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C3 Promotes Expansion of CD8\(^+\) and CD4\(^+\) T Cells in a Listeria monocytogenes Infection\(^1\)

Yumi Nakayama,* Shin-II Kim,† Eui Ho Kim,* John D. Lambris,‡ Matyas Sandor,† and M. Suresh\(^2\)*

It is known that C3 is required for optimal expansion of T cells during acute viral infections. However, it is not yet determined whether T cell responses to intracellular bacterial infections require C3. Therefore, we have investigated the requirement for C3 to elicit potent T cell responses to *Listeria monocytogenes* (LM). We show that expansion of Ag-specific CD8 and CD4 T cells during a primary response to LM was markedly reduced in the absence of C3 activity. Further studies indicated that, unlike in an influenza virus infection, the regulation of LM-specific T cell responses by C3 might not involve the downstream effector C5a. Moreover, reduced T cell responses to LM was not linked to defective maturation of dendritic cells or developmental anomalies in the peripheral T cell compartment of C3-deficient mice. Experiments involving adoptive transfer of C3-deficient CD8 T cells into the C3-sufficient environment of wild-type mice showed that these T cells do not have intrinsic proliferative defects, and a paracrine source of C3 will suffice for clonal expansion of CD8 T cells in vivo. However, stimulation of purified C3-deficient CD8 T cells by plastic-immobilized anti-CD3 showed that C3 promotes T cell proliferation directly, independent of its effects on APC. On the basis of these findings, we propose that diminished T cell responses to LM in C3-deficient mice might be at least in part due to lack of direct effects of C3 on T cells. These studies have furthered our understanding of C3-mediated regulation of T cell immunity to intracellular pathogens. *The Journal of Immunology*, 2009, 183: 2921–2931.

It is known for a long time that complement components form an integral arm of innate immunity (1, 2). However, in recent years, it has become increasingly evident that complement components are also important in both induction and effector phases of adaptive immunity. Specifically, it is well established that B cell activation, Ab production, and some of the effector functions of Abs require complement (3–5). Therefore, complement acts as a bidirectional link in both afferent and efferent phases of humoral immunity. In addition, complement plays an important role in the clearance of Ag/Ab complexes and protects against immune complex diseases (6–8). Although most studies on complement have focused mainly on innate and humoral immunity (3–5, 9), there is emerging evidence that complement component C3 promotes T cell responses to viral infections, including influenza virus and lymphocytic choriomeningitis virus (LCMV)\(^3\) (10, 11). However, our understanding of mechanisms underlying the regulation of T cell responses by complement is incomplete. Experimental models of infections and transplantation have indicated that C3 might regulate T cell responses by distinct mechanisms in a context-dependent fashion. For example, during influenza virus infection of mice, full activation of CD8 T cells require C5a, in addition to C3 (10, 12). However, in the murine visceral leishmaniasis model, induction of CD8 T cell responses by vaccination is dependent upon natural Abs and complement-dependent IL-4 production (13). Additionally, complement has been shown to be important in the genesis of autoimmune myocarditis, and localized production of C3 plays a key role in allogeneic T cell-mediated rejection of renal transplants (14).

Experimental infection of mice with *Listeria monocytogenes* (LM) has provided seminal insights into the mechanisms of innate and adaptive immunity to facultative intracellular bacteria (15). At the cellular level, early killing of LM is dependent upon innate immunity mediated by neutrophils, macrophages, and NK cells, but complete bacterial clearance requires CD8 T cells (16–19). Cytokines TNF-α and IFN-γ play nonredundant roles in controlling bacterial growth because both TNF-α- and IFN-γ-deficient mice are highly susceptible to LM infection (20–23). Importantly, some of the protective effects of TNF-α and IFN-γ against LM might be complement dependent (24, 25). Moreover, it has been reported that LM activates complement, and complement receptor 3 is important for phagocytosis and killing of LM by activated macrophages in vitro (24, 26, 27). Therefore, it is possible that complement receptor 3-dependent phagocytosis could be an important step in Ag processing and presentation to T cells during an LM infection. However, the role of complement component C3 in the elicitation of T cell responses in the context of an intracellular bacterial infection has not been examined. In this study, we have determined the requirement for C3 and C5a in the induction of Ag-specific CD8 and CD4 T cell responses to LM in mice. These studies show that activation and full expansion of CD8 and CD4 T cells during a primary LM infection require C3 but not C5a. To understand the mechanisms underlying the regulation of T cell responses by C3, we have investigated: 1) the effect of C3

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\(^\ddagger\) Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; C5αRa, C5αR1 antagonist; LL0, listeriolysin O; LM, *Listeria monocytogenes*; MFI, mean fluorescence intensity; MHC II, MHC class II; NP, nucleoprotein; PI, postinfection.

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deficiency on the numbers of T cells, B cells, and dendritic cells (DC) in spleen before infection; 2) whether C3 deficiency influences LM-induced maturation of DC in vitro and in vivo; 3) the importance of autocrine and paracrine sources of C3 in driving clonal expansion of CD8 T cells; and 4) whether C3 promotes TCR signaling-induced proliferation of CD8 T cells. These studies further our understanding of the role of C3 in regulating T cell responses to intracellular bacteria and have significant implications in vaccine development and treatment of T cell-dependent immunopathology.

Materials and Methods

Mice
The C3-deficient (C3−/−) and C5a receptor-deficient (C5aR−/−) mice on the C57BL/6 background were provided by Drs. R. Wetsel (University of Texas, San Antonio, TX) and C. Gerard (Harvard Medical School, Boston, MA), respectively (28, 29). Control wild-type (+/+ ) C57BL/6 mice were either littermates or purchased from the National Cancer Institute. Congenic C57BL/6 mice (Ly5.1) were purchased from The Jackson Laboratory. All animal experiments were performed as per institutional animal care guidelines.

LM infection
The rLM that expresses the GP33-41 epitope (rLM/GP33) or nucleoprotein (rLM/NP) of LCMV was generated by Dr. H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) (30). Mice were infected with 5 × 106 CFU of rLM/GP33 or rLM/NP by i.v. injection. Bacterial load in tissues was quantitated by plating tissue homogenates on brain-heart infusion agar plates (30).

DC propagation
Bone marrow-derived DC were generated as described previously (31). Briefly, bone marrow cells were collected from the tibias and femurs of +/+ or C3−/− mice. After RBC lysis, cells were resuspended at a concentration of 107/ml and plated in 6-well plates in RPMI 1640 medium containing 10% FBS and 20 μg/ml murine GM-CSF (PeproTech). DC were cultured for 6 days at 37°C in 5% CO2, and half of the medium containing GM-CSF was replaced on days 2 and 4. On day 6, DC were harvested for infection with LM and T cell priming assay as described below.

Induction of DC maturation by LM infection
Bone marrow-derived DC (105/ml) were plated onto 48-well plates and infected with rLM/GP33 or rLM/NP (multiplicity of infection = 1). LM infection was terminated 4 h later by adding chloramphenicol (10 μg/ml). To examine LM infection-induced maturation of DC, cell surface expression of CD80, CD86, and MHC class II (MHC II) was compared between uninfected and LM-infected DC at 4 days after infection by flow cytometry.

In vitro T cell priming assay
Naive TCR transgenic CD8 T cells were activated in vitro with LM-infected bone marrow-derived DC as described elsewhere (32). Bone marrow-derived DC were infected with rLM/GP33 as described above. As controls, uninfected DC were coated with the MHC I-restricted epitope GP33 peptide (1 μg/ml). After infection with rLM/GP33 or coating with peptide, DC were incubated for an additional 18 h before naive P14 CD8 T cells were added to the culture.

Naive P14 CD8 T cells were purified from the spleens of P14 TCR transgenic mice using T cell enrichment columns (R&D Systems) and labeled with CFSE (Molecular Probes). CFSE-labeled purified P14 CD8 T cells were mixed with LM-infected or peptide-coated DC at a ratio of 10 T cells per DC and cultured for 72 h. After 72 h of stimulation, CFSE fluorescence of P14 T cells was analyzed by flow cytometry.

LM-induced DC maturation in vivo
+/+ or C3−/− mice were infected with rLM/GP33. At days 3 and 4 after infection, single-cell suspensions of splenocytes were prepared after collagenase digestion, as described elsewhere (33). Cells were stained with anti-CD11c, anti-CD11b, anti-CD80, anti-CD86, anti-CD40, anti-Dp (MHC I), anti-I-Ak (MHC II), and anti-B220. The alteration in expression of CD80, CD86, CD40, MHC I, and MHC II molecules on subsets of DC was assessed by flow cytometry.

Adoptive transfer of TCR transgenic P14 CD8 T cells
Ten thousand naive GP33-specific CD8 T cells purified from the spleens of P14/Ly5.1 TCR transgenic mice were adoptively transferred into congenic +/+/Ly5.2 or C3−/−Ly5.2 mice. Twenty-four hours after cell transfer, mice were infected with rLM/GP33.

Treatment of mice with C5aR antagonist (C5aRa)
As a specific C5aRa, cyclic heptapeptide AcF(OPuChawR) was prepared and used as described before (34). Mice were injected i.p. with C5aRa at a dose of 1 μg of peptide/g body weight on days 1, 3, and 5 after LM infection.

Quantitation of LCMV-specific CD8 T cells by MHC I tetramers
D3/GP33 and K/GP34 MHC I Tetramers were prepared as described before (35). Single-cell suspensions of splenocytes were stained with anti-CD8, anti-CD44, and MHC I tetramers for 1 h at 4°C. After staining, cells were fixed in 2% paraformaldehyde and acquired on a FACS Calibur flow cytometer (BD Biosciences). Flow cytometry data were analyzed using the FlowJo software (TreeStar). Foxp3+ regulatory CD4 T cells were visualized by staining splenocytes for cell surface CD4 and intracellular Foxp3 using a commercially available kit (eBioscience).

Quantitation of Ag-specific CD4 and CD8 T cells by intracellular staining
Production of IFN-γ by epitope-specific CD8 and CD4 T cells was assessed by intracellular cytokine staining as described before (35). Briefly, freshly explanted splenocytes were cultured ex vivo for 5 h with the epitope peptide, IL-2, and brefeldin A. After culture, cells were stained for cell surface CD8 or CD4 and intracellular IFN-γ using the Cytofix/Cytoperm kit (BD Pharmingen). Cells were acquired on a FACS Calibur flow cytometer, and data were analyzed using FlowJo software.

Adoptive transfer of CD8 T cells from C3−/− mice into congenic Ly5.1 B6 mice
CD8 T cells were purified from spleens of naive +/+ or C3−/− mice (Ly5.2) by negative selection using magnetic beads (Miltenyi Biotec). A total of 4.8 × 10⁶ purified CD8 T cells were adoptively transferred into congenic C57BL/6Ly5.1 mice by i.v. injection. Twenty-four hours after cell transfer, mice were infected with rLM/GP33 as above.

T cell proliferation assay
CD8 T cells were purified from spleens of +/+ or C3−/− mice by negative selection using a magnetic bead separation technique (Miltenyi Biotec). CD8 T cells (1 × 10⁷) were stimulated with plate-bound anti-CD3 (10 μg/ml) for 48 h in 96-well round-bottom plates (36). Between 24 and 48 h, cells were pulsed with [3H]thymidine, and T cell proliferation was assessed by measuring thymidine incorporation at 48 h.

Statistical analysis
The commercially available software (version 10.2; Systat) was used to analyze data.

Results

C3 is required for optimal expansion of CD8 T cells in a LM infection
In this study, we examined the requirement for C3 in the activation and expansion of CD8 T cells following infection of mice with an intracellular bacterial pathogen, LM. Groups of wild-type +/+ and C3−/− mice were infected with rLM/GP33, which expresses the CTL epitope GP33-41 of LCMV. On day 7 postinfection (PI), activation and expansion of CD8 T cells in spleens of +/+ and C3−/− mice were examined by flow cytometry. First, we compared overall CD8 T cell activation between +/+ and C3−/− mice by quantitating the number of activated (CD44high) CD8 T cells in the spleen. As shown in Fig. 1A, the relative proportion of activated (CD44high) CD8 T cells in spleen of C3−/− mice was reduced by ~50%, as compared with +/+ mice. The differences were more striking when total number of activated (CD44low) CD8 T cells was compared between +/+ and C3−/− mice. Fig. 1B shows that the number of activated but not naive (CD44low) CD8
T cells was substantially lower (3-fold; \( p < 0.0001 \)) in spleens of C3\(^{-/-} \) mice, compared with +/+ mice. These data suggested that optimal activation of CD8 T cells induced by LM infection is dependent upon C3.

Next, we examined the effect of C3 deficiency on activation of Ag-specific CD8 T cells following rLM/GP33 infection. Note that the LCMV CTL epitope GP33-41 present in rLM/GP33 is presented by both D\(^{b} \) and K\(^{b} \) MHC I molecules. On day 7 PI, using MHC I tetramers, we enumerated the number of D\(^{b}/\)GP33- and K\(^{b}/\)GP34-specific CD8 T cells in spleen of +/+ and C3\(^{-/-} \) mice. As illustrated in Fig. 2A, the frequencies of both D\(^{b}/\)GP33- and K\(^{b}/\)GP34-specific CD8 T cells were lower in C3\(^{-/-} \) mice compared with +/+ mice. Fig. 2B shows that the total numbers of CD8 T cells that are specific to these two CTL epitopes were significantly lower (\( p = 0.001 \)) in spleens of C3\(^{-/-} \) mice than in +/+ mice.

We also evaluated the effect of C3 deficiency on the cytokine-producing ability of Ag-specific CD8 T cells ex vivo by intracellular staining for IFN-\( \gamma \). Dot plots of representative mice from each group (Fig. 2C) show Ag-triggered IFN-\( \gamma \) production by GP33- and GP34-specific CD8 T cells. Note that stimulation with the GP33 peptide induces IFN-\( \gamma \) production by both D\(^{b}/\)GP33- and K\(^{b}/\)GP34-specific CD8 T cells. In contrast, stimulation with GP33 peptide only stimulates cytokine production in K\(^{b}/\)GP34-specific CD8 T cells. FACS dot plots in Fig. 2C show that D\(^{b}/\)GP33- and K\(^{b}/\)GP34-specific CD8 T cells from both +/+ and C3\(^{-/-} \) mice produced readily detectable levels of intracellular IFN-\( \gamma \). Fig. 2C also shows that the mean fluorescence intensities (MFI) for IFN-\( \gamma \) staining in C3\(^{-/-} \) CD8 T cells were lower than +/+ CD8 T cells, which suggested that C3 deficiency also affects the quantity of IFN-\( \gamma \) produced by Ag-specific
CD8 T cell responses (Fig. 2), optimal activation and expansion of NP396-specific CD8 T cells also requires C3 activity (Fig. 3). These data suggest that C3 promotes CD8 T cell responses to MHC I-restricted epitopes regardless of whether they are expressed in processed or unprocessed form in APC. Even though CD8 T cell activation was significantly reduced in C3−/− mice, both rLM/GP33 and rLM/NP were cleared from livers in both groups of mice (data not shown) within 7 days PI, which indicated that LM control can occur independently of C3 activity.

**Activation and expansion of CD4 T cells during an LM infection is compromised in C3-deficient mice**

LM infection of C57BL/6 mice is known to elicit strong CD8 and CD4 T cell responses (37). In this study, we examined the requirement for C3 in CD4 T cell expansion during infection of mice with rLM/GP33 or rLM/NP. First, on day 7 after infection with γLM/GP33, we assessed CD4 T cell activation by quantitating the number of activated (CD44high) CD4 T cells in spleens of +/+ and C3−/− mice. The percentages of activated CD4 T cells in spleens of C3−/− mice were lower than in +/+ mice (Fig. 4A). Remarkably, the total number of activated CD4 T cells in C3−/− mice was ~5-fold lower than in +/+ mice (Fig. 4B); C3 deficiency did not affect the number of naive (CD44low) CD4 T cells. Similar differences in CD4 T cell activation were observed when +/+ and C3−/− mice were infected with rLM/NP (data not shown). We also quantitated CD4 T cells that are specific to the MHC II-restricted epitope LLO190-201 present in listeriolysin O (LLO) of LM. Fig. 4C shows the total number of LLO190-201-specific CD4 T cells on day 7 after infection with rLM/GP33 or rLM/NP. These data show that expansion of LLO190-201-specific CD4 T cells was significantly lower (p < 0.001) in C3−/− mice than in +/+ mice. Collectively, data presented in Figs. 1–4 strongly suggested that full activation and expansion of CD8 and CD4 T cells during an LM infection requires C3 activity.

**Requirement of C5aR signaling for CD8 T cell activation in LM infection**

In the complement activation cascade, C3 cleavage leads to the generation of C5 convertase, which in turn cleaves C5 into C5a and C5b (38, 39). Among the two cleavage products of C5, C5a is an anaphylatoxin known to exert potent proinflammatory effects by binding to its receptor on leukocytes and also acts as a chemoattractant for DC (38, 40). Receptors for C5a are known to be expressed on T cells (41, 42), and it has been reported that C5aR signaling might be required for optimal CD8 T cell expansion in influenza virus-infected mice (12). However, it is yet to be determined whether C3-dependent augmentation of T cell expansion in a systemic infection like LM also requires C5aR signaling. Therefore, we next examined whether C5a/C5aR interactions are important for activation of CD8 T cell responses in LM infection. The experimental approach was to block C5a/C5aR interactions in vivo by treating LM-infected mice with a C5aRa, which binds specifically to cell surface C5aR. Treatment of mice with C5aRa has been shown to effectively inhibit C5a-dependent activities in vivo (38, 40). Receptors for C5a are known to be expressed on T cells (41, 42), and it has been reported that C5aR signaling might be required for optimal CD8 T cell expansion in influenza virus-infected mice (12). However, it is yet to be determined whether C3-dependent augmentation of T cell expansion in a systemic infection like LM also requires C5aR signaling. Therefore, we next examined whether C5a/C5aR interactions are important for activation of CD8 T cell responses in LM infection. The experimental approach was to block C5a/C5aR interactions in vivo by treating LM-infected mice with a C5aRa, which binds specifically to cell surface C5aR. Treatment of mice with C5aRa has been shown to effectively inhibit C5a-dependent activities in vivo (12, 34). Cohorts of rLM/GP33-infected C57BL/6 mice were treated with C5aRa or vehicle PBS during expansion phase of the T cell response to LM. On day 7 PI, the activation and expansion of CD8 T cells were analyzed by flow cytometry as described above. As shown in Fig. 5, the number of activated CD44high CD8 T cells and GP33-specific CD8 T cells in the spleen of C5aR-treated mice was comparable to those in control PBS-treated mice. Similar to CD8 T cell responses, C5aRa treatment did not significantly affect the activation of CD4 T cells during an LM infection (Fig. 6). Taken together, data in Figs. 5 and 6 indicated that C5aR...
FIGURE 4. Activation of CD4 T cells is compromised in C3-deficient mice. +/+ and C3−/− mice were infected with rLM/GP33 or rLM/NP, and on day 7 PI, activation of CD4 T cells was assessed in the spleen. A and B, Activation of CD4 T cells. Splenocytes were stained with anti-CD4 and anti-CD44, and the number of activated (CD44high) and naive (CD44low) CD4 T cells was determined by flow cytometry. Dot plots in A are gated on total viable splenocytes, and the numbers are percentages of cells in the respective quadrant of total splenocytes. Data in B are from three to five mice per group ± SD. C, Activation of Ag-specific CD4 T cells. Splenocytes from rLM/GP33- or rLM/NP-infected mice were stimulated ex vivo with the MHC II-restricted peptide LLO190–201, and the number of IFN-γ-producing CD4 T cells was determined by intracellular staining. Each symbol in C represents data from individual mice.

Treatment did not significantly affect either the CD8 or CD4 responses to LM infection in mice.

CD8 and CD4 T cell responses to LM in C5aR-deficient mice

Although less likely, the inability of C5aRa treatment to inhibit T cell responses (Figs. 5 and 6) might be attributed to the ineffectiveness of blocking the binding of C5a to C5aR. To address this issue, we investigated the role of C5a in promoting T cell expansion by infecting +/+ and C5aR-deficient mice (C5aR−/−) with rLM/GP33. On day 7 PI, we assessed activation of CD8 T cells that are specific to the LCMV epitope GP33 or LM epitope LLO296 in the spleen of +/+ and C5aR−/− mice using intracellular cytokine staining. The expansion of GP33- and LLO296-specific CD8 T cells in the spleen of C5aR−/− mice was comparable to those in +/+ mice (Fig. 7A). Analyses using MHC I tetramers provided similar results for the D b/GP33 epitope of LCMV (data not shown). Data in Fig. 7B show that C5aR deficiency had a minimal impact on the activation and expansion of LLO190-specific CD4 T cells. Thus, studies in C5aR−/− mice provided convincing evidence that C5a/C5aR interactions are dispensable for primary activation and expansion of CD8 and CD4 T cells following a systemic infection with LM.

LM-induced maturation of C3-deficient DC in vitro and in vivo

After Ag uptake, under the influence of innate inflammatory signals, immature DC mature into potent APC and present Ag to naive T cells in the secondary lymphoid organs. There is evidence that C3 promotes T cell responses by enhancing the Ag-presenting function of professional APC such as DC and macrophages (40, 43–48). Therefore, we hypothesized that reduced T cell responses to LM in C3−/− mice could be due to impairment in maturation of DC and/or effective Ag presentation to naive T cells. To examine the role of C3 in LM infection-induced maturation of DC, bone marrow-derived DC from +/+ or C3−/− mice were infected with rLM/GP33, and up-regulation of CD80, CD86, and MHC II molecules was assessed by flow cytometry. These studies showed that infected DC from +/+ mice expressed high levels of all three surface molecules, as compared with uninfected controls (data not shown). Importantly, LM-induced up-regulation of CD80, CD86, and MHC II in C3-deficient DC was comparable to +/+ DC (data not shown), which indicated that C3 is not required for LM infection-driven maturation of DC, in vitro.

Next, we investigated the role of C3 in priming of naive CD8 T cells by DC in vitro. CFSE-labeled naive TCR transgenic CD8 P14 T cells were cultured with uninfected, rLM/GP33-infected, or GP33 peptide-coated DC, and Ag-driven proliferation of P14 cells was assessed by flow cytometry. These analyses showed that 40–46% of P14 CD8 T cells divided upon exposure to rLM/GP33 infected DC from +/+ or C3−/− mice. Similarly, 50–53% of P14 CD8 T cells divided when stimulated with +/+ or C3-deficient GP33-coated DC (data not shown). Additionally, only proliferating P14 CD8 T cells present in cultures of LM-infected +/+ or C3−/− DC up-regulated CD44 expression; as expected, all P14 CD8 T cells cultured with uninfected DC maintained their CD44low (naive) phenotype (data not shown).
not shown). These results suggested that the C3 deficiency did not affect the ability of DC to process and/or present Ags to naive CD8 T cells in vitro.

Infection with LM has been shown to induce maturation of DC (49). Therefore, we assessed whether C3 deficiency affected DC maturation induced by LM infection in \( \text{C3}^{+/+} \) and \( \text{C3}^{-/-} \) mice in vivo. Plasmacytoid and conventional DC isolated from spleen of LM-infected mice at days 3 and 4 PI were assessed for maturation as above directly ex vivo. The expression levels for MHC I, MHC II, CD40, CD80, and CD86 on plasmacytoid or conventional DC from naive/uninfected \( \text{C3}^{-/-} \) mice were largely similar to those in spleen of \( \text{C3}^{+/+} \) naive/uninfected mice (Fig. 8). LM infection triggered up-regulation in the expression of these molecules, especially on conventional DC, from both \( \text{C3}^{+/+} \) and \( \text{C3}^{-/-} \) mice at days 3 (data not shown) and 4 PI (Fig. 8). Notably, the levels of MHC I, CD40, CD80, and CD86 on plasmacytoid or conventional DC from naive/uninfected \( \text{C3}^{-/-} \) mice were lower but not significantly different, as compared with DC isolated from \( \text{C3}^{+/+} \) mice. These data suggested that C3 deficiency did not significantly affect the maturation of DC in vivo, following an acute LM infection.

**Activation and expansion of wild-type monoclonal TCR transgenic CD8 T cells in C3-deficient mice**

Next, we tested whether C3-deficient APC are able to activate and expand wild-type Ag-specific CD8 T cells in vivo. We adoptively transferred \( 10^7 \) purified wild-type naive/Ly5.1 GP33-specific TCR transgenic P14 CD8 T cells into congenic \( +/+ \) and \( \text{C3}^{-/-} \) Ly5.2 mice, which were subsequently infected with rLM/GP33. Seven days after rLM/GP33 infection, the activation and expansion of donor P14/Ly5.1 CD8 T cells in spleens of \( +/+ \)/Ly5.2 and \( \text{C3}^{-/-} \)/Ly5.2 recipient mice were assessed by flow cytometry. As
shown in Fig. 9, A and B, the frequencies and total number of P14/Ly5.1 CD8 T cells in spleens of C3−/− mice tended to be lower but not significantly different compared with those in +/+ mice. Thus, deficiency of C3 in non-T cells did not significantly compromise the activation and expansion of wild-type CD8 T cells following infection with rLM/GP33. These data also suggested that C3−/− APC are capable of activating wild-type CD8 T cells in vivo.

Characterization of T cells, B cells, and DC in spleen of naive uninfected C3−/− mice

It is possible that a defect in the peripheral T cell compartment of C3−/− mice might underlie ineffective activation and expansion of T cells during an immune response. To address this issue, we compared the number of T cells, B cells, and DC in spleens of uninfected +/+ and C3−/− mice. As shown in Fig. 10A, the numbers of naive (CD44low) or activated (CD44high) phenotype CD8 and CD4 T cells in spleen of C3−/− mice were similar to those in wild-type mice. Additionally, the numbers of B cells and Foxp3+ regulatory CD4 T cells were unaffected by C3 deficiency. Moreover, C3 deficiency did not cause detectable alterations of DC subsets in spleen (Fig. 10B). Taken together, these data suggested that C3 deficiency did not affect the number of T cells, B cells, or DC in the periphery. Therefore, lower CD8 and CD4 T cell responses to LM cannot be linked to a deficiency in the number of T cells or DCs in C3−/− mice, before infection.

Activation and expansion of C3−/− CD8 T cells in a C3-sufficient lymphoid environment of wild-type mice

Although the numbers of naive and activated phenotype T cells in C3−/− mice were similar to those in +/+ mice, it is possible that T cells that have matured in a C3-deficient environment might have intrinsic defects in activation and expansion following antigenic stimulation. In this study, we tested whether CD8 T cells from C3−/− mice respond to antigenic stimulation in a C3-sufficient environment. We adoptively transferred purified CD8 T cells from C3−/− (Ly5.2) or +/+ (Ly5.2) mice into congenic +/+ Ly5.1 mice, which were subsequently infected with rLM/GP33. At day 7 after infection, we quantitated the activation and expansion of donor Ly5.2+ and recipient (Ly5.1+) GP33-specific CD8 T cells in the spleens of LM-infected Ly5.1 mice. LM infection triggered strong activation of GP33-specific CD8 T cells in Ly5.1 recipient mice. The numbers of endogenous Ly5.1+ GP33-specific CD8 T cells in spleens of mice that were recipients of C3−/− CD8 T cells were similar to those in spleens of mice that were recipients of +/+ CD8 T cells (Fig. 11). Importantly, the activation and expansion of donor Ly5.2+ C3−/− GP33-specific CD8 T cells was comparable to those of Ly5.2+ +/+ GP33-specific CD8 T cells in adoptively transferred recipients. These data illustrated that CD8 T cells from C3−/− mice are not intrinsically defective but are fully capable of Ag-driven activation and expansion in a C3-sufficient environment.
It was of interest to examine whether C3 regulates T cell proliferation directly, independent of its effects on APC. To explore this possibility, we asked whether C3 is required for CD8 T cells to undergo proliferative expansion in vitro upon stimulation via the TCR, independent of the contribution of C3 derived from APC. As illustrated in Fig. 12, the proliferation of purified C3-deficient CD8 T cells induced by plastic-immobilized anti-CD3 was significantly lower, as compared with +/+ CD8 T cells. These findings show that C3 promotes proliferation of CD8 T cells induced by signaling via the TCR. Taken together, these data suggested that suboptimal CD8 T cell responses to LM in C3−/− mice might be linked at least in part to lack of C3 effects on T cells.

Discussion

In response to infections, complement system can be activated by three different pathways, namely classical, alternative, and lectin. Regardless of the pathway involved, activation of C3 is a necessary event in the stepwise progression of the cascade of enzymatic reactions that results in the generation of biologically active molecules, which exert distinct effects during the immune response (8, 39, 50). In addition to the well-studied role in B cell activation,
several studies have shown that complement does regulate T cell responses (10, 11, 51). Although complement activation has been shown to occur in mice infected with LM (26), the role of complement components in the elicitation of T cell responses is unknown. In this study, we show that complement component C3 is essential for optimal activation and expansion of Ag-specific CD8 and CD4 T cells during infection of mice with LM. Further studies to understand the mechanisms demonstrate that reduced T cell responses to LM in C3−/− mice is not linked to defective maturation of DC or a deficiency for C5a in vivo. By performing adoptive transfer of wild-type CD8 T cells into C3−/− mice or C3−/+ CD8 T cells into wild-type mice, we show that autocrine or paracrine sources of C3 might be sufficient to drive clonal expansion of CD8 T cells in vivo. Finally, we report that C3 augments AgR-triggered proliferation of purified CD8 T cells in vitro, which would suggest that lack of direct effects of C3 on T cells might blunt T cell responses of C3−/− mice to LM infection.

In this study, complete absence of C3 resulted in a marked reduction in the expansion of Ag-specific CD4 and CD8 T cell responses to LM in mice. Similarly, optimal expansion of virus-specific CD8 T cells during infections with influenza virus and LCMV requires C3 activity (10, 11). Additionally, T cell-dependent acute rejection of renal grafts is promoted by C3 activity (14). The mechanism(s) that are involved in promoting T cell expansion by C3 is not completely understood. It has been reported that CD8 T cell responses to influenza virus in mice is significantly reduced by deficiency of either C3 or C5a, which suggested that C3 might promote T cell responses via C5a (12). In contrast to studies with influenza infection, our studies clearly showed that both CD4 and CD8 T cell responses to LM were normal in the apparent absence of C5aR signaling. These findings indicate that requirement for C5a/C5aR interactions might be dictated by the nature of the infecting organism and the associated pathogenesis. Since C5aR has been found to be expressed locally in tissues such as lung and liver, C5a/C5aR signaling might be more critical for tissue-specific host defense in the peripheral tissues such as lung during an influenza virus infection but not in a systemic LM infection (29, 52, 53).

It has been reported that complement might augment vaccine-induced CD8 T cell immunity to leishmaniasis via natural Abs and IL-4 (13), and complement activation products iC3b/C3dg can bind to Ag/natural Ab complexes and promote Ag uptake by APC (54, 55). Since absence of B cells did not appear to affect CD8 T cell responses to LM in mice, it is unlikely that C3 promotes T cell responses to LM by Ab-dependent mechanisms (56).

It is known that innate immune mechanisms, especially those mediated by neutrophil- and macrophage-mediated phagocytosis, are important in control of LM infection (19, 57). Complement receptor 3 has been implicated in opsonization, phagocytosis, and killing of C3b-bound LM by lystericial macrophages. Therefore, in C3−/− mice, lack of C3b-dependent uptake of LM by phagocytes might impede efficient Ag processing and presentation to T cells, resulting in suboptimal expansion of T cells. This hypothesis is supported by reports which show that: 1) efficiency of Ag processing and presentation by professional APC to T cells is greatly enhanced by tagging Ags to C3 fragments (58, 59); 2) complement factors can directly interact with T cells and modulate the function of Ag-presenting T cells, which are crucial for T cell expansion (10, 11, 46, 60, 61); 3) C3 deposition on APC augments T cell proliferation (62); and 4) C3-deficient macrophages and DC do not effectively stimulate alloreactive T cells in vitro. Our studies clearly show that LM infection-induced maturation of C3-deficient DC was comparable to +/+ DC. Notably, the ability of LM-infected DC from C3−/− mice to activate naive CD8 T cells was similar to those of +/+ DC. Therefore, C3 deficiency does not appear to impair Ag processing and/or presentation of listerial Ags to naive CD8 T cells at least in vitro. Similarly, C3-deficient DC infected with *Mycobacterium bovis* strain bacille Calmette-Guérin did not exhibit detectable defects in activation of naive T cells (data not shown). Moreover, the maturation of DC induced by LM infection appears to be largely normal in C3−/− mice. Importantly, normal activation and expansion of adoptively transferred TCR transgenic CD8 T cells in LM-infected C3−/− mice support our interpretation that APC-derived C3 may not be required for optimal Ag processing and/or presentation in vivo. It is unknown why DC require C3 to optimally stimulate alloreactive T cells but not after LM infection. One possibility is that LM could trigger in infected DC a broad spectrum of pattern recognition receptors whose downstream effects are redundant with complement-mediated effects. Hence, in LM-infected DC, C3 function could become redundant and therefore dispensable during T cell activation.

Apart from their ability to enhance Ag presentation by professional APC, anaphylatoxins C3a and C5a have potent proinflammatory and chemotactic effects. Activated T lymphocytes have been shown to express functional receptors for C3a (C3aR) that are known to influence immune cell trafficking in inflammation (50, 61, 63). Hence, it is possible that deficiency of C3a in C3−/− mice disrupts proper trafficking of DC and T cells in vivo during the T cell response to LM. This in turn could adversely influence the recruitment and activation of naive T cells in the secondary lymphoid organs.

Binding of complement activation products to complement receptors CR1 and CR2 is a strategy by which complement modulates immune responses. CR1/CR2 are predominantly expressed on APC, including B cells, and CR1/CR2 signaling in B cells has been shown to regulate activation threshold, Ag uptake, processing and presentation, isotype switching, and generation of memory B cells (4, 58, 64, 65). In addition to B cells, CR1/CR2 expression on T cells has been reported and believed to mediate stable binding between the APC and T cells via C3 (62, 66). Hence, C3 could regulate T cell responses by interacting directly with T cells or indirectly via APC. In other models of infection, C3-promoted CD8 T cell responses are minimally affected by CR1/CR2 deficiency (10, 11). It remains to be determined whether C3 promotes T cell responses to LM via CR1/CR2.

CD46, membrane cofactor protein is a cell surface receptor that is expressed on all nucleated cells, and ligands for CD46 include complement components C3b and C4b. The effects on T cell expansion induced by binding of C3b or C4b depends on the isoform of CD46. Only when both isoforms of CD46 are coexpressed on a T cell, stimulatory effects appear to dominate over the suppressive effects (67). Therefore, it is possible that in LM-infected mice, C3 deficiency led to abrogation of CD46/C3b interactions and reduced expansion of Ag-specific CD8 T cells. However, an emerging role of CD46-induced regulatory T cells makes it more difficult to separate the effect on T cell activation and regulation (45, 68).

To reiterate, reduced expansion of CD8 and CD4 T cells in LM-infected C3−/− mice could be due to defects in T cells and/or non-T cells. Data presented in this article show that activation and expansion of adoptively transferred wild-type TCR transgenic CD8 T cells were largely intact in LM-infected C3−/− mice, which indicated that C3 deficiency in non-T cells did not significantly affect Ag processing/presentation to wild-type CD8 T cells in vivo. We explored whether impaired T cell responses in C3−/− mice (11, 12) could be a sequel to developmental defects in the peripheral T cell repertoire. However, the peripheral T and B cell compartment, including Foxp3+ regulatory T cells and DC, are unaffected by C3 deficiency. Additionally, C3-deficient CD8 T cells do not appear to be intrinsically defective because they exhibit normal
activation and expansion upon transfer into wild-type mice. These two lines of evidence strongly suggest that lower T cell responses to LM in C3−/− mice are not likely linked to a defective T cell compartment.

How do C3 deficiency blunt T cell responses to LM? First, C3 produced by APC themselves or other cells could augment their Ag-presenting abilities. Second, C3 derived from APC could act on T cells directly to augment their proliferation. Third, T cell-derived C3 (41) could activate themselves and/or APC in an autocrine or paracrine fashion, respectively. Our studies indicated that: 1) CD8 T cell activation and expansion can occur when responding T cells can produce C3 but not the APC; and 2) responding CD8 T cells are not dependent upon autocrine C3 for activation and proliferation. It is likely that regardless of the cellular source, autocrine or paracrine C3 will support full expansion of CD8 T cells in vivo. However, it is unclear whether direct effects of C3 on T cells promote T cell responses in vivo. Our in vitro studies showed that C3 might promote the proliferative responses of purified CD8 T cells to TCR stimulation by a mechanism that is distinct from its effect on professional APC. This finding suggests that lower CD8 T cell responses in LM in C3−/− mice might be linked at least in part to the lack of C3-induced effects on Ag-stimulated T cells. Future studies using C3R-deficient CD8 T cells might be able to resolve this question in vivo.

In summary, in this article, we provide strong evidence that C3 plays a critical role in enhancing T cell responses to an intracellular bacterial infection by promoting proliferative expansion of Ag-triggered CD8 T cells. These findings have implications in rational design of effective vaccines and treatment of T cell-dependent autoimmune disorders.

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Disclosures
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References