Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissue

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Edited by Laurie H. Glimcher, Harvard School of Public Health, Boston, MA, and approved December 1, 2010 (received for review April 16, 2010)

Monocytes patrol various tissues for signs of infection and inflammation. Inflammatory monocytes enter peripheral tissues at sites of microbial infection and differentiate into dendritic cells and macrophages. Here, we examined the importance of monocytes in primary mucosal infection with herpes simplex virus 2 (HSV-2), and demonstrate that monocyte-derived APCs are required to elicit IFN- γ secretion from effector Th1 cells to mediate antiviral protection. However, monocyte-derived APCs were dispensable for the generation of Th1 immunity and for the restimulation of memory Th1 cells during secondary viral challenge. These results demonstrate that distinct APC subsets are dedicated for CD4 T cell priming, elicitation, and memory recall responses to a given viral pathogen within the same mucosal tissue and reveal a specialized role for monocyte-derived APCs in the emergency response to infection.

migration | sexually transmitted infection | antigen presentation

Dendritic cells (DCs) play a crucial role in the induction and regulation of adaptive immune responses (1). Recent studies have begun to unravel the importance of DCs in the restimulation of effector and memory T cell responses. DCs are required for secondary CD8 T cell responses to viral infections within the lymphoid organs (2). Other studies have revealed the importance of DCs in the execution phase of the adaptive immune responses at the site of infection. DCs that migrate into the site of infection are required to restimulate CD8 T cells to proliferate during primary influenza infection (3, 4) and during reactivation of HSV-1 (5).

Monocytes differentiate in the bone marrow and circulate throughout the body in anticipation of inflammation and infection. Two distinct types of monocytes have been defined: Ly6C⁺ inflammatory monocytes that are rapidly recruited to the site of inflammation, and Ly6C⁻ monocytes that patrol the blood vessels (6). Once inside the tissue, inflammatory monocytes can give rise to various DC and macrophage subsets (7). Monocyte-derived DCs are found in peripheral tissues and in secondary lymphoid organs. They provide antimicrobial defense by at least three separate mechanisms (8): by secreting antimicrobial factors (TNF- α , NO); by inducing stimulation of naive lymphocytes within the lymphoid organs; and by eliciting recall responses from effector and memory T cells in the periphery. First, after infection, Ly6C⁺ monocytes egress massively from bone marrow to the bloodstream in a CCR2-dependent fashion and differentiate into DCs that produce TNF- α and NO (9, 10). These cells, known as Tip-DCs [for TNF- α /inducible nitric oxide synthase (iNOS)-producing DCs], are required for lysis and clearance of *Listeria monocytogenes* (10) and *Toxoplasma gondii* (11) following primary infection. Second, monocyte-derived DCs migrate from the skin to the draining lymph nodes and prime naive CD4 T cells after *Leishmania major* infection (12). In contrast, monocytes do not participate directly in antigen presentation after *Salmonella* infection, although monocyte recruitment to the skin is required for CD4 T cell priming (13). Third, monocyte-derived DCs are responsible for stimulating effector CD8 T cells at the peripheral sites of infection following influenza virus (3) and HSV-1 (5) infection. Although various roles have been assigned

to DCs and monocyte-derived DCs, the relative importance of tissue-resident DCs and inflammatory monocyte-derived DCs in the induction and execution of adaptive immune responses to a given pathogen remains unclear.

Most natural infections with pathogens begin by invasion in local peripheral tissues such as mucosal surfaces. HSV-2 infection is the primary cause of genital herpes, which affects 45 million persons in the United States (14). Upon entering the genital mucosa, HSV-2 replicates rapidly within the vaginal epithelial cells and establishes latency in the innervating sacral ganglia. The immunological mechanism of protection requires robust CD4 and CD8 T cell responses (14). Studies using the murine model of genital herpes showed that robust Th1 immunity is required for protection against primary and secondary HSV-2 challenge (15). We have previously demonstrated that memory Th1 cells form foci along the genital tract after infection with an attenuated thymidine kinase (TK)-defective HSV-2 (16). IFN- γ secreted from such local memory Th1 cells acts on the stromal compartment (likely the epithelial cells) to provide protection against secondary challenge. We found that the antigen presenting cells (APCs) responsible for restimulating memory Th1 cells to secrete antiviral IFN- γ were not the infected epithelial cells themselves but, rather, were the B cells and DCs. However, it remains unclear what type of APCs mediate restimulation of effector Th1 cells during primary HSV-2 infection. This question is particularly important in vaccine design, as vaccines against HSV-2 must rely on a small number of circulating memory T cells that enter the vaginal mucosa to control viral replication and spread to the innervating ganglia.

Here, we examine the role of inflammatory monocytes after physiological primary viral infection with HSV-2. We assessed the requirement for inflammatory monocytes and monocyte-derived APCs in CD4 T cell priming and effector functions, and provide evidence that tissue-resident DCs and inflammation-induced monocyte-derived APCs play distinct biological roles. These results reveal that priming and elicitation of primary effector CD4 T cells are mediated by distinct subsets of DCs suited for their distribution and antigen-presenting capacities.

Results

CCR2-Deficient Mice Fail to Control Primary Genital HSV-2 Infection.

To examine the role of inflammatory monocytes in local antiviral immune protection, we used a murine model of genital herpes infection in which protection is provided by effector Th1 cells in the genital mucosa (15). Because inflammatory monocytes require CCR2 for egress from the bone marrow (9), we examined the ability of CCR2^{-/-} mice to defend against challenge with HSV-2. Naive C57BL6 (WT) and CCR2^{-/-} mice were chal-

Author contributions: N.I. and A.I. designed research; N.I. and L.M.M. performed research; N.I. and A.I. analyzed data; and N.I. and A.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1005201108/-DCSupplemental.

tissue-resident DC phenotype that we find at steady state (Fig. S5A). These data suggest that monocytes are recruited as emergency responders to the infected vaginal tissue, peaking around day 4, and that although the majority of these cells disappear after clearance of viral infection, some give rise to tissue-resident DCs that patrol the tissue at steady state.

To determine the nature of CD11b⁺ APCs, we examined their surface phenotype. Both CD11b⁺ CD11c⁺MHC II⁺ and CD11b⁺ CD11c[−] MHC II⁺ cell types expressed CD86, Sca-1, and CD107b (Mac-3) but not CD8α, CD103, and CD207 (Fig. S5C). In functional studies, both CD11c[−]CD11b⁺ and CD11c⁺CD11b⁺ cells isolated from day 5 HSV-2-infected vaginae induced minimal activation of naive allogeneic T cell proliferation, in contrast to the robust proliferation induced by splenic conventional DCs (Fig. S5D). These data indicate that monocyte-derived APCs in the vagina are likely macrophages that express MHC II, as they lack the ability to elicit naive T cell activation.

Inflammatory Monocytes Require CCR2 for Entry into HSV-2-Infected Tissue. Thus far, our data indicate that CCR2-dependent presence of monocytes and monocyte-derived cells in the local tissue is a prerequisite for immune protection against primary HSV-2 genital infection. However, the mechanism by which CCR2 mediates this effect is unclear. Although it is known that CCR2 is required for monocyte egress from the bone marrow (9), this receptor may also be used by monocytes to enter inflamed tissue. To determine whether the CCR2 expressed on Ly6C^{hi} monocytes is required for their entry into the infected genital mucosa, we coadoptively transferred monocytes from WT (CD45.1⁺CD45.2⁺) or CCR2^{−/−} (CD45.2⁺) mice into the same congenic CD45.1⁺ WT hosts 1 d after HSV-2 infection. Four days after HSV-2 challenge, recruitment of donor-derived cells to the various tissues was analyzed. With both WT and CCR2^{−/−} donors, we found equivalent numbers of donor monocyte-derived cells (total CD11b⁺ cells) in the recipient spleen and bone marrow (Fig. S6A and B), as previously described (9). Minimal recruitment of WT or CCR2^{−/−} donor-derived CD11b⁺ cells was observed in the draining lymph nodes (Fig. S6B). Notably, donor-derived cells were present only in the vaginae of groups that received monocytes from WT, and not CCR2^{−/−} mice (Fig. 3). These data indicated that CCR2 expression on inflammatory monocytes was required for entry into the infected genital mucosa.

Next, to determine whether monocytes are sufficient to confer protection, WT monocytes were adoptively transferred into congenic CCR2^{−/−} mice 1 d after ivag HSV-2 challenge; 4 d p.i.,

recruitment of donor-derived monocytes to the indicated tissues was analyzed. Unexpectedly, entry of WT monocytes into the vaginal tissue of CCR2^{−/−} hosts was diminished compared with entry into WT hosts (Fig. S7A). As a result, CCR2^{−/−} mice transplanted with WT monocytes were unable to control viral replication (Fig. S7B). Therefore, although we were unable to demonstrate the sufficiency of monocytes in protection against HSV-2, these data indicated the requirement for CCR2⁺ cells in priming the vaginal tissue for entry by monocytes.

Type I IFNs Induce CCR2 Ligands and Recruit Monocytes to Infected Tissue.

To determine the mechanism by which CCR2 mediates monocyte entry into the inflamed genital tissue, we examined the expression of the CCR2 ligands, CCL2, CCL7, CCL8, and CCL12. HSV-2 infection resulted in up-regulation of mRNA for all of these chemokines (Fig. S7A). CCL2 protein was rapidly secreted in vaginal mucosa of both WT and CCR2^{−/−} mice (Fig. S7B), demonstrating that CCR2 ligand expression occurred independently of CCR2 expression. Given the rapid onset of CCL2 secretion (within 24 h of HSV-2 challenge), we reasoned that CCR2 ligands must be induced by innate signals. It is known that local type I IFNs are secreted rapidly following HSV-2 infection (19). In addition, type I IFNs have been shown to induce CCR2 ligand expression and recruitment of monocytes (20). Notably, CCR2 ligand production was almost undetectable in the genital mucosa of HSV-2-infected IFN-αβR^{−/−} mice (Fig. S7). Next, to examine whether IFN responsiveness is required for the recruitment of CD11b⁺ monocytes into vaginal tissues following HSV-2 challenge, monocytes from WT CD45.1⁺ mice were adoptively transferred into CD45.2⁺ WT or IFN-αβR^{−/−} mice. Five days after HSV-2 challenge, donor monocyte-derived cells were counted. There was a severe deficiency of endogenous Ly6C^{hi} monocyte-derived cells in IFN-αβR^{−/−} mice (Fig. 4A and B, “Recipient”), indicating that IFN-induced signals were required for monocyte recruitment and their subsequent differentiation in situ. Moreover, even the WT (IFNαβR⁺ CCR2⁺) donor monocytes failed to migrate into the vaginal tissue of the IFN-αβR^{−/−} recipient mice (Fig. 4A and B, “Donor”). To exclude the possibility that uncontrolled virus infection in the IFN-αβR^{−/−} mice leads to destruction of monocyte-derived cells rather than impairing their recruitment, we adoptively transferred WT (CD45.1⁺) Ly6C^{hi} monocytes into CD45.2⁺ WT or CD45.2⁺IFN-αβR^{−/−} mice 5 d after HSV-2 challenge. Twelve hours later, donor-derived cells in genital tissues were analyzed by flow cytometry. Donor monocyte entry into the vaginal tissues was impaired in the IFN-αβR^{−/−} hosts (Fig. S8). Instead, more donor monocytes were retained in the peripheral blood of the IFN-αβR^{−/−} recipient. These data indicated that Ly6C^{hi} monocytes use CCR2 to enter the vaginal tissues following genital herpes infection by responding to chemokine ligands that are induced by local nonmonocyte cells in response to type I IFNs.

Monocyte-Derived APCs Are Dispensable for Priming and Recruitment of Th1 Cells to Infected Tissue.

To determine the mechanism by which monocyte-derived cells mediate antiviral protection, we examined the following possibilities. First, monocyte-derived APCs might be required to prime protective Th1 responses in the draining lymph nodes. Second, monocytes might be required to recruit effector Th1 cells to the genital mucosa. To examine the first possibility, we measured CD4 T cell priming in CCR2^{−/−} mice. Following HSV-2 ivag infection, IFN-γ-secreting CD4 T cells specific for HSV-2 antigens were found in equal frequency and number in the draining lymph nodes (Fig. S8A). In addition, IFN-γ secretion in response to HSV-2 antigen from CD4 T cells isolated from WT and CCR2^{−/−} mice were comparable (Fig. S8B). These data indicated that CD4⁺ T cell priming occurred normally in CCR2^{−/−} hosts. Next, we examined recruitment and function of effector Th1 cells in the infected

Fig. 3. CCR2 is required for tissue migration of monocytes following genital HSV-2 infection. Ly6C^{hi} monocytes (2×10^6 cells per mouse) isolated from the BM of CD45.1⁺CD45.2⁺ WT mice or CD45.2⁺ CCR2^{−/−} mice were adoptively transferred into CD45.1⁺ WT mice ($n = 3$) 1 d after ivag HSV-2 infection. Four days after ivag HSV-2 infection, recruitment of CD45.1⁺CD45.2⁺ CD11b⁺ monocyte-derived cells to the vagina (A) was analyzed. (B) Bar graphs represent absolute numbers of donor-derived total CD11b⁺ cells, CD11b⁺CD11c⁺, and CD11b⁺CD11c[−] APCs in vagina. * $P < 0.05$, ** $P < 0.01$. Results are representative of two similar experiments.

Fig. 4. Type-I IFN-mediated up-regulation of CCR2 ligands is required for blood monocyte recruitment. (A and B) CD45.2⁺ WT mice ($n = 3$) or CD45.2⁺ IFN-αβR^{-/-} mice ($n = 3$) that had been transplanted with CD45.1⁺ CD11b⁺ monocytes (2×10^6 cells per mouse) were challenged with HSV-2 ivag. Five days later, donor (CD45.1⁺)-derived CD11c⁺MHC class II⁺ cells and recipient (CD45.2⁺)-derived CD11c⁺MHC class II⁺ cells in vaginal tissues were counted (B). * $P < 0.05$. Results are representative of two similar experiments.

tissue. Lymphocytes in the HSV-2-infected vaginal tissue were isolated and restimulated ex vivo, and the percentage and frequency of IFN-γ-secreting CD4 T cells and CD8 T cells were assessed using intracellular IFN-γ staining (Fig. S8C) and enzyme-linked immunosorbent spot (ELISPOT) (Fig. S8D). Both of these analyses showed that similar numbers of CD4⁺ T and CD8⁺ T cells capable of secreting IFN-γ were present in the HSV-2-infected genital mucosa in WT and CCR2^{-/-} mice. These data indicate that priming, recruitment, and effector potential of Th1 cells remains intact in CCR2^{-/-} hosts.

Monocyte-Derived APCs Are Required to Restimulate Th1 Cells to Secrete IFN-γ in Infected Tissue. Because Th1 priming, migration, and effector functions remain intact in CCR2^{-/-} mice, we next examined whether monocyte-derived cells might be required to restimulate effector Th1 cells within the infected tissue to mediate IFN-γ-dependent immune protection in situ. Vaginal infection with HSV-2 generates two waves of IFN-γ in mucous secretion; the first wave of IFN-γ is secreted by NK cells at 2 d p.i., whereas CD4⁺ T cells produce IFN-γ starting at 4 d p.i. (21). Notably, in CCR2^{-/-} hosts ivag infected with HSV-2, IFN-γ in the vaginal mucous secretion was severely diminished throughout the course of infection (Fig. 5A). The diminished IFN-γ secretion at 2 d p.i. is consistent with reduced recruitment of NK cells at this time point (Fig. S2). In addition, significantly reduced IFN-γ levels were detected in the vaginal tissue at day 5 p.i. in CCR2^{-/-} hosts (Fig. 5B). These data suggested that monocyte-derived cells are required for restimulation of effector Th1 cells in situ. To test this possibility, we determined the antigen pre-

Fig. 5. Monocyte-derived APCs reactivate Th1 cells to secrete IFN-γ within the virally infected tissue. (A) IFN-γ levels in vaginal wash in WT mice ($n = 9$) and CCR2^{-/-} mice ($n = 9$) was measured at the indicated days following genital herpes infection (WT HSV-2, 10^4 pfu per mouse). (B) IFN-γ levels in tissue homogenates in WT mice ($n = 4$) and CCR2^{-/-} mice ($n = 4$) was detected at day 5 p.i. (C) Monocyte-derived APCs predominantly present viral antigen to effector CD4 T cells in the vaginal tissues. CD11c⁺CD11b⁺, CD11c⁺CD11b⁻, and CD11c⁻CD11b⁺ subsets among live lineage⁺ cells were FACS-sorted from vaginal tissues at 5 d p.i. and cocultured with effector CD4⁺ T cells isolated from draining LN (day 7) in the presence of mock or viral antigen. IFN-γ secreted from T cells (Left) and T cell proliferation (Right) was measured by ELISA and thymidine incorporation, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. These results are representative of three similar experiments (mean ± SEM).

sending capacity of these monocyte-derived cell populations in the vaginal tissue. CD11c⁺CD11b⁺, CD11c⁻CD11b⁺, CD11c⁺CD11b⁻, and CD11c⁻CD11b⁻ cell populations were FACS sorted, and incubated with effector CD4 T cells in the presence (positive control) or absence of exogenously added viral antigens. CD11b⁺CD11c⁺ APCs induced robust CD4 T cell IFN-γ secretion and proliferation (Fig. 5C). In contrast, CD11b⁻CD11c⁺ and CD11b⁺CD11c⁻ cells induced only minimal CD4 T cell activation. Taken together, these data indicate that monocyte-derived CD11b⁺CD11c⁺ APCs in the vaginal tissue are required for restimulating effector Th1 cells to secrete IFN-γ in situ.

Discussion

Our results demonstrated that monocytes play a key role in antiviral defense against HSV-2 infection. We found that monocytes are recruited to the infected genital mucosa in a CCR2-dependent manner through their responsiveness to type I IFN-induced chemokines. Tissue resident DCs in the vagina were rapidly outnumbered by monocyte-derived APCs within 5 d of infection and returned to steady-state levels by 7 d. Monocytes and their derivatives were found to be dispensable for priming of naive CD4 T cells, in that CCR2^{-/-} hosts achieved WT levels of CD4 T cell expansion, differentiation, and migration into inflamed tissue. Instead, upon entering the infected tissue, inflammatory monocyte-derived CD11b⁺CD11c⁺ APCs were required to restimulate effector Th1 cells in situ. In CCR2^{-/-} mice, inflammatory monocytes failed to repopulate the HSV-2-infected genital mucosa, restimulate Th1 cells to secrete IFN-γ and

provide protective immunity. Collectively, these data revealed the importance of monocyte-derived APCs in eliciting Th1-mediated antiviral effector functions during primary infection with HSV-2.

The chemokine receptor CCR2 is expressed on Ly6C^{hi} monocytes and a subset of B cells, NK cells, NKT cells, pDC, and activated T cells (22–25). However, following HSV-2 infection, the number of B cells, NKT cells, pDC, and activated T cells in the vaginal mucosa of CCR2^{−/−} mice were found to be comparable to that of WT mice (Fig. 24). We observed a temporal impairment in the recruitment of NK cells and granulocytes early during the infection. The striking phenotype of the CCR2^{−/−} mice was the almost complete lack of monocytes and monocyte-derived cells to the HSV-2-infected vaginal mucosa. We demonstrated that CCR2 was required for the entry of monocytes from circulation into mucosal tissues by adoptive transfer of WT and CCR2^{−/−} monocytes into congenic WT mice. Thus, in addition to being required for egress from the bone marrow as reported previously, CCR2 expression by the monocyte is also required for monocyte entry into inflamed tissues. In addition, our data revealed the importance of CCR2 expression on the host cells for entry of CCR2⁺ monocytes into the vaginal mucosa. Our data further demonstrated that the ligands for CCR2 are critically controlled by type I IFNs. Although the importance of type I IFNs in innate antiviral defense against HSV is well known (26), the exact effector mechanism(s) responsible for such protection remains unclear (27). Our data revealed one aspect of how type I IFNs can orchestrate antiviral defense, namely, through the induction of monocyte-recruiting chemokines.

Previously, we demonstrated that B cells and DCs maximize Th1-mediated immune protection against secondary ivag challenge with HSV-2 in previously immunized mice (16). Here, we found that monocytes do not play a significant role in immune protection at the memory phase. Therefore, our data indicate that the APCs responsible for eliciting IFN- γ secretion from primary effector Th1 cells (monocyte-derived cells) vs. secondary challenge (B cells and DCs) are distinct. We speculate that the distinct usage of different APCs to elicit IFN- γ secretion from Th1 cells represents strategic advantages for the host. Unlike B cells and DCs, monocytes can be recruited to inflamed tissues rapidly in very high numbers by responding to type I IFN-induced chemokines through CCR2. This inducible expansion in APC number allows immigrant effector Th1 cells to quickly scan for viral antigens and to become restimulated to secrete IFN- γ . In contrast, during memory phase, long after the clearance of primary viral infection, Th1 cells establish multiple foci within the vaginal mucosa. Such foci contain B cells and DCs that are poised to take up viral antigens upon secondary challenge and to stimulate Th1 cells locally. For this phase of immune response, inflammatory monocytes are not as well suited to act as APCs as pre-established localized defense foci, as monocytes have no antigen specificity and require time to differentiate into DCs.

It remains unclear how tissue-resident DCs and newly recruited inflammatory monocyte-derived APCs coordinate T cell activation. Our results revealed that steady-state tissue-resident DC are primarily involved in priming of naive CD4 T cells (28), whereas inflammatory monocyte-derived APCs regulate cytokine production from effector T cells at the site of infection. Of note, the latter APCs are not capable of activating naive T cells, suggesting that these cells are dedicated for supporting the function of effector T cells. Whether monocyte-derived APCs contribute to priming vs. effector phase of T cell responses depends on the infection model. In a cutaneous *Leishmania* infection model, monocyte-derived DCs in the dermis were shown to take up parasitic antigens, migrate to the draining lymph nodes, and prime CD4 T cell responses (12). In the inflamed skin, CD11b⁺ dermal DCs were found to stimulate IFN- γ and IL-10 secretion from Th1 and Tregs, respectively (29). In the

case of HSV-1 infection, peripheral tissue-resident memory T cells are restimulated to undergo rapid proliferation by infiltrating monocyte-derived DCs (5). Specifically, T cells associated with HSV-1-infected dorsal root ganglia were restimulated by infiltrating monocyte-derived DCs to undergo expansion *in situ* (5). Although effector functions of peripheral tissue memory T cells were not examined, these data indicated that monocyte-derived DC activate expansion of memory T cells to combat HSV-1 infection. Our current study demonstrated that monocyte-derived CD11b⁺CD11c⁺ APCs restimulate cytokine production by effector CD4 T cells at the site of infection. However, they were not required for the priming stage of Th1 immune responses. Interestingly, monocyte-derived DCs in the dermis accumulate over weeks of *Leishmania* infection and present antigen to CD4 T cells 4 wk into chronic infection (12), whereas HSV-2 is cleared from the genital mucosa within 5–6 d of infection (16). In addition, monocyte-derived DCs were directly infected by *Leishmania* (12), whereas HSV-infected DCs are rendered incapable of migrating to the lymph node to prime T cell responses (30–34). Therefore, the relative contributions of monocyte-derived cells in immune priming vs. restimulation likely depend on the chronicity of infection as well as the target tropism of a given pathogen.

In summary, our findings provide insight into the regulation of antiviral immunity mediated by tissue-resident vs. inflammatory DCs. Tissue-resident DCs migrate to the draining lymphoid tissues to trigger activation of naive T cell priming. In contrast, inflammatory monocyte-derived APCs are responsible for restimulation and cytokine production from effector CD4⁺ T cells at the site of infection. These findings provide an important foundation for the development of mucosal vaccines and therapeutic approaches against pathogen and cancer.

Methods

Mice. Six- to 8-week-old female C57BL/6 (CD45.2⁺), B6.SJL-PtprcaPep3b/BoyJ (B6.Ly5.1) (CD45.1⁺), B6.129-H2^{dIA1-Ea}/J (MHC class II^{−/−}), CCR2^{−/−} (B6.129S4-Ccr2tm1lf/J), CD11c-DTR transgenic mice (B6.FVB-Tg [Itgax-DTR/GFP] 57Lan/J) and IFN- α BR^{−/−} mice were purchased from The Jackson Laboratory. All procedures used in this study complied with federal guidelines and institutional policies by the Yale animal care and use committee.

Viruses. 186syn[−] TK[−] (35) and 186syn⁺ (36) were generous gifts of David Knipe (Harvard Medical School, Boston, MA). All viruses were propagated and titered on Vero cells as previously described (37).

Viral Challenge. Intravaginal infection with HSV-2 was carried out as previously described (37). Briefly, 6- to 8-wk-old female mice were injected s.c. in the neck ruff with progesterone (Depo Provera, Pharmacia Upjohn) at 2 mg per mouse in a 100- μ L volume. These mice were immunized ivag with 1.2×10^2 pfu or 10^4 pfu of HSV-2 (186syn⁺) in a 10- μ L volume. The severity of disease was scored as follows (38): 0, no sign; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; 4, hind-limb paralysis; 5, premoribund. Due to humane concerns, the animals were killed before reaching moribund state.

Vaginal Viral Titers and Cytokine Measurement. Vaginal fluids were collected between days 0 and 7 of HSV-2 infection using calcium alginate swabs and PBS. Viral titers were obtained by titration of vaginal wash samples on Vero cell monolayer as described previously (39, 40). IFN- γ levels were detected from vaginal fluids (19) or tissue (41) as previously described. ELISPOT assays to determine IFN- γ -secreting CD4 T cells in the vaginal tissue were performed as previously described (16).

Adoptive Transfer of BM Monocytes. Single-cell BM suspensions from CD45.1⁺C57BL6 mice were stained with 7-AAD, Ly6C/CD11b, lineage markers (CD90.2, B220, DX5, CD11c, MHC class II, Ly6G, and CCR3); then 7-AAD[−]Ly6C⁺(CD11b⁺) Lin[−] cells were isolated by sorting on a FACS Aria flow cytometer (BD Bioscience) and injected into recipient mice.

Detection of Blood Monocyte-Derived APCs in Vaginal Tissues. FITC-labeling of blood monocytes and liposome containing PBS or clodronate (250 μ L per

mouse) injection was previously demonstrated (18). Clodronate-liposomes and PBS-liposomes were a gift from Roche and liposomes were generated as previously described (42).

Statistical Analysis. Survival curve was analyzed using the log-rank test. For other data, statistical significance was determined using two-tailed unpaired *t* test, where appropriate. A *P* value of 0.05 or less was considered statistically significant. All calculations were performed using the Prism software

program for Macintosh (GraphPad Software) or Microsoft Excel. Error bars in all graphs indicate SEM and represent biological replicates.

ACKNOWLEDGMENTS. The authors thank M. Sasai for technical help, Z. Zhao and G. Lyon for cell sorting, and E. Foxman for critical reading of the manuscript. This study was supported by Grants AI054359, AI062428, and AI064705 from the National Institute of Allergy and Immunology. A.I. is a recipient of the Burroughs Wellcome Investigators in the Pathogenesis of Infectious Disease award.

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