Macrophage-Derived Complement Component C4 Can Restore Humoral Immunity in C4-Deficient Mice

Mihaela Gadjeva, Admar Verschoor, Mark A. Brockman, Heather Jezak, Li Ming Shen, David M. Knipe and Michael C. Carroll

J Immunol 2002; 169:5489-5495; doi: 10.4049/jimmunol.169.10.5489
http://www.jimmunol.org/content/169/10/5489

References
This article cites 50 articles, 35 of which you can access for free at:
http://www.jimmunol.org/content/169/10/5489.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Macrophage-Derived Complement Component C4 Can Restore Humoral Immunity in C4-Deficient Mice1

Mihaela Gadjeva,*† Admar Verschoor,‡ Mark A. Brockman,§ Heather Jezak,* Li Ming Shen,* David M. Knipe,§ and Michael C. Carroll2*†‡

Mice with a disrupted C4 locus (C4−/−) have an impaired immune response to thymus-dependent Ags. To test the role of bone marrow-derived C4 in humoral immunity, we reconstituted deficient animals with wild-type bone marrow or an enriched fraction of bone marrow-derived macrophages. C4 chimeras were immunized with 4-hydroxy-3-nitrophenyl conjugated to keyhole limpet hemocyanin (NP5-KLH) or infected with HSV-1, and the Ab response was evaluated. Wild-type bone marrow rescued the humoral immune response to both Ags, i.e., the soluble Ag and HSV-1, demonstrating that local C4 production is sufficient for humoral responses. Although the C4 chimeric animals lacked detectable C4 in their sera, C4 mRNA was identified in splenic sections by in situ hybridization, and C4 protein deposits were identified in the germinal center areas of splenic follicles by immunofluorescence staining. Macrophages derived from bone marrow produced sufficient C4 protein to restore the humoral response to NP5-KLH in C4-deficient animals when administered along with Ag. Cell-sorting experiments, followed by C4-specific RT-PCR, identified splenic macrophages (CD11b+ , CD11c+) as a cellular source for C4 synthesis within the spleen. The Journal of Immunology, 2002, 169: 5489–5495.

The impairment in T-D responses to Ags administered i.v. can be restored by passive administration of purified C3 or C4 along with Ag (24, 25). Although the liver is the major source of serum C3 and C4, certain bone marrow (BM)-derived cells are also capable of producing complement proteins (26, 27). Notably, reconstitution of C3-deficient animals with wild-type (WT) BM can restore the humoral response to T-D Ag administered i.v (28) or intradermally (i.d.) (29). BM-derived cells are also a source of C1q (30); thus, myeloid cells can provide a local source for complement, both in the spleen and lymph nodes. These studies raise important questions regarding the source and regulation of myeloid-derived complement.

To determine whether local synthesis is sufficient to restore the humoral response, C4-deficient mice were engrafted with WT BM or with an enriched fraction of BM-derived macrophages. BM engraftment was sufficient to restore the humoral response to a T-D-soluble Ag 4-hydroxy-3-nitrophenyl conjugated to keyhole limpet hemocyanin (NP5-KLH) or infectious virus (HSV-1). Local C4 was produced by CD11b+/CD11c+ splenic macrophages, which also synthesized C1q and C3. Moreover, an enriched population of BM-derived macrophages was capable of restoring the B cell response to NP5-KLH in deficient animals. Taken together, these data suggest that upon stimulation, macrophages within the secondary lymphoid compartment produce early complement components, which allows for triggering of the complement cascade via the classical pathway, leading to enhancement of humoral responses to T-D Ags.

Materials and Methods

Mouse strains

C4−/− mice were generated by homologous recombination, as described previously (7), and backcrossed with C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice for five generations (>95% C57BL/6). Animals were bred and maintained at the Warren Alpert Animal Facility at Harvard Medical School. STAT-1−/− and IFN-γ−/− mice were purchased from Taconic (Germantown, NY) and The Jackson Laboratory, respectively.

Copyright © 2002 by The American Association of Immunologists, Inc.
Generation of BM chimeras

BM cells were collected by flushing the femurs and tibias with cold PBS/0.1 mMEDTA, followed by depletion of erythrocytes by lysis with 0.15 M NH₄Cl, 10 mM KHCO₃. At 7–10 wk of age, C₄⁻/⁻ or C57BL/6 mice were lethally irradiated using two 650-rad doses and reconstituted i.v. with 10 × 10⁶ BM cells derived from gender- and age-matched C57BL/6 mice.

Immunization protocol

After transplantation, animals were rested for 6 wk and then either immunized with 100 µg NP₃-KLH i.v. or infected with 2 × 10⁶ PFU HSV-1 (KOS1.1 strain) i.d. (16, 31). After each immunization, mice were rested for 3 wk and then boosted. Animals were sacrificed 1 wk after the third boost. Serum samples were collected weekly.

**Serum C4 ELISA**

Immulon 1B microtiter plates (DYNEX Technologies, Chantilly, VA) were coated overnight with rat anti-murine C₄ mAb 16D2 (a kind gift from E. Kremmer, GFN National Research Center for Environment and Health, Munich, Germany) in carbonate buffer. After blocking with 5% dry milk in PBS and 0.01% Tween 20, serial dilutions of mouse serum in blocking buffer were applied to the wells and incubated for 2 h at 37°C. Murine C₄ was detected with rabbit anti-human C4c (DAKO, Glostrup, Denmark) and then followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO). Plates were developed by adding Sigma-Aldrich alkaline phosphatase substrate 104, and absorbance was measured at 405 nm.

Ab titers

Immulon 1B microtiter plates (DYNEX Technologies) were coated overnight with NP₃-haptenated BSA (32) (Sigma-Aldrich), blocked as described above, and serial serum dilutions were applied and incubated for 3 h at 37°C. Murine IgG was detected by alkaline phosphatase-conjugated rat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). Plates were developed by adding Sigma-Aldrich alkaline phosphatase substrate 104, and absorbance was measured at 405 nm.

**In situ C4 hybridization**

A BamHI-KpnI restriction fragment representing the 5′ terminal end of C₄ cDNA (a kind gift from R. Ogata, Torrey Pines Institute for Molecular Studies, San Diego, CA) was subcloned into pBluescript II KS⁺ (Stratagene, La Jolla, CA). Antisense digoxigenin (DIG)-labeled mC4 transcripts were produced by linearizing the pBSTnC4 plasmid with XbaI and transcribing with T3 RNA polymerase. DIG-labeled probes were produced with a DIG RNA labeling kit (SP/T7) (Roche Diagnostics, Indianapolis, IN) per manufacturer’s instructions. RNase-free sections (4 µm) were cut, and in situ hybridization was performed, as described previously (28).

**BM-derived macrophages**

BM-derived macrophages were grown in L929 (American Type Culture Collection, Manassas, VA)-preconditioned DMEM (Life Technologies, Rockville, MD) medium and supplemented with 10% FCS, 5% horse serum (Sigma-Aldrich), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Life Technologies) until the cells were confluent. Cells were scraped with a cell lifter (Fisher Scientific, Pittsburgh, PA), replated at 1 × 10⁵ cells/well in six-well cell culture Costar plates (Corning Glass, Corning, NY), and stimulated with 5000 IU rIFNa (R&D Systems, Minneapolis, MN) for 12 h.

Immunofluorescence

Splenocytes were snap frozen in OCT (Tissue Tek Sakura, Torrence, CA)-filled molds (VWR, West Chester, PA) and stored at −80°C until cryosections were cut. Sections (5 µm) were fixed for 4 min in ice-cold acetone. Sections were blocked with 2% BSA, 2% FCS, and PBS. The following steps were conducted in the presence of the blocking buffer: sections were stained with anti-C₄ rat mAb 15H12 or 16D2 (kind gifts from E. Kremmer) conjugated to Cy5 (Amersham, Arlington Heights, IL) or biotin- and FITC-conjugated peanut agglutinin (PNA; EY Laboratories, San Mateo, CA), as described (9). Both mAbs produced similar results when used for immunofluorescence. The biotinylated 16D2 was visualized using avidin-PE (BD PharMingen, San Diego, CA).

**FACS analysis and sorting**

Splenocytes were incubated on ice with anti-FcR (2.4G2), before treatment with Abs specific for CD45.1, CD45.2, CD11b, or CD11c (all from BD PharMingen), and analyzed using a FACScalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA), or sorted using a FACSVantage. When necessary, the splenocytes were depleted from B and T lymphocytes by magnetic cell sorting with microbeads before FACS analysis or sorting. For depletion purposes, biotinylated Abs specific for CD19 or CD3 (BD PharMingen) were used, followed by streptavidin MACS beads (Miltenyi Biotec, Auburn, CA). The separation was performed on MACS LD-separation columns (Miltenyi Biotec), and the unbound cells were collected and analyzed further. Cell viability was accounted for by propidium iodide (PI) staining and subsequent gating out of PI positively stained cells during the analysis.

**RT-PCR**

Total RNA was purified from sorted cells or splenic tissue per Qiagen (Valencia, CA) RNeasy mini kit instructions. First strand synthesis was performed with SuperScript RT (Life Technologies). C₄ RT-PCR yielded a 472-bp-specific band after 30 rounds of amplification with the following primer set: sense (GGTTCTGAAGGTGCCTGTCC) and antisense (GTGAAGGCCAATGACCAAAAGG). C₃ message was amplified after 35 cycles with the following set of primers: sense (GGCTGACTCTGTGTGGGT) and antisense (TCTCTGGTTCCTTCAACCTCT).

**Results**

**Humoral responses are restored in the WT BM→C₄⁻/⁻ chimeric animals**

To determine whether myeloid-derived C₄ was important in the T-D humoral response, C₄-deficient mice were reconstituted with WT BM (WT BM→C₄⁻/⁻). Three cohorts of chimeric animals were analyzed in these studies: 1) WT BM→WT; 2) WT BM→C₄⁻/⁻; and 3) C₄⁻/⁻ BM→C₄⁻/⁻. C57BL/6 (CD45.2) and C₄⁻/⁻ mice on C57BL/6 (CD45.2) background were used as recipients for donor marrow from C57BL/6 (CD45.1) mice. The congenic CD45.1 C57BL/6 strain was used to facilitate tracking of the donor-derived cells bearing the allogeneic CD45.1 marker in the recipient mice. Mice were immunized i.v. with 100 µg of soluble haptenated NP₃-KLH at days 0 and 21. This dose of Ag was previously shown to be fully immunogenic (28). Adjuvants were omitted from the immunization protocol to avoid circumventing the role of complement. NP-specific IgG titers were determined by ELISA (Fig. 1a). Alternatively, mice were infected i.d. with 2 × 10⁶ PFU of infectious HSV-1 (Fig. 1b). Consistent with previous studies using C₄-deficient mice, the immune response of C₄-deficient chimeras to both soluble Ag (7) and infectious HSV (16) was ~8- to 18-fold lower than that of WT chimeras. By contrast, nearly normal primary and secondary Ab responses were observed in C₄-deficient mice reconstituted with WT BM.

To further characterize the humoral response in the chimeric animals, splenic sections were prepared, and the number of GCs was determined by PNA staining, followed by immunofluorescence analysis. At least five animals per group were analyzed 7 days after the tertiary immunization (Table 1). The percentage of GCs per follicle was similar to that observed in splenic sections of WT BM→C₄⁻/⁻ chimeras and WT BM→WT chimeras (Table 1, Fig. 1). By contrast, the percentage of GCs in the splenic sections of C₄⁻/⁻ BM→C₄⁻/⁻ chimeras was reduced. Immunohistochemical analysis of splenic sections identified detectable C₄ deposits in the GC areas in all of the WT chimeras, compared with ~50% in WT BM→C₄⁻/⁻ chimeras (Table 1, Fig. 2b). C₄-specific staining in the spleen of all primary sections was found in the GC areas and appeared to be network-like, consistent with the presence of adducts of C₄ and Ag/C₃ protein captured by the FDCs.

**The cellular source of complement C₄**

The WT BM→C₄⁻/⁻ chimeric animals lacked measurable C₄ protein in their sera based on ELISA analysis (Fig. 2a), but C₄ was detected in splenic sections by immunofluorescence, as discussed...
above (Fig. 2b). Moreover, C4 RNA was identified in splenic sections by in situ hybridization (Fig. 2c). The C4-producing cells were detected with a DIG-labeled C4 antisense RNA probe derived from C4 cDNA. Comparison of splenic sections from the three groups of chimeras immunized with NP5-KLH revealed a similar distribution of C4 mRNA-positive cells throughout the white pulp and some individual cells in the red pulp. As expected, no C4 mRNA-positive cells were observed in sections prepared from C4−/− BM→C4−/− animals (Fig. 2c). In summary, reconstitution of C4−/− mice with WT BM restored C4 synthesis within the spleen and lymph nodes, as measured by protein staining (Fig. 2b), in situ hybridization (Fig. 2c), and RT-PCR (data not shown).

To determine the extent of engraftment and identify the cellular source of donor-derived C4, single cell suspensions of spleen cells

Table I. Reconstitution of GC formation in secondary lymphoid tissue of C4 BM chimeric animals  

<table>
<thead>
<tr>
<th></th>
<th>Nonimmunized</th>
<th>Immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 5)</td>
<td>WT BM→WT (n = 5)</td>
</tr>
<tr>
<td>Number of follicles</td>
<td>19 ± 4</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Number of GC</td>
<td>2 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>C4 presence in GC</td>
<td>2 ± 3</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

*Engraftment of WT BM into C4−/− mice restores formation of GC in splenic tissue. Spleens were harvested from nonimmunized WT (C57BL/6) or chimeric mice (WT BM→WT, WT BM→C4−/−; or C4−/− BM→C4−/−) 1 wk after final immunization with 100 μg NP5-KLH i.v. Cryosections were prepared as described in Fig. 2, and the frequency of PNA + GCs was determined by immunofluorescence. The results identify a similar frequency of GCs in the spleenic follicles of immunized WT BM→WT and WT BM→C4−/− mice, in contrast to negligible GCs in the follicles of C4−/− BM→C4−/− chimeras.
were prepared from chimeras and analyzed by FACS. Over 90% of the CD11b population in the spleen was of donor origin, based on expression of the CD45.1 donor allotype (Fig. 3). This compartment includes myeloid-derived cells, such as splenic macrophages, dendritic cells, and neutrophils. The CD11b splenocytes were further subdivided based on CD11c staining and forward and side scatter characteristics. CD11b R2- and CD11b R3-gated cells were sorted (Fig. 4a), and cytospins were prepared. Nuclear morphology determined with Giemsa Wright staining showed that the R2 gate included granulocytes, whereas the R3 gate was comprised primarily of monocytes (macrophages). To establish whether the sorted cells produced not only C4 and C3 complement components, but also C1q, total RNA was prepared from the sorted populations and analyzed by RT-PCR. Although both granulocytes and monocytes synthesize C3 (Fig. 4b), only the monocytic population was positive for C4 and C1q mRNA. Thus, a similar population of macrophages produced C1q, C4, and C3.

**BM-derived macrophages can produce sufficient C4 to restore humoral immunity to NP-KLH**

To test the ability of macrophages to produce complement C4, monocytic cells were derived from cultured C57BL/6 BM. FACS analysis of the cultured cells revealed a high frequency of macrophages (CD11b+, F480+CD11c+, and I-A+ cells; data not shown). The CD11b profile (R3) is based on combined R1 and R2 gates. Representative histograms are shown from WT BM→WT, WT BM→C4⁻/⁻, and C4⁻/⁻ BM→C4⁻/⁻ chimeras 14 wk after BM transplantation. a, Mean level of chimeraism in WT BM→WT and WT BM→C4⁻/⁻ is determined by the frequency of CD45.1 allotype-marked donor cells. Significantly, >90% of CD11b+ cells are donor derived. Results represent analysis of splenocytes harvested from five mice per group. Note that C4⁻/⁻ BM donor cells are CD45.2; therefore, the degree of chimeraism could not be determined.
local complement synthesis that includes not only C3, but also other complement proteins. To test this possibility, C4−/− mice were engrafted with WT BM or an enriched population of BMM. We found that BM engraftment leads to restoration of humoral response in C4-deficient animals despite an apparent absence of C4 protein in the serum.

Ab responses to two different T-D Ags were evaluated (soluble protein Ag and infectious HSV-1). In both cases, WT BM engraftment led to reconstitution of humoral responses as measured by Ab titters (Fig. 1). Peripheral infection with HSV-1 represents a physiologically relevant model to study the role of complement activation. Previous analysis of mice deficient in C3, C4, or CD21/35 following infection with HSV-1 revealed an impaired secondary response characterized by low Ab titters. These results suggest that the classical pathway is important in initiating complement activation leading to the activation of C3. The effects of C3 are dependent on CD21/CD35, a similar impairment is observed in CD21/CD35-deficient mice (16). Previously, we demonstrated that WT BM engraftment in C3−/− mice restored the impaired Ab response to HSV-1 (29). WT BM→C4−/− chimeric mice have a phenotype similar to WT BM→C3−/− mice with regard to HSV-1 Ab titters, indicating that local complement production is adequate to enhance the antiviral response and that BM-derived cells can produce complement components C3 and C4. These findings implicate locally produced complement as important to the humoral immune response and may be helpful in the design of future vaccines.

In contrast to WT BM→C1q−/− chimeras (30), in which WT BM engraftment led to reconstitution of circulating C1q serum levels, engraftment did not restore C4 serum levels in WT BM→C4−/− chimeras (Fig. 2a); this finding is similar to previous observations in WT BM→C3−/− mice (28). These variations could be explained by the differences in regulation of C1q vs C3 and C4. Macrophages, a major cellular source of C1q, are responsible for circulating C1q levels, while hepatocytes are the predominant source of serum C3 and C4 (26, 27, 30).

C4 synthesis by BM-derived cells was detected by in situ hybridization (Fig. 2c). C4 mRNA localized randomly throughout the splenic white pulp, suggesting that the monocytic-producing cells were not clustered within a subregion, such as the follicular zone. A similar pattern was previously observed for C3 mRNA (28). In this study, the pattern of C3 mRNA expression correlated with MOMA-2+ cells based on immunohistochemistry of serial sections. Moreover, MOMA-2+ cells were identified as a major source of C3 by RT-PCR analysis of an enriched fraction of cells. We used a similar approach to sort MOMA-2+ cells by magnetic beads from a suspension of splenocytes; RT-PCR analysis identified C4 mRNA in MOMA-2+ cells, but not B cell controls (results not shown). Thus, the MOMA-2+ population of macrophages can express both C3 and C4.

Previous experiments have identified numerous sites for C3 synthesis in addition to the liver. In humans, extrapathic C3-producing cells include monocytes/macrophages, fibroblasts, capillary endothelial cells, T cells, endometrium, adipocytes, osteoblasts, and intestinal epithelial cells (33–38). Similar extrapathic sources have been identified in the mouse (39, 40). Although studied less intensely, C4 synthesis appears to be more limited than C3. Murine C4 synthesis was identified in peritoneal and resident kidney macrophages (41, 42). Bone marrow-derived macrophages (CD11b+CD11c−) are a cellular source of C4 in the spleen and lymph nodes (Figs. 4 and 5, and data not shown). The CD11b+ cells in WT BM→C4−/− chimeras were over 98% donor derived, as judged by the frequency of the allotypic marker (Fig. 3). Splenic populations were sorted based on CD11b vs CD11c expression,
size, and granularity (43). C4 and C1q mRNAs were detected only in the monocytic/macrophage-sorted population (Fig. 5b), whereas granulocytes and monocytes both expressed C3. This pattern of differential synthesis of complement may reflect the various physiological activities of the cell types. Macrophages appear to produce all the necessary complement components to enhance B cell activation, whereas neutrophils might contribute more to alternative pathway-mediated inflammatory reactions.

Notably, engraftment of WT BM led to sufficient C4 synthesis to allow for local complement activation. As a consequence of complement activation, C4 deposits were captured on FDCs (Fig. 2b). The C4-specific staining in the splenic sections colocalized with PNA (Fig. 2b) and Ag (data not shown). The frequency of C4 deposits was low in BM and splenic sections by immunofluorescence staining. The data are consistent with previous published reports identifying the presence of activated products of complement (C1q, C3, C4, and C5) within the GCs (6, 28, 44, 45). Taylor et al. (46) recently reported that the FDC-restricted epitope, FDC-M2, is complement C4 and that its localization in the spleen follows complement activation and is in the form of immune complexes. Complement facilitates trapping of Ag-Ab complexes within the FDC network. C4 deposits on FDCs can be explained by direct interaction with CD35 (9, 15). Alternatively, C4 capture follows complement activation and is in the form of immune complexes that are detected by the immunofluorescent staining most likely represents C4-bearing immune complexes. These data are consistent with previous published reports identifying the presence of activated products of complement (C1q, C3, C4, and C5) within the GCs (6, 28, 44, 45).

Adoptive transfer experiments of BM-derived macrophages in C4−/− mice suggest a physiological role for macrophage-produced C4 (Fig. 5b). Low levels of macrophage C4 suffice to facilitate complement activation, leading to Ag tagging with C3/C4 fragments, capture, and retention on FDCs. The data are consistent with the hypothesis that complement activation via the classical pathway is required to enhance T-D responses to inert protein Ags delivered i.v.

In summary, WT BM or BMM engraftment can provide sufficient C4 to allow for complement activation and restoration of humoral responses in WT BM→C4−/− chimeric mice. Thus, enhancement of humoral immunity to pathogens is an important function of locally produced early complement.

Acknowledgments

We thank Drs. R. Barrington and M. Zhang for helpful comments during preparation of the manuscript; Dr. T. Schneider for assistance with cell-sorting experiments; A. Burton and J. Xia for genotyping the mice; and M. Ottaviano for technical assistance.

References