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Myeloid C3 Determines Induction of Humoral Responses to Peripheral Herpes Simplex Virus Infection

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The complement system, in addition to its role in innate immunity, is an important regulator of the B cell response. Complement exists predominantly in the circulation and although the primary source is hepatic, multiple additional cellular sources have been described that can contribute substantially to the complement pool. To date, however, complement produced by these secondary sources has been deemed redundant to that secreted by the liver. In contrast, using a bone marrow chimeric model, we observed that C3 synthesis by myeloid cells, a relatively minor source of complement, provided a critical function during the induction of humoral responses to peripheral HSV infection. Anti-viral Ab, as generated in an efficient humoral response, has been associated with protection from severe consequences of HSV dissemination. This report offers insight into the generation of the adaptive immune response in the periphery and describes a unique role for a nonhepatic complement source.


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Hepatic cells constitutively secrete complement C3, which is maintained at ~1 mg/ml in the circulation (16). Nonhepatic cells, such as keratinocytes (17), kidney tubular epithelial cells (18), and macrophages (19), provide additional sources of C3. The contribution to the circulation from these secondary sources varies greatly depending on the system, from clearly detectable (20, 21) to negligible (22, 23). In certain experimental models, nonhepatic complement sources can have profound effects, such as described for kidney transplants where allograft rejection is significantly tempered in the absence of renal C3 production (24). Nonhepatic sources for complement factors other than C3 have also been described (25–27), and in cases such as C4 or C6 are associated with demonstrable effects (28, 29). Nonetheless, these reports indicate only that complement produced by secondary sources could substitute for or contribute to the normal function of hepatic complement. They do not describe an essential role for complement produced by a nonhepatic source.

Animals genetically deficient in complement factors have defective Ab and memory responses to i.v. inoculation that can be restored through passive transfer of the missing component (30, 31). In addition, bone marrow (BM) transplantation experiments have demonstrated that myeloid C3 is sufficient for enhancement of the humoral response to i.v. administered protein (22) or peripheral HSV-1 infection (23). It remains unclear whether this myeloid C3 serves a distinct function or whether it too represents a redundancy in the complement system. To address whether myeloid C3 has an essential biological role, we adopted a radiation chimera approach in which all C3 sources are intact except for BM-derived cells that are genetically C3 deficient. Circulating C3 levels are normal in these mice and, indeed, they respond normally to virus or inert protein presented via the circulation. However, further characterization revealed a striking defect in the humoral response to peripheral HSV infection. These results show a functional requirement for myeloid cells as a source of complement.

Materials and Methods

Mice and BM chimeras

Mice deficient in complement component C3 (C3−/−) were maintained on a C57BL/6 background (backcrossed 10 generations; CDA,2 allotype). C57BL/6 mice of the CD45.1 allotype served as wild type (WT). All mice
were housed at Harvard Medical School in a specific pathogen-free facility. Studies were performed in compliance with institutional guidelines set forth by the Center for Blood Research and Harvard Medical School Animal Care and Use Committees. Mice received food and water ad libitum. Mice 6–8 wk of age were lethally irradiated (2 × 650 rad), anesthetized, infused with BM cells (1 × 10⁷), and rested for 5 wk before experiments as described earlier (22, 23).

**Virus and inoculations**

WT HSV-1 strain KOS1.1 (32) was used for all experiments. KOS1.1 is an attenuated, non-neurovirulent HSV-1 strain that at 2 × 10⁶ PFU intradermally (i.d.) reproducibly generates a robust host immune response that protects the animal against virulent challenge (33–35). A cell-free virus stock was prepared and mice were inoculated as described previously (36). For i.v. inoculations, mice were injected with 2 × 10⁶ PFU of KOS1.1 or 50 μg of β-galactosidase (β-gal) protein (Sigma-Aldrich, St. Louis, MO) into the retro-orbital sinus.

**Serum collection and ELISA**

Blood was collected from the tail vein of each animal and IgG Ab titers were determined by ELISA, as described previously (23, 36), except that HSV-1 strain KOS1.1 or β-gal were used as coating Ags. IgG titers represent the final 2-fold dilution, resulting in an Ab titer >2 U above a control dilution of naive serum, and they are expressed as the mean reciprocal dilution ± SEM. Serum C3 concentrations were determined by ELISA, as described previously (23).

**ELISPOT assay**

Single-cell suspensions were prepared from draining lymph nodes (LN) of mice 7 days after i.d. HSV infection. ELISPOT assays were completed essentially as described by Sedgwick and Holt (37).

**CD4⁺ cellular proliferation assay**

At day 7 following primary or secondary infection or mock treatment, single-cell suspensions were prepared from the draining LN of individual mice. CD4⁺ T cells isolated by magnetic separation using Dynabeads Mouse CD4 (L3T4) and DETACHaBEAD Mouse CD4 (Dynal Biotech, Lake Success, NY). Cells were washed, counted, and suspended in DMEM/10. Purity was verified by FACS analysis and was always >99%. Splenocytes from uninfected C57BL/6 mice were prepared for use as stimulator cells 1 day before LN harvest. T lymphocytes were depleted by magnetic separation using Dynabeads Mouse Pan T (Thy1.2; Dynal Biotech) according to the manufacturer’s direction and erythrocytes were lysed using the Mouse Erythrocyte Lysing System (R&D Systems, Minneapolis, MN). One-half of the cells were infected with HSV at a multiplicity of infection of 3 for 1 h at 4°C and washed with PBS. Infected and uninfected cells were each suspended at 1 × 10⁶ cells/ml in DMEM/10 and incubated for 20 h at 37°C with 5% CO₂. After incubation, cells were treated with 50 μg/ml mitomycin C (Sigma-Aldrich) for 1 h at 37°C, washed five times with 50 ml of PBS, and resuspended in DMEM/10. Infected and uninfected stimulator cells were plated at 3 × 10⁵ cells/well in a black well, clear bottom 96-well plate (Costar, Cambridge, MA) in a 100-μl vol. Quadruplicate samples of CD4⁺ cell preparations were added to the stimulator plates at 5 × 10⁴ cells/well in a 50-μl vol. Plates were incubated at 37°C with 5% CO₂ for 5 days, then the plates were labeled for 8 h with 5-bromo-2'-deoxyuridine (BrdU). BrdU incorporation into the DNA of proliferating cells was measured using the Cell Proliferation ELISA-Chemiluminescent kit (Roche, Indianapolis, IN).

**FACS analysis of surface markers and intracellular C3**

Single-cell suspensions from spleen, LN, or peripheral blood were incubated with anti-FeR (2.4G2). For intracellular staining, cells were permeabilized with saponin (Sigma-Aldrich) in PBS and incubated with monoclonal rat anti-mouse C3 conjugated with Cy5. Subsequent to intracellular staining, cells were washed extensively in saponin buffer and rescaled in PBS. The following monoclonal anti-mouse surface marker stains were used: CD45.1, CD45.2, B220, CD3e, CD4, CD8α, CD11b, and CD11c (BD Pharmingen, San Diego, CA). Flow cytometry was performed on a FACSCalibur, and cell sorting was done on FACSVantage.

**Histology**

Cryosections of OCT (Tissue-Tek, Torrance, CA)-embedded LN and skin were prepared as described elsewhere (23) and treated with mAbs specific for CD35 (BD Pharmingen) followed by mouse adsorbed anti-rat Ig-PE (BD Pharmingen). Areas of CD35 staining were measured using Openlab software (Improvision, Lexington, MA). C3 was detected in tissues using rabbit anti-human-C3-dFITC (DAKO, Carpinteria, CA) enhanced with anti-rabbit-Ig-Alexa488 (Molecular Probes, Eugene, OR). Other reagents used on skin cryosections included anti-mouse-CD3e, CD4, B220, and CD11b (BD Pharmingen). H&E staining was used on paraffin-embedded skin to evaluate overall virus-induced infiltration and pathology.

**RT-PCR**

Total RNA was isolated from skin tissue or sorted cell populations using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis from 1 μg of total RNA was performed using an oligo(dT) primer (Invitrogen) and Moloney murine leukemia virus-reverse transcriptase (Invitrogen) for 1 h at 42°C. To detect a 706-bp mouse C3 product, 35 cycles (94°C for 30 s, 52°C for 1 min, and 72°C for 1 min) of PCR with Taq Supermix (Invitrogen) were completed using primers C3 sense (GGC TGACTCTGTGTTGCTTG) and C3 antisense (TCTCTGGTCTTCTTCAAC TCT). A control 379-bp β-actin product was detected using 30 cycles (94°C for 30 s, 50°C for 1 min, 72°C for 1 min) with primers β-actin sense (CCCTAAGGCAACCGTGAAGAGTAGCACG) and β-actin antisense (CCAGGAGGAGAAGGATGGCG).

**Results**

C3 synthesis by BM-derived cells is necessary for humoral response to peripheral HSV-1 infection

BM chimeras provide an effective approach to measure the relative importance of BM-derived C3 vs that derived from other tissues. To examine the role of this source in enhancing the humoral response, chimeric mice were prepared by reconstituting lethally irradiated WT or C3⁻/⁻ mice with 2 × 10⁶ BM cells isolated from either WT or C3⁻/⁻ mice. Donor-derived cells (both myeloid and lymphoid) can be distinguished from recipient cells by the allodeuterminants CD45.1 or CD45.2. FACS analysis of lymphocytes in peripheral blood and CD11b⁺ cells within the inguinal peripheral lymph nodes (pLN) confirmed that reconstitution of lymphoid and myeloid compartments was >90% (data not shown).

As earlier reports indicated (23, 33), mice with a disrupted C3 gene (C3⁻/⁻) had a severe defect in their humoral response to dermal HSV infection relative to WT mice (Fig. 1a). This impairment could be corrected by reconstituting C3⁻/⁻ mice with WT BM (WTBM→C3⁺/⁺; Fig. 1b), despite negligible levels of serum C3 (23) (Fig. 1c). To assess whether BM-derived C3 was not sufficient but also necessary for responses to an i.d. infection, WT mice were reconstituted with BM from C3⁻/⁻ animals (C3⁻/⁻ BM→WT). These mice, referred to as “reverse chimeras,” had normal C3 levels in their blood, as expected (Fig. 1c). In reverse chimeras, all C3 sources are intact, except those that are BM-derived. Despite this, the humoral response to i.d. infection in these mice was greatly diminished when compared with that of WTBM→C3⁺/⁺ (normal chimeras) and WTBM→WT controls (Fig. 1b). This finding suggested that C3 in the circulation produced by non-BM-derived sources was not sufficient to enhance humoral immunity to peripheral infection.

**Reduced number of Ab-forming cells (AFC) correlates with impaired humoral response**

To investigate the frequency of HSV-specific B cells, draining pLN were harvested 7 (primary) and 28 (secondary) days postinfection and cells were prepared for ELISPOT assays. Comparing the number of HSV-specific IgG AFC in the four chimeric groups following primary infection revealed a significant reduction in the two groups reconstituted with C3⁻/⁻ BM relative to those reconstituted with WT BM, i.e., ~10 vs 50 AFC per 100,000 lymphocytes, respectively (Fig. 2a). These results suggested that naive B cell activation within draining pLN was limited in the absence of myeloid-produced C3. Analysis of the secondary response indicated a similar impairment in the number of IgG AFC between the chimeric groups reconstituted with C3⁻/⁻ BM and those receiving WT BM.
WT BM, i.e., 10–20 vs 50–100 AFC per 100,000 lymphocytes, respectively (Fig. 2a). Thus, the frequency of AFC in the four groups of immunized chimeras was consistent with their respective Ab titers (Fig. 2b).

Reduced FDC area in follicles of pLN of infected reverse chimeras

Intradermal HSV infection induces a robust GC response within the follicles of draining pLN of WT, but not C3−/− mice (33). Expansion of FDC, which trap and retain C3-coated immune complexes via complement receptor CR1 (CD35), is a hallmark of the GC response (38, 39). FDC are important not only for Ag retention, but also for maintaining GC B cell survival (5, 39, 40). To examine the size of FDC areas within pLN of infected mice, LN were harvested 1 wk after tertiary infection and cryosections were prepared and stained for murine CD35 and C3. Confocal microscopy analysis of LN sections prepared from the two WT BM chimeras identified prominent CD35+ FDC staining that correlated with C3 deposition (Fig. 2b). In contrast, the FDC areas identified within sections of the C3−/− BM chimeras were relatively small (Fig. 2b). When normalized to the total surface area of the section, the FDC area of LN prepared from mice reconstituted with WT BM (WTBM→C3−/− and WTBM→WT) was more than twice that of chimeras receiving C3−/− BM (C3−/− BM→WT and C3−/− BM→C3−/−). Thus, a relative lack of C3 binding and a reduced size of FDC areas correlated with an impaired IgG response in the reverse chimeras, despite an abundance of C3 in the serum.

CD4+ T lymphocyte responses are intact in reverse chimeras

The absence of anti-viral Ab responses in the reverse chimeras could represent an incomplete functional reconstitution of the lymphoid compartment. To test whether T lymphocyte responses were intact, WT, C3−/−, C3−/− BM→WT, and WTBM→C3−/− mice were infected i.d. with HSV and pLN were harvested 3 wk later. CD4+ T cells were isolated from draining inguinal pLN and examined in an in vitro proliferation assay. CD4+ cells isolated from all infected groups expanded comparably in response to viral Ag, based on incorporation of BrdU (data not shown). The results were consistent with previous studies reporting that CD4+ cells are primed normally in C3−/− mice infected with a similar dose of HSV (33). Further support for normal T cell priming was obtained by immunohistochemical analysis of skin sections prepared from infected mice. HSV inoculation resulted in infiltration of CD4+ T cells (a characteristic of HSV infection (41–44)) to the site within 7 days. All four chimeric groups developed extensive infiltration of CD4+ cells, whereas negligible infiltration was observed in the skin of mice injected with PBS (data not shown).

Humoral responses to HSV-1 administered i.v. are normal in reverse chimeras

Previous studies have reported that reconstituting C3−/− guinea pigs with WT guinea pig serum restored their humoral response to thymus-dependent Ags injected i.v. (30, 45). These findings suggest that C3 present in the circulation is sufficient for Ab responses to proteins delivered systemically. To determine whether enhancement by BM-derived C3 depended on the route of infection, naive chimeras were inoculated i.v. at days 0, 21, and 42 with HSV. Our ELISA analysis of the IgG response identified an apparently normal response in the reverse chimeras (C3−/− BM→WT) similar to that of WTBM→WT and WTBM→C3−/− (Fig. 3a). As expected, chimeras deficient in C3 (C3−/− BM→C3−/−) failed to respond (Fig. 3a). Additional experiments were performed in which the reverse chimeras that failed to respond to a previous i.v. HSV infection regimen, subsequently received multiple inoculations i.v. with an immunogenic dose (50 μg) of the Escherichia coli protein β-gal in PBS. Now, both reverse chimeras and mice reconstituted with WT BM responded normally following the secondary and tertiary i.v. injection, while mice deficient in C3 again failed to respond, as expected (Fig. 3b). These combined results demonstrate that C3 derived from either the circulation or secreted by BM-derived cells is sufficient to support a humoral response to Ags administered i.v., whereas a response following peripheral exposure depends on the latter. Moreover, they verified that the reverse chimeric mice did not have an inherent defect in their ability to respond to thymus-dependent Ags.

Infiltration of CD11b+ cells into the infected dermis is normal in reverse chimeras

An alternate explanation for the impaired response observed in the reverse chimeras is that myeloid-derived C3 is essential for induction of an inflammatory response in the skin and subsequent transport of Ag to draining pLN. To examine cellular infiltration into the site of infection, chimeric mice were infected on days 0 and 21. Tissues were collected at day 28 and prepared for cryo-,
sections and RT-PCR analysis. Light microscopy analysis of H&E-stained, paraffin-embedded sections revealed limited infiltrating leukocytes in the dermis of mock-infected mice, as expected (Fig. 4, a and b). By contrast, an intense infiltrate of mononuclear cells into the dermis and underlying adipose tissue was observed in all mice infected with HSV. Inflammatory cells were largely lymphocytes and monocytes, in approximately equal numbers, with granulocytes comprising the remaining 5% of the population. The low frequency of granulocytes corresponds to the fact that the sections were harvested relatively late, at 7 days after the second infection with virus. Significantly, a similar level of infiltration was observed for all groups of infected mice. Representative sections are shown for the responding WTBM→C3−−/− and nonresponding C3−−/−BM→WT groups (Fig. 4, c and d). The robust inflammation observed in the reverse chimeras and C3-deficient animals correlated with their normal T cell response to virus and appeared to be complement independent. Thus, despite an impaired humoral response in the reverse chimeras, mononuclear cell migration into the site of infection was similar to that observed in WT controls.

To further analyze the types of cells infiltrating the skin and to detect the presence and location of C3 protein, cryosections were stained for CD11b and C3. Confocal microscopy of these sections revealed that a high frequency of the infiltrating cells was positive for CD11b (Fig. 4, e and f). In chimeras generated with WT BM, these CD11b+ cells also stained positive for C3, indicating that they could serve as a local C3 source (Fig. 4g), whereas corresponding CD11b+ cells in C3−−/−BM-derived chimeras were negative for C3 protein (Fig. 4h). RT-PCR analysis of inflamed skin tissues from normal chimeras (WTBM→C3−−/−) infected with HSV verified this finding because the only potential C3 source in these mice was derived from the BM (Fig. 4i). In addition to WT BM-derived cells, epidermis-lining keratinocytes also stained positive for C3 in chimeras with WT-derived stromal cells, i.e., reverse chimeras, but not in those with a C3−−/− background (Fig. 4, j and k). The overall amount of C3 observed in tissues of infected reverse chimeras and WT mice was considerably high (Fig. 4f). This finding most likely represents C3 derived from keratinocytes or from serum that leaked from the vasculature and is consistent with edema of an ongoing inflammatory response. These results suggest that BM-derived cells capable of producing C3 are present in the skin, but also that C3 is not normally a limiting factor within the inflamed dermis. As such, these observations do not explain the impaired humoral response in reverse chimeras.

Leukocyte infiltration into inflamed pLN is normal in reverse chimeras

Another potential explanation for the impaired B cell response in the reverse chimeras is that myeloid-derived C3 is required for efficient trafficking of leukocytes to draining pLN. To examine this possibility, inguinal LN were harvested from mice 7 days after primary or secondary infection or mock treatment. At the primary time point, we observed a 4-fold increase in total leukocyte numbers in infected WT or C3−−/− mice. The overall number of leukocytes observed in WT and C3−−/− mice was not substantially different, although the number in C3−−/− mice was slightly lower, i.e., 9.91 ± 0.90 million cells for WT vs 7.86 ± 0.82 million cells for C3−−/− upon primary immunization. Interestingly, the total number of leukocytes in WT and C3−−/− mice following secondary infection was only 3-fold greater than in mock-infected mice. A similar increase in total cell numbers (2- to 3-fold) was observed when comparing normal and reverse chimeras following primary and secondary infection. FACS analysis of lymphocytes revealed a modest increase in the frequency of B cells (B220+) in infected vs mock-treated mice in all four groups (1.5- to 2-fold). Interestingly, the frequency of CD4+ and CD8+ T cells in the pLN of all groups was reduced by ~10% in infected mice as compared with mock animals. This reduction could reflect the migration of activated T cells from pLN into the site of infection. Importantly, no
I). In summary, we observed no significant changes in lymphocyte frequencies were observed between any of the infected groups, arguing against a trafficking defect as the root cause of impaired responses in the reverse chimeras.

Analysis of DC and macrophage frequencies indicated comparable increases in the numbers of cells following primary infection in all four groups, although the overall number of macrophages found in WT mice was slightly higher than in C3<sup>−/−</sup> mice (Table 1). In summary, we observed no significant differences in the number of APC (DC and macrophages) or lymphocytes (B cells and T cell subsets) in the draining LN following HSV infection that could explain the differences in the ability of mice to develop an efficient humoral response.

Identification of myeloid C3 production within the pLN

We next considered whether the lack of response in the reverse chimeras might indicate that local C3 synthesis within pLN is necessary to enhance B cell activity. In a previous report, in situ and RT-PCR analysis implicated the MOMA-2<sup>+</sup> subset of macrophages as a source of C3 within the spleen (22). To further characterize BM-derived C3 within the lymphoid compartment, we used a combined approach of intracellular FACS analysis to identify potential C3-producing cells within draining pLN. These cells were then FACS sorted and their C3 expression was verified by RT-PCR. For this, single-cell suspensions were prepared from individual pLN from the groups of mice (WT, C3<sup>−/−</sup>, WTBM→C3<sup>−/−</sup>, and C3<sup>−/−</sup>BM→WT), permeabilized, and stained with an anti-C3 Ab. Cells were then surface stained for CD11b and CD11c. Further analysis for C3 staining showed that the C3<sup>pos</sup> cells were restricted to the CD11b<sup>high</sup>CD11c<sup>−</sup> population in the two groups of mice generated with WT BM (WTBM→WT and WTBM→C3<sup>−/−</sup>) relative to the two chimeric groups containing C3<sup>−/−</sup> BM (C3<sup>−/−</sup>BM→WT and C3<sup>−/−</sup>BM→C3<sup>−/−</sup>) (Fig. 5a). Given the high degree of chimerism, we were not surprised that this population remained negative for C3 staining in the reverse chimeras.

To confirm that the observed C3 stained representing cell synthesis and not uptake via complement receptors, we cell sorted CD11b<sup>high</sup>CD11c<sup>low</sup> LN cells from infected WT mice. Cells were subdivided into two populations based on size and granularity. Light microscopy analysis of each purified population identified them as granulocytes and monocytes, respectively (data not shown). From these populations, total RNA was isolated for RT-PCR analysis of C3 message expression (Fig. 5b). We observed that the isolated granulocytes and monocytes were able to synthesize C3 mRNA, while control B and T lymphocyte populations were negative for C3 product. Thus, C3 intracellular staining observed by FACS for the CD11b<sup>high</sup>CD11c<sup>low</sup> population corresponded to C3 synthesis. These results identify macrophages and granulocytes as potentially critical sources of C3 within draining pLN.

Discussion

The humoral response to HSV is dependent on an intact classical pathway of complement, and the effect is mediated via complement receptors CD21/CD35 (33). Although the liver is the primary

Table 1.  Professional APC numbers in draining inguinal LN of responding, nonresponding, chimeric, or non chimeric mice<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Mock (PBS)</th>
<th>Primary (HSV-1)</th>
<th>Secondary (HSV-1)</th>
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<tr>
<td></td>
<td>DC (&lt;10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>DC (&lt;10&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>WT</td>
<td>18.3 ± 1.49</td>
<td>273 ± 88</td>
<td>41.8 ± 14.03</td>
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<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>C3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>7.7 ± 1.64</td>
<td>188 ± 64</td>
<td>30.4 ± 7.53</td>
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<tr>
<td></td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>WT BM→C3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11.0 ± 2.15</td>
<td>536 ± 340</td>
<td>22.5 ± 8.77</td>
</tr>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>C3&lt;sup&gt;−/−&lt;/sup&gt; BM→WT</td>
<td>11.0 ± 1.42</td>
<td>366 ± 155</td>
<td>21.8 ± 4.78</td>
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<tr>
<td></td>
<td>(n = 3)</td>
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<sup>a</sup>Mice were immunized i.d. on weeks 0 (mock and primary HSV) and 3 (secondary HSV) and LN were harvested 1 wk after receiving the last inoculum. FACS analysis was used on single-cell suspensions from two draining inguinal LN per mouse. B220<sup>−</sup> and CD3<sup>−</sup>positive cells were excluded from total cell analysis, while the remaining cells were plotted on the basis of CD11b vs CD11c expression. DC were defined as CD11c<sup>high</sup> and macrophages as the least granular DC11b<sup>high</sup> population on a granularity vs size plot. Results as mean ± SD.

<sup>a</sup>M<sub>0</sub>, Macrophage.
source of circulating complement, recent studies have demonstrated that local C3 or C4 production by BM-derived cells is sufficient to induce B cell responses to inert proteins or infectious virus (22, 23, 29). These findings established the potential utility of local synthesis, but they could not rule out the influence of serum complement in adaptive immunity (30, 31). Further analysis of leukocyte infiltration, mainly to the adipose tissue layer, and keratinocyte C3 production, as illustrated for WTBM WT (J) vs C3<sup>-/-</sup> animals regardless of the BM they received (illustrated in f). Colocalization of C3 (green) and CD11b<sup>+</sup> infiltrating cells, while remaining absent in that of mock-infected controls (data not shown). Colocalization of C3 (green) and CD11b<sup>+</sup> in animals that received WT BM (g), but not C3<sup>-/-</sup> BM (h), suggested BM-derived myeloid cells produce C3 locally. This was confirmed by C3 mRNA-specific RT-PCR on skin of infected WTBM<->C3<sup>-/-</sup> mice (i). Infected skin from C3<sup>-/-</sup> BM->C3<sup>-/-</sup> and WTBM->WT served as negative and positive controls, respectively (two independent experiments; M, marker; W, water control). Importantly, C3 protein was identified throughout the dermis of WT animals regardless of the BM they received (illustrated in f) for the nonresponding C3<sup>-/-</sup> BM->WT group). These animals have normal liver-derived serum C3 levels and, limited to the epidermis, keratinocyte C3 production, as illustrated for WTBM->WT (J) vs C3<sup>-/-</sup> BM->C3<sup>-/-</sup> control animals (k) (K, keratinocyte layer; S, skin surface, dead cell layer).

FIGURE 4. Analysis of leukocyte infiltration and C3 presence in the dermis of BM chimeric mice (a–d). H&E staining of skin tissue sections from responding (c) and nonresponding (d) chimeric HSV-infected animal groups revealed extensive leukocyte infiltration, mainly to the adipose tissue layer, relative to mock-infected counterparts (a and b). In all cases, polymorphonuclear cells made up <5% of infiltrating cells, while lymphocytes and monocytes accounted equally for the remainder. e–k, Immunohistochemistry revealed substantial infiltration of CD11b<sup>+</sup> cells (red) into the dermis of all HSV-infected groups (illustrated in e and f), while remaining absent in that of mock-infected controls (data not shown). Colocalization of C3 (green) and CD11b<sup>+</sup> in animals that received WT BM (g), but not C3<sup>-/-</sup> BM (h), suggested BM-derived myeloid cells produce C3 locally. This was confirmed by C3 mRNA-specific RT-PCR on skin of infected WTBM<->C3<sup>-/-</sup> mice (i). Infected skin from C3<sup>-/-</sup> BM->C3<sup>-/-</sup> and WTBM->WT served as negative and positive controls, respectively (two independent experiments; M, marker; W, water control). Importantly, C3 protein was identified throughout the dermis of WT animals regardless of the BM they received (illustrated in f) for the nonresponding C3<sup>-/-</sup> BM->WT group). These animals have normal liver-derived serum C3 levels and, limited to the epidermis, keratinocyte C3 production, as illustrated for WTBM->WT (J) vs C3<sup>-/-</sup> BM->C3<sup>-/-</sup> control animals (k) (K, keratinocyte layer; S, skin surface, dead cell layer).

Reconstitution of C3<sup>-/-</sup> mice with WT BM restored B cell response to cutaneous HSV infection (Fig. 1b). By contrast, chimeric mice prepared in the reverse manner (i.e., C3<sup>-/-</sup> BM->WT mice) failed to respond similarly to the virus (Fig. 1b) despite normal circulating levels of C3 (Fig. 1c). Consequently, absence of genetically C3-sufficient BM likely also results in absence of antiviral Ab that various studies have shown to be protective against disseminated HSV disease (46–49). Although Ab is not associated with peripheral clearance of HSV, it does function to attenuate viral spread, presumably through Ab-dependent cellular cytotoxicity and complement-dependent (50) or -independent (51–53) viral neutralization. Loss of this activity may make one prone to potentially fatal complications of HSV infection most commonly seen in neonates and immunocompromised individuals (54–56). Additional experiments using a more virulent strain of HSV would be required to verify this assumption.

The diminished Ab response in reverse chimeras was characterized by a reduced number of IgG AFC (following both primary and secondary infections) and reduced FDC areas within the follicles of draining pLN (Fig. 2). Because T cells responded normally, the impairment was not due to a general failure to restore functionality of the lymphoid compartment in the reverse chimeras. The finding that the reverse chimeras responded normally when HSV was administered i.v. provided further evidence that their adaptive immune system was intact and that the defect was limited to the periphery (Fig. 3). The observance of normal responses to i.v. Ag agrees with earlier studies exploring the role of serum complement in adaptive immunity (30, 31). Further analysis showed that leukocyte trafficking to the site of infection (Fig. 4) or draining pLN (Table I) was similar in the four chimeric groups, suggesting that differences in C3 expression had little impact on these events. Furthermore, the degree of chimerism in the four groups of mice was >90%, and we confirmed restoration of the myeloid and lymphoid compartment by donor marrow. Thus, the impaired humoral response observed in the reverse chimeras could...
C3 protein was identified in the skin of WT BM. The bar graph represents the mean fluorescence intensity (MFI) levels for C3 ± SD, and a sample mean fluorescence intensity histogram for each chimera group is shown in black, overlaying a C3−/− BM→C3−/− negative control in gray. A, CD11b(high)CD11c(low) were further divided on the basis of size and granularity, FACS sorted, and identified as polymorphonuclear (P) and monocytes/macrophages (M), respectively (data not shown). C3 synthesis within the polymorphonuclear and monococyte/macrophage fractions was confirmed by RT-PCR on total RNA from these sorted populations. B, T, and H2O represent B, T cell, and H2O controls, while + and − signify PCR with or without reverse transcription reaction, respectively.

not be explained by defects in T cell activation or leukocyte trafficking, but rather appeared to be due to a more fundamental role of BM-derived C3.

The complement system influences B cell responses in two major ways: localization of Ag within the lymphoid compartment and coreceptor signaling. Both roles require activation of classical pathway and covalent binding of C3 to viral Ags. Upon activation of C3, the internal thioester within the α-chain is exposed and rapidly forms a covalent linkage via nucleophilic attack on suitable acceptor sites on neighboring proteins or carbohydrates (57, 58). This reaction is highly inefficient because the competing reaction is hydrolysis by water. Therefore, effective binding of C3 to viral Ags requires close interaction at the site of activation and a relatively high concentration of C3. In peripheral infection, the skin and draining pLN are two plausible sites for C3 activation and covalent attachment.

Several sources of C3 were identified in the inflamed dermis, i.e., keratinocytes, infiltrating myeloid cells, and serum C3 (Fig. 4). Immunohistochemical examination of dermal tissues from the four chimeric groups after infection identified substantial amounts of C3 in the tissues of both responding WTBM→WT and nonresponding C3−/−BM→WT mice (Fig. 4). By comparison, low levels of C3 protein were identified in the skin of WTBM→C3−/− mice, which do respond normally (no C3 protein was found in C3−/−BM→C3−/− mice) (Fig. 4). Given the relatively high amount of C3 present in the cutaneous tissues of reverse chimeras (C3−/−BM→WT), it seems unlikely that the defect in B cell activation is due to limited levels of complement in the skin.

In contrast to infected dermis, C3 levels appeared to be limited in the pLN. Immunohistochemical staining of reverse chimera pLN identified C3 within vessels, but there was little evidence of serum C3 leakage into the follicular or T cell zones (Fig. 2b). It is more probable that highly targeted local myeloid C3 synthesis is required within the pLN, where naive B cells are first exposed to Ag and become activated to initiate the humoral response. By this model, viral Ag drags or is transported into pLN where it is bound by pre-existing natural Ab. Low-affinity IgM binding would activate local production of classical pathway components and focus the attachment of C3 to the Ag (as reviewed in Refs. (59 and 60). Indeed, although the number and frequency of myeloid cells within the draining pLN was normal in all chimeras, C3 synthesis was detected only in pLN of responding mice (i.e., those that received WT BM) (Fig. 5). Therefore, both cell- and serum-derived complement appeared to be limiting in the nonresponsive reverse chimera pLN. This may indicate a distinguishing feature of pLN as compared with edematous dermal tissues or the highly blood-perfused spleen. However, a definitive role for myeloid-derived C3 in the skin cannot be discounted and it will be important in future experiments to examine viral Ag trafficking into the draining pLN in the presence or absence of C3.

Our results emphasize a role for local synthesis of C3 by myeloid cells. It will be important to determine the source of other complement proteins that are required for C3 activation, i.e., C1 (C1q, C1r, C1s), C2, and C4. Macrophages are the major source of C1q (27, 61) and they are known to produce other early classical pathway components (62, 63). In particular, C4 produced by macrophages is sufficient to restore the humoral response to Ag administered i.v. in C4−/− mice (29, 64). Therefore, a developing model is that activation of macrophages induces the secretion of C1, C2, C4, and C3 locally in levels sufficient to ensure efficient C3 coupling to pathogens. This autonomous role for macrophages might be critical in sites where complement proteins within the circulation are not readily available for activation and binding of C3 to foreign Ags, such as pLN.

Because myeloid cells are not the only nonhepatic source of C3, the question is raised what other cell types, such as keratinocytes, contribute to the overall immune response, if not the humoral response? The complement system has multiple roles including its long-recognized functions in innate immunity. Keratinocyte C3 appears to have no role in enhancing the humoral response (see C3−/−BM→WT animals); it might instead be particularly important as a first-line defense by mediating nonspecific lysis through the membrane attack complex or enhancement of phagocytosis. This role seems reasonable given the fact that keratinocytes strategically line the skin surface. High levels of serum C3 might play a similar role, protecting the body from rapid, systemic spread of pathogens.

In summary, we have identified a critical role for myeloid-derived complement C3 in the Ab response to a peripheral infection by HSV. This demonstrates that serum complement may not be
able to fulfill every in vivo activity and that other secondary sources of complement may play similar essential roles in defined biological niches. This study provides a novel insight into the induction of adaptive immunity to microbial infection and it supports a more general model in which macrophages secrete all of the early components of classical pathway complement necessary to ensure C3 activation and binding to Ags within the peripheral lymphoid compartment.

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