this proliferation was only to a maximum of three divisions. There were no P-14 T cells accumulating in the terminal division peak, nor were there any T cells seen between division peak three and eight. Evidence will be presented below that some of this very limited response may be due to cross-presentation by host APCs. Therefore, not all MHC class I-expressing cells can stimulate naive T cells, even when the APCs home to the T cell areas in the lymphoid organs.

MΦ and DCs stimulate the generation of effector T cells

Effective immune responses require that T cells not only proliferate but also express effector functions. Under some conditions, T cells can be stimulated to undergo several rounds of cell division but become anergic and fail to express effector functions (40). To determine whether both peptide-pulsed DCs and MΦ stimulated naive P-14 T cells to become effector cells in vivo, we first analyzed whether 3–4 days after immunization the proliferating T cells were producing the effector cytokine IFN-γ. Both MΦ and DCs stimulated similar numbers of naive P-14 T cells to produce IFN-γ (Table I). Similar results were obtained in three experiments. Consistent with previous reports (41), only T cells that have undergone multiple rounds of division were making IFN-γ.

We next analyzed the ability of both MΦ and DCs to induce cytolytic function using in vivo cytolyis assays as described previously (39). Table I shows data from a representative experiment. In replicate animals, both DCs and MΦ stimulated specific lysis of peptide-pulsed targets (85–87 vs 48–60% specific lysis, respectively). Similar results were observed in four experiments with both APCs stimulating robust killing, although the DCs stimulated stronger responses than MΦ (p = 0.00003). Therefore, both the
intracellular IFN-γ and in vivo cytolysis assays indicate that MΦ and DCs are able to stimulate naive P-14 T cells to become fully functional effector cells.

**MΦ and DCs can stimulate the generation of memory**

To evaluate whether MΦ also stimulated the formation of memory cells, we analyzed mice MΦ40 days after they received injections with CSFE-labeled Tg P-14 T cells and primed with peptide-pulsed or unpulsed APCs. Because i.v. inoculation of APCs led to a larger pool of effectors, we chose this route of immunization for these experiments. Memory cells were identified as transferred T cells that had undergone a complete loss of CSFE and survived ≥40 days (42). Figure 7 shows the results from replicate mice in a representative experiment. MΦ and DCs both induced small but similar numbers of long-lived memory cells. Similar results were observed in three experiments. Although there was some mouse to mouse variability in the number of memory cells that were generated, the level of memory cells induced by peptide-pulsed MΦ and DCs was not statistically different (p = 0.123). In contrast, very few, if any, CSFE-negative P-14 T cells were identified in the mice injected with DCs or MΦ that were not coated with peptide, as expected. These data demonstrate that MΦ and DCs prime similar levels of memory cells.

We next examined whether memory cells primed by MΦ were functional. We stimulated these cells ex vivo with peptide and performed intracellular IFN-γ cytokine staining. Results from a typical experiment are shown in Table II. Animals that received peptide-pulsed MΦ or DCs had a substantial population (between 18 and 35%) of IFN-γ-secreting Tg P-14 T cells. In contrast, animals receiving unpulsed APCs had <1.5% of the Tg T cells making IFN-γ. Similar results were obtained in three experiments. Therefore, MΦ and DCs stimulate the generation of similar levels of functional memory cells.

To determine whether MΦ could also prime endogenous polyclonal non-Tg T cells, C57BL/6 mice were immunized i.v. with either MΦ or DCs pulsed with 10 μg/ml gp33 peptide and rested for 40 days so that memory T cells, if generated, would be present. The mice were then challenged with LCMV, and 4 days later viral titers in the spleen were quantified. Immunization with either MΦ

![Table I. MΦ and DC stimulate P-14 T cells to become effector cells](image-url)

<table>
<thead>
<tr>
<th>Stimulating APC</th>
<th>Percentage of P-14 Cells Secreting IFN-γ</th>
<th>Percentage of Lysis of gp33-Pulsed Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ag, mouse 1</td>
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<td>4</td>
</tr>
<tr>
<td>No Ag, mouse 2</td>
<td>N.D.</td>
<td>0.3</td>
</tr>
<tr>
<td>DC, 10 μg/ml peptide mouse 1</td>
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<tr>
<td>DC, 10 μg/ml peptide mouse 2</td>
<td>31.5</td>
<td>85</td>
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<td>MΦ, 10 μg/ml peptide mouse 1</td>
<td>30.1</td>
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</tr>
<tr>
<td>MΦ, 10 μg/ml peptide mouse 2</td>
<td>27.3</td>
<td>48</td>
</tr>
</tbody>
</table>

* CSFE-labeled CD45.1+ P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, the mice were immunized i.v. with MΦ or DCs pulsed with 10 μg/ml. Three days after immunization, splenocytes were stained for CD45.1, CD8, and intracellular IFN-γ as described in Materials and Methods. The percentage of CD8+ T cells producing the IFN-γ from individual mice is shown. This experiment is representative of three experiments with similar results. N.D., Not determined.

* Another cohort of mice were immunized as described above. Four days after immunization, the mice were injected i.v. with target cells prepared as described in Materials and Methods. After 18 h, the spleens were removed, stained for CD45.1, and analyzed by FACSCalibur. Percentage lysis was calculated as described in Materials and Methods and is displayed for individual mice. This experiment is representative of three experiments with similar results.

![FIGURE 7. Priming of P-14 T cells by MΦ and DCs leads to memory cell formation](image-url)
or DCs led to a 95% reduction in viral titers in these mice (Fig. 8), which is a level of protection that is similar to what is observed in animals that are directly immunized with KAVYNFATCGIFA (which is cross-primed on host APCs) (34). This experiment was repeated three times with similar results. These data demonstrate that MΦ can prime naïve T cells and that these primed T cells can offer protection to a viral challenge.

**MΦ and DCs directly prime T cell responses**

We next sought to determine whether the transferred MΦ were stimulating T cells directly or indirectly, by transferring their peptide to other APCs in the mice (cross-presentation). To distinguish between the possibilities, we used mice whose bone marrow-derived APCs lacked the appropriate MHC I molecule needed to present any peptide transferred from the injected MΦ. In one set of experiments, we used radiation bone marrow chimeras. H-2<sup>s</sup> × H-2<sup>s</sup> (bxs) F<sub>1</sub> mice were lethally irradiated and reconstituted with bone marrow from mice that were either H-2<sup>b</sup> or H-2<sup>s</sup>. APCs from H-2<sup>s</sup> mice are unable to present the gp33 peptide, whereas H-2<sup>b</sup> APCs can present this peptide. The chimeric mice were then allowed to rest for 3–4 mo, and the replacement of their APCs was tested by adoptively transferring CFSE-labeled TCR Tg T cells and immunizing with an Ag that can be cross-presented on H-2<sup>D</sup> (the LCMV 13-mer peptide KAVYNFATCGIFA) (34). P-14 Tg T cells were stimulated to proliferate in F<sub>1</sub> mice that were reconstituted with either MΦ or DCs pulsed with 10 μg/ml gp33 were given i.v. Forty days later, spleens and lymph nodes were removed and stained for CD8, CD90.1, and intracellular IFN-γ as described in Materials and Methods. The data are represented as percentage of CD8<sup>+</sup> CD90.1<sup>+</sup> CFSE<sup>+</sup> cells secreting the cytokine from individual mice. This experiment was repeated three times with similar results.

<table>
<thead>
<tr>
<th>APC Type</th>
<th>Percentage of IFN&lt;sup&gt;+&lt;/sup&gt; P-14 Memory Cells in the Lymph Node</th>
<th>Percentage of IFN&lt;sup&gt;+&lt;/sup&gt; P-14 Memory Cells in the Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ag</td>
<td>1.02</td>
<td>1.03</td>
</tr>
<tr>
<td>DC, 10 μg/ml</td>
<td>18.4</td>
<td>35.1</td>
</tr>
<tr>
<td>MΦ, 10 μg/ml</td>
<td>24.2</td>
<td>32.6</td>
</tr>
</tbody>
</table>

* CFSE-labeled CD8<sup>+</sup> P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, MΦ or DCs pulsed with 10 μg/ml gp33 were given i.v. Forty days later, spleens and lymph nodes were removed and stained for CD8, CD90.1, and intracellular IFN-γ as described in Materials and Methods. The data are represented as percentage of CD8<sup>+</sup> CD90.1<sup>+</sup> CFSE<sup>+</sup> cells secreting the cytokine from individual mice. This experiment was repeated three times with similar results.

**FIGURE 8.** Both MΦ and DCs induce protective immunity. C57BL/6 mice were immunized with either MΦ or DCs pulsed with 5 μg/ml gp33 peptide. Mice were rested for 40 days to allow for the formation of memory T cells. The mice were then challenged with 5 × 10<sup>3</sup> PFU of LCMV. Four days later, spleens were harvested and homogenized, and plaque assays were performed. The data are represented as log<sub>10</sub> PFU. Student’s t tests were performed, and the data were statistically significant (p = 0.000004 for DCs; and p = 0.000067 for MΦ) when compared with the animals immunized with unpulsed APCs. The difference in protection offered between the two APC types was not statistically significant (p = 0.56). This experiment is representative of three experiments with similar results.

**FIGURE 9.** MΦ and DCs directly stimulate naive P-14 T cells in bone marrow chimeras. A. A total of 3.8 × 10<sup>6</sup> CFSE-labeled CD8<sup>+</sup> P-14 T cells was transferred into S→BXS (top panels) or B→BXS (bottom panels) bone marrow chimeric mice. One day later, the mice were immunized with the indicated APCs (0.5 × 10<sup>6</sup>) in the footpad. Unpulsed APCs and the LCMV 13-mer were used as the negative and positive control, respectively. Four days later, the draining lymph nodes were removed and stained with APC-conjugated anti-CD90.1 and PerCP-conjugated anti-CD8. The data display the CFSE profiles of CD8<sup>+</sup> and CD90.1<sup>+</sup> live cells. The different lines represent individual animals in the same experiment. This experiment was repeated four times with similar results. B. Similar to A, except the cells were assayed for the production of intracellular IFN-γ as described in Materials and Methods. The data are plotted as CFSE on the x-axis and IFN-γ on the y-axis. The numbers in the left-hand corner are the percentage of Tg cells producing IFN-γ. This experiment is representative of three experiments with similar results.

**FIGURE 9A.** MΦ and DCs stimulate P-14 T cells within the chimeric mice to produce IFN-γ. A total of 3.8 × 10<sup>6</sup> CFSE-labeled CD8<sup>+</sup> P-14 T cells was transferred into S→BXS (top panels) or B→BXS (bottom panels) bone marrow chimeric mice. One day later, the mice were immunized with the indicated APCs (0.5 × 10<sup>6</sup>) in the footpad. Unpulsed APCs and the LCMV 13-mer were used as the negative and positive control, respectively. Four days later, the draining lymph nodes were removed and stained with APC-conjugated anti-CD90.1 and PerCP-conjugated anti-CD8. The data display the CFSE profiles of CD8<sup>+</sup> and CD90.1<sup>+</sup> live cells. The different lines represent individual animals in the same experiment. This experiment was repeated four times with similar results. B. Similar to A, except the cells were assayed for the production of intracellular IFN-γ as described in Materials and Methods. The data are plotted as CFSE on the x-axis and IFN-γ on the y-axis. The numbers in the left-hand corner are the percentage of Tg cells producing IFN-γ. This experiment is representative of three experiments with similar results.
whether they were producing IFN-γ by intracellular staining. Priming with both MΦ and DCs stimulated P-14 Tg T cells to differentiate into IFN-γ-secreting effector T cells in s↔bxs chimeras (Fig. 9B). Furthermore, if these primed animals were rested for 40 days, P-14 memory cells were also present and making IFN-γ (Table III).

To further test whether cross-priming might account for responses, we transferred CSFE-labeled P-14 T cells into β2-microglobulin-deficient mice, whose bone marrow-derived APCs and other cells lack MHC I molecules. This experimental system does not require irradiation and does not have any host APCs that should be able to cross-present Ag. The Tg T cells did not proliferate in the β2-microglobulin-deficient mice when they were immunized with gp33 13-mer, KAVYNFATCGIFA (the peptide that can be cross-presented), but cells did proliferate in wild-type mice injected with this peptide (data not shown). Therefore, as expected, endogenous APCs in the β2-microglobulin-deficient mouse cannot cross-present Ag. In this system, peptide-pulsed MΦ and DCs were both able to stimulate P-14 T to proliferate (Fig. 10A). This experiment was repeated four times. To demonstrate that the P-14 T cells directly stimulated by MΦ became effectors, intracellular IFN-γ staining was performed as described above. Injecting both MΦ and DCs s.c. led to the differentiation of P-14 T cells into IFN-γ-secreting effectors (Fig. 10B). In these experiments, we pulsed the MΦ with 10-fold more peptide than DCs because we had shown (Fig. 3A) that this is necessary to have equivalent T cell responses after s.c. injection of the same number of MΦ and DCs. Under these conditions, the percentage of terminally divided P-14 T cells making IFN-γ is similar irrespective of the stimulating APC. Together with the chimera data, these results demonstrate that MΦ can directly stimulate naïve T cell responses.

Because limited proliferation was seen when T cells pulsed with KAVYNFATC were used as APCs in wild-type mice (Fig. 6B), we repeated these experiments in β2-microglobulin-deficient mice. The limited ability of T cells to stimulate P-14 T cells to divide was even further reduced in the class I-deficient mice (data not shown), suggesting that some of the limited proliferation of Tg T cells observed in wild-type mice stimulated by peptide-pulsed T cells was due to cross-presentation.

### Table III. P-14 CD8 T cells stimulated by MΦ and DC differentiate into functional memory cells in bone marrow chimeras

<table>
<thead>
<tr>
<th></th>
<th>Percentage of IFN-γ in Lymph Node</th>
<th>Percentage of IFN-γ in P-14 T Cells in the Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6→ bxs 13-mer, 5 μg</td>
<td>59.5</td>
<td>63.2</td>
</tr>
<tr>
<td>B6→ bxs DC nag</td>
<td>1.49</td>
<td>1.75</td>
</tr>
<tr>
<td>B6→ bxs DC, 10 μg/ml</td>
<td>13.6</td>
<td>35.7</td>
</tr>
<tr>
<td>B6→ bxs MΦ, 10 μg/ml</td>
<td>22.4</td>
<td>36.2</td>
</tr>
<tr>
<td>WT DC nag</td>
<td>1.02</td>
<td>1.13</td>
</tr>
<tr>
<td>WT DC, 10 μg/ml</td>
<td>18.4</td>
<td>35.1</td>
</tr>
<tr>
<td>WT MΦ, 10 μg/ml</td>
<td>24.2</td>
<td>32.6</td>
</tr>
<tr>
<td>S→bxs 13-mer, 5 μg</td>
<td>2.19</td>
<td>1.12</td>
</tr>
<tr>
<td>S→bxs DC, 10 μg/ml</td>
<td>10.3</td>
<td>40.8</td>
</tr>
<tr>
<td>S→bxs MΦ, 10 μg/ml</td>
<td>18.6</td>
<td>34.5</td>
</tr>
</tbody>
</table>

*CD90.1* CSFE-labeled P-14 T cells were adoptively transferred into bone marrow chimeras. One day later, the mice were immunized s.c. with 1 × 10^7 MΦ or DC pulsed with 10 μg/ml peptide or without Ag (nag) or 5 μg/ml 13-mer as a positive control. Forty days later, inguinal lymph nodes and spleens were removed and stimulated in vitro with 5 μg/ml gp33 in the presence of Brefeldin A. The samples were then stained for CD90.1, CD8, and intracellular IFN-γ. These data are shown as percentage of P-14 T cells containing IFN-γ. This experiment is representative of three experiments with similar results. WT, wild-type.

Discussion

In vitro, there is abundant evidence that MΦ acquire Ag and stimulate primed CD4 T cells (20, 23, 43–46) and CD8 T (27, 30, 47–55). Similarly, MΦ have been isolated from animals injected with viruses (56, 57) or soluble Ag (58) and shown to present Ag to primed T cells ex vivo. Interestingly, in some other studies DCs are the only Ag-bearing APCs isolated from Ag-injected animals that stimulate T cells in vitro (10, 59–62). This may indicate that in vivo MΦ acquire and present some, but not all, Ags. However, we have found that MΦ can be more difficult to liberate and recover from lymphoid tissue than DCs so that it is possible to underestimate their contribution to Ag presentation using ex vivo assays. In vivo, the presentation of Ag by MΦ to primed T cells is clearly important for CD4 and CD8 T cell-mediated type IV hypersensitivity responses and clearance of intracellular pathogens. In all of these situations, MΦ are stimulating previously activated (effector) T cells or T cell clone/hybridomas.

Naïve T lymphocytes have more stringent requirements for activation than effector T cells and hybridomas. The issue of what APC initially stimulates these cells to initiate immune responses is
an important one. It is well established that DC can stimulate primary immune responses (7, 10, 19, 25, 26, 63, 64), and our data confirm this point. However, our data show that MΦ can also stimulate T cells in vivo to proliferate, express effector functions, and mature into memory cells. These responses are due to direct stimulation of T cells by MΦ and not cross-presentation by or contamination with small numbers of DCs.

Our findings are surprising, because it has been argued previously that DCs are the only APCs that initiate primary immune responses (7, 25, 26, 65) and that MΦ play no role in this process. However, the published evidence supporting this view is relatively limited. Much of the evidence comes from in vitro experiments where DCs, but not MΦ, were found to stimulate primary T cell responses in MLRs or to foreign Ags (65–68). However, it is unclear whether the in vitro experiments accurately model the in vivo situation; e.g., it is often difficult to stimulate primary T cell responses to soluble Ags in vitro (even with DCs), whereas these same Ags stimulate strong immunity in vivo. The strongest evidence that DCs play a key role in stimulating primary T cell responses in vivo comes from Tg mice that express the receptor for diphtheria toxin under the control of the CD11c promoter (a DC-specific promoter). When these mice received injections with diphtheria toxin, CD11c-positive DCs were eliminated, and the generation of CTLs to some Ags (e.g., Listeria monocytogenes and Plasmodium yoelii) was inhibited (26). Whether responses to other Ags are similarly dependent on DCs is still an open question. It is possible that some Ags are preferentially presented on DCs, whereas others are also presented on MΦ (see above). Also, a caveat in the diphtheria-toxin studies was the possibility that the ability of MΦ to stimulate T cells was impaired by the toxin, e.g., when MΦ ingested the toxin during phagocytosis of dying DCs. MΦ that lack receptors for toxins can nevertheless be affected by these agents when the toxins enter the phagosome and are transferred to the cytosol (30).

In a set of experiments designed to visualize the APCs that interacted with naive T cells in situ, Norbury et al. (25) infected mice with a vaccinia virus expressing enhanced green fluorescent protein. They observed clustering of TCR-Tg CD8+ T cells around GFP-positive (infected) DCs but not MΦ. Although this was interpreted to show that only infected DCs were stimulating the T cells, cell stimulation was being inferred. What was actually measured, T cell-APC clustering, is influenced by strength of adhesion, chemokines, and potentially other factors. In fact, DCs can cause T cells to cluster, even in the absence of specific Ag (67). This analysis would fail to detect single T cells that were stimulated and/or ones that detached from an APC. Moreover, a substantial component of the T cell response to vaccinia occurs through cross-priming (5, 69, 70) and the APCs involved in this process would not express GFP from the vaccinia recombinants. Consistent with a cross-priming mechanism, T cells were observed to cluster with GFP-negative mononuclear cell, many of which lacked a DC marker. These findings suggested that in addition to DCs, other mononuclear APCs were stimulating T cells in vivo.

In summary, DCs can stimulate primary T cell responses and may be particularly potent in doing so. However, the evidence that they are the only cells that can prime responses and that MΦ lack this capability is limited. It is possible that DCs are the principal APCs for presenting some Ags (e.g., Listeria and malaria) (26). In contrast, our data unambiguously demonstrate that MΦ can also stimulate naive CD8+ T cells in vivo. Whether MΦ are also sufficient to prime naive CD4 T cell responses remains to be determined.

It has been reported that monocytes in peripheral tissues can convert into DCs after they migrate into the lymph node (71). Is it possible that the ability of MΦ to stimulate immune responses in our experiments is due to their conversion to DCs? It should be noted that even if such a conversion were to occur, our data would still indicate that in tissues, MΦ (i.e., before conversion to DCs) are another APC that function in tissues as sentinel cells for immune surveillance. However, such a conversion is unlikely because MΦ, as opposed to monocytes, are believed to be a terminally differentiated cell. Moreover, we find that stable MΦ clones are also able to directly prime naive T cells.

Our studies have used peptide-pulsed MΦ to demonstrate the potential of these APCs to initiate immune responses. Is it likely that MΦ can stimulate primary T cell responses to other kinds of Ag? Although we have not investigated this issue, in vivo MΦ acquire other kinds of Ags either directly in lymph nodes and spleen (72, 73) or in tissues and then migrate to lymph nodes (56, 57). In the lymphoid organs, the MΦ present acquired Ags on both MHC class I and II molecules (58). Given this, and our finding that on a per cell basis MΦ and DCs that have migrated to lymphoid organs have a similar T cell stimulatory over a range of peptide concentrations, it is likely that MΦ will contribute to the generation of T cell responses in vivo, at least in some situations.

Are MΦ and DCs the only cells that can initiate T cell responses? Kundig et al. (74) have shown that fibroblasts transfected with viral proteins directly induced the generation of viral-specific CTL when injected directly into the spleen. It has also been shown that fibrocytes can migrate to the lymph node and present Ag with an efficiency approaching that of DCs (75). These experiments have suggested that any Ag-bearing cell that migrates to lymph nodes can initiate T cell responses. However, because many experiments have demonstrated that bone marrow-derived APCs are necessary for the initiation of immune responses (4–6, 13, 76), fibroblasts are unlikely to play a role in the priming of T cell responses in physiological situations. In addition, our experiments using T cells as APCs demonstrate that the ability of an Ag-bearing cell to migrate into the lymph nodes is not a sufficient property to initiate responses. It is possible that B cells can prime T cell responses in vivo, although this has been controversial (77–88). How do MΦ compare with DCs in stimulating T cell responses? In vitro DCs are reported to be 100-1000 more potent APCs than MΦ. In vivo, we also find differences in the potency of these MΦ compared with DCs in some situations. Thus, when the MΦ are injected s.c., it takes 10-fold more Ag to prime naive T cells compared with DCs. However, when the APCs are injected via an i.v. route, they migrate in similar numbers and are of equal potency. Moreover, if we inject more MΦ s.c. to normalize for the number of cells migrating to lymph nodes, then the MΦ stimulate similar responses as DCs. Therefore, the difference in potency between the MΦ and DCs in our system surprisingly appears to be most related to the ability of these cells to migrate to lymphoid tissues. An important implication of these findings is that the number of MΦ vs DCs present at a site of Ag deposition may influence which of these cells will contribute more to priming T cells. During inflammation, there is a large increase in the number of MΦ from recruitment and proliferation. Under these conditions, MΦ are much more numerous than DCs. Therefore, MΦ could be the dominant APC in certain situations, particularly infection.

The finding that MΦ can initiate immune responses is an important one because bone marrow APCs play an essential role in detecting infection and initiating responses. Our observations raise many important questions. In what setting do MΦ initiate immune responses? Are there different APC requirements for the priming of CD4+ vs CD8+ T cells? Because these two APCs may make different mediators and thereby influence responses in different ways, the initiating APC may play a role in determining whether
protective immunity is generated. Are the T cells primed by Mβ functionally different from the T cells primed by DCs? It will also be of interest to examine the interactions and synergies between different APCs.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

References

[2080] MMö STIMULATE NAIVE CD8+ T CELL RESPONSES
Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice.


