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Dendritic Cell Synthesis of C3 Is Required for Full T Cell Activation and Development of a Th1 Phenotype

Qi Peng, Ke Li, Hetal Patel, Steven H. Sacks, and Wuding Zhou

Previous studies have found that deficiency of complement component C3 is associated with reduced T cell responses in several disease models including viral infection, autoimmune disease, and transplantation. However, the underlying mechanism is unclear. In this study, we demonstrate that dendritic cells (DCs) are able to synthesize C3 and this synthesis is required for the capacity of DCs to stimulate alloreactive T cell responses in vitro and in vivo. Compared with C3-producing DCs, C3-nonproducing DCs exhibit reduced potency to stimulate an alloreactive T cell response, favor the polarization of CD4+ T cells toward Th2 phenotype, and have regulatory T cell-driving capacity. In addition, priming mice with C3-deficient DCs compared with wild-type DCs led to delayed skin allograft rejection. Our findings that nonproduction of C3 by DCs significantly reduced T cell stimulation and impaired allograft rejection provide a potentially important explanation of how C3-deficient mice develop reduced T cell responses and how C3-deficient donor kidney is protected from T cell-mediated graft rejection. The Journal of Immunology, 2006, 176: 3330–3341.
alloresponse (25, 26), this raises the possibility that local synthesis of C3 by DCs is needed for T cell activation.

However, to date, it is unclear whether DCs, the most potent APCs, are able to synthesize C3. Given the importance of C3 for both the pathological and physiological regulation of nonspecific and specific immune responses, DC synthesis of C3 could be an important contributory factor in DC activation and their other diverse functions.

In this study, we explored the hypothesis that DCs are able to synthesize C3 and this synthesis can modulate DC functions, therefore contributing to T cell responses. We examined C3 synthesis in murine bone marrow-derived DCs by RT-PCR, ELISA, and immunohistochemical staining. Using a mouse model of T cell alloreactivity, with either C3−/− or C3+/+ mice as the stimulator strain, we studied the effect of DC synthesis of C3 on DC functions in alloreactive T cell responses, including T cell stimulation, T cell polarization, regulatory T cell development, and skin allograft rejection.

Materials and Methods

Mice

Homozygous C3−/− mice were generated by Prof. M. Carroll and colleagues (11) using homologous recombination in embryonic stem cells. These mice were then backcrossed in our laboratory onto the C57BL/6 parental strain for 11 generations. Skin grafts between the backcrossed C3−/− and congenic strains showed long-term graft survival (>100 days). C3+/+ mice (C57BL/6), BALB/c, and B10.Br were purchased from Harlan. Male mice (6–7 wk old) were used in all experiments. All animal procedures were conducted within the Animals Act U.K. (Scientific Procedures, 1986).

DC cultures

DCs were cultured from bone marrow progenitor cells using a modified protocol of a previously described method (27). In brief, bone marrow cells were harvested from three to four mice. Washed bone marrow cells were prepared in DC culture medium (RPMI 1640 medium, 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 20 ng/ml GM-CSF), at a density of 1 × 10^6 cells/ml, then plated in 24-well plates at 1 ml/well. Culture medium was refreshed with fresh medium every 2 days. At day 6, dislodged cells were collected and purified with CD11c MicroBeads (Miltenyi Biotec). Purified cells were cultured for an additional 2 days in addition of LPS (1 μg/ml) to allow the cells to mature, unless otherwise specified.

Preparation of peritoneal macrophages

Peritoneal macrophages were prepared from mice inoculated i.p. with 1 ml of 3% thioglycollate. After 6 days, the peritoneal cell population was harvested and purified with CD11b MicroBeads (Miltenyi Biotec).

Immunohistochemical staining

DCs cultured for 8 days on coverslips were fixed with paraformaldehyde, and then stained for C3 using an indirect method. The Abs used were: goat anti-mouse C3 (ICN Pharmaceuticals) and FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories).

Flow cytometry

For analysis of surface molecule expression, 2 × 10^5 DCs or T cells were stained with FITC or PE-conjugated Ab or the appropriate isotype control Ab, at 4°C for 30 min followed by washing three times in 2 ml of PBS with 1% BSA. The cells were then fixed in 400 μl of 1% paraformaldehyde in PBS. For analysis of foxp3 expression, cells were first stained for the expression of surface molecule (CD4) and, after fixation and permeabilization, were incubated with PE-conjugated Ab based on the manufacturer’s recommendation. The stained cells were analyzed by flow cytometry (FACScan; BD Biosciences). Ab reagents used in flow cytometry (reflecting 0 flow cytometry) follows. FITC-conjugated mouse anti-mouse MHC class I (H-2D^b, CTD; Serotec); PE-conjugated rat anti-mouse MHC class II (I-A^d, E, MS/I14.15.2), PE-conjugated rat anti-mouse CD40 (3/23), PE-conjugated Armenian hamster anti-mouse ICAM-1 (CD54, 3E2), PE-conjugated Armenian hamster anti-mouse CD40 (3/23), PE-conjugated Armenian hamster anti-mouse C3 (ICN Biomedicals) and HRP-conjugated goat anti-mouse C3 (Nordic). The standard used here was pooled normal mouse serum, which has a reported C3 concentration of 0.7 mg/ml (28).

Conventional RT-PCR

Total RNA was extracted from the cell pellets and subsequently used for semiquantitative PCR. PCR was conducted with 2 μl of diluted cDNA (reflecting 0.2 μg of total RNA), 12.5 pmol of each 3rd and 5′ primer pair, either for each testing gene or GAPDH gene (Table I), in 25 μl of reaction buffer (Promega). The PCR cycle consisted of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. Amplified PCR products were visualized after electrophoresis on 1.5 or 2% agarose gel containing ethidium bromide. GAPDH primers, 12.5 pmol each, were added in every reaction as an internal control.

RT-quantitative PCR (RT-qPCR)

Real-time RT-qPCR was performed with an MJ Research PTC-200 Peltier Thermal Cycler and DyNaMo HS SYBR Green qPCR kit (MJ BioWorks). PCR setup was in low-profile microplates containing 10 μl of master mix, 2 μl of diluted cDNA (reflecting 0.2 μg of total RNA), 10 pmol of each 3rd and 5′ primer pair, either for each testing gene or GAPDH gene (Table I), in a 20-μl reaction volume. Amplification was performed according to manufacturer’s cycling protocol and done in triplicate. Gene expression was expressed as 2^(-ΔΔCt) (29), where Ct is cycle threshold, ΔΔCt =

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^a All primers were designed in our laboratory using Oligo 4 software. There are intronic sequences between primers 1 and 2.
sample 1Δ(Ct) − sample 2Δ(Ct); Δ(Ct) = GAPDH (Ct) − testing gene (Ct).

Preparation of T cells

Naive allogeneic T cells were derived from splenocytes of normal BALB/c mice. Usually, splenocytes from four to five mice were used for the T cell preparation. CD3⁺, CD4⁺, and CD8⁺ T cells were prepared from splenocytes using the Spin-Sep Enrichment Cocktail kit (StemCell Technologies). Following the preparation, the purity of the T cell preparation was routinely >90%, as determined by flow cytometry.

Analysis of allogeneic T cell response in vitro

A total of 5 × 10⁵ irradiated (2000 rad) DCs and 2 × 10⁵ purified allogeneic T cells (CD3⁺ or CD4⁺ or CD8⁺) were cocultured in T cell culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin) for up to 11 days, unless otherwise specified. Culture supernatants were collected at various time points and used to perform ELISA. T cell proliferation was assessed at 96 h after coculture by measuring the incorporation of [³H]thymidine.

Mixed lymphocyte reaction

Splenocytes (2 × 10⁵) derived from immunized BALB/c mice or B10BrH-2k mice, at day 0, were given 5 × 10⁵ irradiated (2000 rad) donor DCs (C57BL/6H-2b, either C3⁻/⁻ or C3⁺⁺). Fourteen days after administration, mice were killed. The spleen was used for the assessment of T cell response ex vivo by MLR.

Analysis of immune response in vivo

BALB/cH-2d or B10BrH-2k mice, at day 0, were given 5 × 10⁵ irradiated (2000 rad) donor DCs, CD3⁺, CD4⁺, or CD8⁺, respectively. Mice were killed 11 days after administration, and the lymph nodes were harvested for assessing immune response ex vivo by MLR.

Skin grafting

Tail skin from donor animals was grafted onto the left flank of recipients under isoflurane anesthesia. The graft site was covered with paraffin-emulsified gauze, a dressing of dry gauze and secured with Transpore 3M adhesive tape. The dressing was removed on day 5, and the grafts were evaluated for signs of rejection on a daily basis for 11 days, unless otherwise specified. The rejection was determined as the day on which >80% of the graft area was necrotic. The groups were assessed in a blinded fashion.

C3 gene silencing

C3⁻/⁻ DCs (5 × 10⁵) were transfected with a range of C3 small interfering RNA (siRNA) concentrations (sense sequence: 5'-GGAAUUCUACUCUA GAUAAAGt-3', antisense sequence: 5'-CUAUAGGUGAUUAGAUU Cct-3') and silencer negative control siRNAs (Ambion) using siRNA transfection kit (Qiagen). Forty-eight hours after transfection, culture supernatants were analyzed for C3 by ELISA. DCs were irradiated (2000 rad) and used for T cell stimulation. DCs (5 × 10⁴) were cocultured with naive allogeneic CD4⁺ T cells (2 × 10⁵) for 3 days. Culture supernatants were then analyzed for IFN-γ using ELISA.

Statistical analysis

ELISA or T cell proliferation assays were performed in three to six replicates wells of the cocultures. Results were expressed as a mean ± SEM and subjected to statistical analysis. Student’s t test or two-way ANOVA or one-way ANOVA was used where appropriate to determine significant differences between samples. The graft survival data were analyzed using the Mantel-Haenszel test. All experiments were repeated at least three times.
Results

Synthesis of C3 by DCs

Bone marrow-derived cells from WT mice were cultured in DC culture medium for 6 days. Purified DCs were cultured for an additional 2 days in the presence of LPS. After culturing for 8 days, the majority of cells expressed high levels of MHC class I, MHC class II, and costimulatory molecules CD40, ICAM-1, B7.1, and B7.2. Staining of all DC preparations was negative for the macrophage marker F4/80, suggesting that contamination with macrophages was minimal (data not shown).

To investigate whether these WT DCs have the ability to synthesize C3, we performed RT-PCR, ELISA, and immunochemical staining. Using RT-PCR, we detected mRNA transcript for C3 (282 bp), which had identical electrophoretic mobility to that from macrophages (positive control), but which was not detected in DCs derived from C3−/− mice (Fig. 1A).

To detect C3 protein secreted by DCs, we cultured DCs from WT mice and purified them at day 6 with CD11c beads. A total of 2 × 10^5 purified cells were then further cultured for 3 days in 0.2 ml of DC culture medium, in the presence of LPS. Culture supernatants were analyzed for C3 using ELISA. We also collected supernatants from peritoneal macrophages (2 × 10^5) cultured for 3 days for the positive control and supernatants from C3−/− DC cultures for the negative control. As shown in Fig. 1B, C3 was detected in the supernatants of WT DC and macrophage cultures (at 192 ± 1.8 and 113 ± 9.6 ng/ml, respectively), but not detected in the supernatants of C3−/− DC cultures. To examine the effect of LPS on DC synthesis of C3, we cultured DCs from WT mice and purified them at day 6 with CD11c beads. A total of 2 × 10^5 purified cells were then further cultured for up to 7 days, either in the presence or absence of LPS. Culture supernatants were analyzed for C3 using ELISA. We found that DCs were able to produce C3 in the absence of LPS; however, the production was significantly increased in the presence of LPS (Fig. 1C).

We also performed cellular immunochemical staining for C3 on WT DCs cultured for 8 days. DCs were uniformly stained with an anti-mouse C3 Ab, but staining was negative on DCs derived from C3−/− mice (Fig. 1D). Thus, cultured BM DCs have the ability to synthesize and secrete C3, and this synthesis is up-regulated by LPS.

C3−/− DCs have reduced potency to stimulate alloreactive CD4+ T cells in vitro

We next tested the hypothesis that local synthesis of C3 by DCs influences the potency of DCs to stimulate alloreactive T cells. For this purpose, we used responder T cells, including CD4+, CD3+, and CD8+ T cells, prepared from the spleens of naive BALB/cH-2d mice, and stimulator DCs prepared from allogeneic C57BL/6H-12b
mice (either C3\(^{+/+}\) or C3\(^{-/-}\)). Purified CD4 T cells were also prepared from C57BL/6 H-2b as syngeneic controls. The T cells were cocultured with irradiated DCs for up to 7 days. T cell activation was then assessed by measuring the level of IFN-\(\gamma\) and IL-2 in the culture supernatant using ELISA. We found that the production of IFN-\(\gamma\) and IL-2 was significantly lower in allogeneic CD4\(^{+}\) T cells stimulated by C3\(^{-/-}\) DCs compared with those stimulated by C3\(^{+/+}\) DCs. The effect of the C3 status of DCs on the T cell response was more pronounced in CD4\(^{+}\) T cells and undetectable in CD8\(^{+}\) T cells (Fig. 2). Thus, deficient production of C3 by DCs rendered them less potent to stimulate allogeneic CD4 T cells but not CD8 T cells. These data suggest that the effect of C3 was primarily on the MHC class II-dependent pathway of donor Ag presentation. Syngeneic controls exhibited only a very low level of cytokine production (data not shown), showing the observed T cell response was specific for donor Ag.

**Reduced capacity of C3\(^{-/-}\) DCs for immune stimulation in vivo**

Next, we determined whether C3\(^{-/-}\) DCs have a reduced ability to elicit an allogeneic T cell response in vivo. At day 0, BALB/c\(^{H-2d}\) or B10Br\(^{H-2k}\) mice were given 5 \times 10^5 donor DCs (either C3\(^{+/+}\) or C3\(^{-/-}\)) by i.p. injection. On day 14, mice were killed and their splenocytes were used for analysis of the allogeneic T cell response in vivo using MLR, where splenocytes derived from the above DC administered BALB/c or B10Br mice were used as the responders, and in which allogeneic splenocytes from C57BL/6 mice were used as stimulators. T cell activation was assessed by measuring IFN-\(\gamma\) and IL-2 in the culture supernatant using ELISA. The T cell response in mice (both BALB/c and B10Br) that had received C3\(^{-/-}\) DCs was impaired, with significant lowering of IFN-\(\gamma\) and IL-2 production, compared with mice that had received C3\(^{+/+}\) DCs (Fig. 3). Thus, administration of C3\(^{-/-}\) DCs resulted in defective T cell activation. Both BALB/c and B10Br mice received C3\(^{-/-}\) DCs developed weaker T cell activation, suggesting that the effect of complement was allo- but not strain-dependent.

**C3\(^{-/-}\) DCs favor the polarization of CD4\(^{+}\) T cells toward Th2 phenotype**

Because DCs are not only able to initiate activation of naive T cells, but also play an important role in determining the direction of the immune response, we asked whether DC synthesis of C3 could also have an effect on its T cell-polarizing capacity. To test the hypothesis, we cocultured irradiated C3\(^{+/+}\) or C3\(^{-/-}\) DCs with purified naive allogeneic CD4\(^{+}\) T cells for up to 11 days. Then, we measured the production of IFN-\(\gamma\) and IL-4 in the culture supernatants using ELISA. The production of IFN-\(\gamma\) (Th1 cytokine) in T cells stimulated by C3\(^{-/-}\) DCs was consistently lower than that stimulated by C3\(^{+/+}\) DCs (from days 5 to 11 of coculture) (Fig. 4A). In contrast, the production of IL-4 (Th2 cytokine) in T cells stimulated by C3\(^{-/-}\) DCs was higher than that stimulated by C3\(^{+/+}\) DCs, at 7–11 days of coculture (Fig. 4B). Thus, C3\(^{+/+}\) DCs and C3\(^{-/-}\) DCs exhibited different T cell-polarizing capacity, where C3\(^{+/+}\) DCs favor the polarization of CD4\(^{+}\) T cells toward a Th1 phenotype, and C3\(^{-/-}\) DCs favor the polarization of CD4\(^{+}\) T cells toward a Th2 phenotype.

A unique function for DCs is to express a selective set of T cell-polarizing molecules that determine the balance between Th1 and Th2 development. Therefore, we sought to determine whether there is differential expression of Th1-polarizing cytokine (IL-12) in C3\(^{+/+}\) and C3\(^{-/-}\) DCs. We cultured DCs from WT or C3\(^{-/-}\) mice, and after purification at day 6 with CD11c beads, cultured them for an additional 2 days in the presence of LPS. Culture supernatants were analyzed for IL-12 using ELISA. We found that the production of IL-12 was significantly lower in C3\(^{-/-}\) DC cultures compared with C3\(^{+/+}\) DC cultures (Fig. 4C).

**C3\(^{-/-}\) DCs have regulatory T cell driving capacity**

As IL-10 has been implicated as a (co-)factor responsible for the induction of regulatory T cell development (31, 32), we studied...
whether the C3 status of DCs would have an effect on IL-10 production by CD4+ T cells. We cocultured irradiated C3+/+ or C3−/− DCs with purified naive allogeneic CD4+ T cells (2 × 10⁵) from BALB/c mice for up to 11 days. Culture supernatants were analyzed for IL-10 using ELISA. We found that the production of IL-10 was significantly higher in T cells stimulated by C3−/− DCs than that stimulated by C3+/+ DCs, at 7–11 days of coculture (Fig. 5A).

Increasing evidence indicates that DCs are able to expand Ag-specific CD4+CD25+ regulatory T cells (32, 33). Therefore, we asked whether C3+/+ and C3−/− DCs have different abilities to elicit the development of regulatory T cells. Regulatory T cells specifically express forkhead transcription factor gene (foxp3), which is thought to be a marker for CD4+CD25+ regulatory T cells (34). We cocultured CD4+ T cells with allogeneic C3+/+ or C3−/− DCs for 9 days and then performed conventional RT-PCR and RT-qPCR for the detection of foxp3 gene expression in stimulated T cells. We detected mRNA transcript for foxp3 (118 bp) in both groups of stimulated T cells (Fig. 5B). However, the level was increased ~4-fold in T cells stimulated by C3−/− DCs compared with those stimulated by C3+/+ DCs (Fig. 5C). In another set of experiments, we cocultured CD4+ T cells with allogeneic C3+/+ or C3−/− DCs for 9 and 18 days, then examined intracellular foxp3 protein expression in these CD4+ T cells using flow cytometry. We found the percentage of foxp3+ T cells was about 2-fold higher in CD4+ T cells stimulated by C3−/− DCs than in that stimulated by C3+/+ DCs (Fig. 5D). Thus, CD4+ T cells primed by C3−/− DCs in vitro showed elevation of IL-10 production and foxp3 expression, suggesting that the generation of regulatory T cells was dependent on C3.

Suppression of the alloreactive T cell response by C3−/− DC-stimulated CD4+ T cells

To further investigate whether C3−/− DC-stimulated CD4+ T cells could have regulatory T cell function, thus modulating the alloreactive T cell response, we performed a T cell suppression assay. Enriched CD4+CD25+ T cells were prepared in vitro by coculturing CD4+ T cells with allogeneic DCs (either C3+/+ or C3−/−) for 18 days as described in Materials and Methods. The T cell suppression assay was setup by adding enriched CD4+CD25+ T cells to a coculture of CD4+ T cells (H-2b) and irradiated C3+/+ DCs (H-2b), at a ratio of 1:1:4 for enriched CD4+CD25+ T cells, DCs, and CD4+ T cells. Inhibition of the T cell response was assessed at 4 days after coculture by cytokine measurement and incorporation of [3H]thymidine. Results showed that enriched CD4+CD25+ T cell preparation generated by C3−/− DC stimulation significantly inhibited T cell activation and T cell proliferation, but this effect was less pronounced when using enriched CD4+CD25+ T cells generated by C3+/+ DC stimulation (Fig. 6).

C3−/− DCs elicited delayed skin allograft rejection

As demonstrated above, C3−/− DCs have an impaired potency to stimulate an alloreactive T cell response, favor the polarization of CD4+ T cell toward Th2 phenotype and have regulatory T cell-driving capacity. This suggests that C3−/− DCs can downregulate the alloimmune response. Next, we investigated whether the immunization of recipient mice with donor C3−/− DCs could delay allograft rejection in a murine skin transplant model, compared with the use of WT DCs. Bone marrow-derived immature DCs were prepared from either C3+/+ or C3−/− mice (H-2b) and irradiated. B10Br mice (H-2b) (n = 15/group), at day 0, were given either C3+/+ or C3−/− DCs (H-2b) by t.p. injection; 14 days after the administration of DCs the mice received a C57BL/6 (H-2b) skin graft (derived from C3−/− mice). Skin grafts (n = 11/group) were monitored day 5 onwards after skin transplantation. T cell responses in the remaining B10Br mice (n = 4/group) were measured ex vivo by MLR at day 7 after skin grafting. Our results showed that mice immunized with C3−/− DCs had significantly delayed graft rejection and had lowered T cell responses ex vivo (with reduced [3H]thymidine uptake and lower level of IFN-γ and higher level of IL-10 production) compared with mice that had received C3+/+ DCs (Fig. 7). Although the difference in graft survival between mice administered with C3+/+ and C3−/− DCs is relatively small, we used a fully MHC-mismatched strain combination in which the window for skin graft rejection is small, usually within 2 days (35). Given this stringency, prolongation of skin graft survival even by 2 or 3 days is significant and can reflect a reduced T cell response in the host. In support, we demonstrated a clear difference in alloreactive T cell responses between mice primed with C3+/+ and C3−/− DCs. Taking together the skin graft survival studies and T cell response data, our results indicate that defective T cell priming with C3−/− DCs can result in delayed allograft rejection.
C3 gene-silenced DCs have lowered ability to stimulate alloreactive T cells

To verify the importance of C3 on the T cell stimulatory ability of DCs, we performed inhibitory studies using WT DC. We inhibited C3 gene expression in \textit{C3}^{-/-}/H11001/H11001 DCs using siRNA, and measured the ability of these DCs to stimulate alloreactive T cells compared with control-treated DCs. As shown in Fig. 8, treatment with \textit{C3} siRNA, at the concentrations of 50 or 100 nM, significantly reduced the amount of C3 secreted, and co-coordinately lowered the level of T cell activation elicited by these DCs. However, treatment with irrelevant siRNA at the concentration of 100 nM did not significantly reduce the amount of C3 produced or lower the level of T cell activation. These data provide evidence that endogenous production of C3 by DCs is required for DCs to function as potent APCs. Our data also suggest that the reduced potency of \textit{C3}^{-/-}/H11002/H11002 DCs to stimulate alloreactive T cells is not due to an artifact of the homologous recombination procedure.

\textbf{C3}^{-/-}/H11002 DCs have no intrinsic gene defect for MHC class II and costimulatory molecules

There is a possibility that the recombinant DNA procedure used to generate the \textit{C3}^{-/-}/H11002 mice interfered with nontargeted genes that are
critical for immune stimulation. To investigate this prospect, we examined the gene expression of MHC class II, CD40, ICAM-1, and B7.2. DCs were cultured for 7 days without LPS stimulation or for 8 days where LPS was present on the final 2 days of culture.

Using conventional RT-PCR and RT-qPCR, we found that all of these genes were transcribed in both C3<sup>+/+</sup> and C3<sup>-/-</sup> DCs (Fig. 9A). The level of gene expression in C3<sup>-/-</sup> DCs showed no defect when compared with that of C3<sup>+/+</sup> DCs in both 7- and 8-day cultures. In fact, the level of gene expression of MHC class II, CD40, and ICAM-1 was slightly elevated in C3<sup>-/-</sup> DCs of 8-day cultures (Fig. 9, B and C). This data is consistent with our observations in C3<sup>-/-</sup> macrophages (data not shown). The reason for this elevation is not clear, but perhaps reflects a compensatory response to defective Ag presentation.

Reduced cell surface expression of MHC class II and B7.2 in C3<sup>-/-</sup> DCs

Next, we examined the protein expression for MHC class II and costimulatory molecules to determine whether there had been altered cell surface expression of these important immune regulators in C3<sup>-/-</sup> DCs. The results of flow cytometric analysis of cell surface MHC class II, CD40, ICAM-1, and B7.2 expression in LPS-stimulated DCs are presented in Fig. 9D. We found that the level of surface expression of MHC class II and B7.2 was consistently lower (21–25% and 21–30%, respectively) in C3<sup>-/-</sup> DCs than in C3<sup>+/+</sup> DCs. Although this reduction is relatively small, this observation was made in three independent experiments, where DCs were prepared from three mice in each case. In addition, we obtained similar results on macrophages and lymphocytes derived from C3<sup>-/-</sup> mice (data not shown). Given that surface expression of MHC molecules is a key determinant for the ability of APCs to stimulate T cells, and B7.2 is a critical factor for the amplification of T cell responses, the reduced surface expression of MHC class II and B7.2 molecules on C3<sup>-/-</sup> DCs could contribute to their weaker effect in allograft rejection.

C3<sup>-/-</sup> DCs have no hyporesponsiveness to LPS stimulation

LPS treatment of BM-derived DCs can increase the proportion of mature cells and therefore enhance the capacity of DCs for T cell
stimulation. As C3−/− DCs have reduced ability to stimulate alloreactive CD4+ T cells, we sought to determine whether C3−/− DCs also exhibit hyporesponsiveness to TLR4 ligand (LPS) stimulation. We assessed the gene expression and functional activity of TLR4 in C3+/+ and C3−/− DCs following LPS stimulation. Using conventional RT-PCR and RT-qPCR, we found that C3−/− DCs have no gene expression defect for TLR4. Surprisingly, the level of TLR4 mRNA in C3−/− DCs was elevated compared with C3+/+ DCs. The production of TNF-α by C3−/− DCs was comparable to that with C3+/+ DCs, in the presence or absence of LPS in the DC culture medium (Fig. 10). Therefore, C3−/− DCs have no hyporesponsiveness to LPS stimulation.

Discussion

Identifying specific stimulatory pathways and molecules that determine the diverse functions of DCs is critical for understanding and manipulating adaptive immunity. Although it is becoming clear that microbial and viral components acting on TLRs lead to DC activation, endogenous and physiologic molecules that could modulate the activation states of DCs remain mostly unidentified. The work presented here shows that murine BM-derived DCs are able to synthesize C3 and this synthesis is required for DCs to develop a fully activated phenotype capable of eliciting a Th1 response.

Our data show that DCs cultured from WT mice are capable of synthesizing a substantial amount of C3 and this synthesis is up-regulated by LPS stimulation. The quantity of C3 released by DCs is comparable to that produced by macrophages, which are considered to be an important extrahepatic source of C3 (19). Our study therefore identifies a novel property of BM-derived DCs, namely the ability to synthesize and secrete C3. In addition, our findings offer insight into the regulation of the T cell response by local production of C3.

To investigate the functional relevance of DC synthesis of C3 in the adaptive T cell response, we used a transplant model, where alloantigen was synthesized and presented by donor DCs to alloreactive T cells. DCs cultured from C3+/+ and C3−/− mice exhibited different properties, in terms of stimulating and regulating specific T cell responses. Compared with C3+/+ DCs, C3−/− DCs displayed reduced surface expression of MHC class II and B7.2 on the cell surface, and showed reduced production of the Th1-polarizing molecule, IL-12, which is a potent inducer of IFN-γ production leading to the development of Th1 responses. Furthermore, C3−/− DCs elicited significantly lower alloreactive T cell responses in vitro and in vivo. In contrast to C3+/+ DCs, C3−/− DCs induced naive CD4+ T cells to produce a higher level of Th2 cytokine (IL-4) and a lower level of Th1 cytokine (IFN-γ). These data suggest that DCs require the expression of C3 to elicit a fully activated Th1 response. In addition, it appears from our measurements of IL-10 and foxp3, that the regulatory T cell driving capacity of DCs was also dependent on C3.

To verify the role of C3 suggested in the studies with C3 knockout DCs, we performed a gene-silencing experiment using WT DCs. The finding of reduced T cell stimulation with C3-inhibited DCs establishes a causal relationship between C3 expression and the T cell stimulatory capacity of DCs. However, in addition to deficient C3 production, the DCs derived from the knockout mice also exhibited reduced cell surface expression of MHC class II and B7.2 molecules, suggesting that the reduced capacity of these cells to elicit a T cell response may have been due to lack of Ag (allo-MHC) and costimulatory molecules, in addition to lack of C3. However, there was no apparent defect of gene expression for MHC class II and B7.2, indicating that the lack of cell surface immunoregulatory molecules in the deficient DCs was not the result of associated gene defects, but may have been secondary to C3 deficiency. Taken together, our data with C3 knockout DCs and C3-inhibited DCs suggest that C3 is the primary defect underlying the weakened capacity of these cells for T cell stimulation.

The property of DCs to synthesize C3 is compatible with the known physiological and pathological functions of DCs in vivo. In the normal steady state (absence of inflammation or “danger” signal), DCs appear to produce only a small amount of C3, which is perhaps consistent with DCs having a limited ability to stimulate an immune response. Low level production of C3 may also benefit the elimination of abnormal tissue or cells, such as damaged, apoptotic, and mutant cells from the host, by the innate immune system (1). In contrast, in some pathological conditions, DC synthesis of C3 may be up-regulated by microbial factors such as LPS and by nonspecific inflammatory stimuli such cytokines, ischemia/reperfusion injury, and surgery. These nonspecific inflammatory stimuli have been shown to up-regulate C3 synthesis in vitro and in vivo (19, 36–38). Given the observations that DC synthesis of C3 is an important characteristic of DC activation, up-regulation of DC synthesis of C3 during inflammation and infection could enhance the DC’s Ag-presenting capacity.

The results presented here have important implications for organ transplantation. Alloantigen is a unique Ag that is synthesized by donor APCs and includes alloantigenic MHC molecules and MHC/peptide complexes (39). The principal effector mechanism underlying acute organ transplant rejection is the vigorous adaptive immune response mounted by recipient T cells against donor alloantigen (25, 40). CD4+ T cells play a central role in mediating graft rejection by producing cytokines that direct the proliferation and differentiation of effector cells, such as T cells and macrophages. It has been proposed that a Th1-driven response mediates
the destruction of the graft, while a Th2-driven response may favor the induction of tolerance to the graft (41, 42). More recently, a study of mouse skin allograft rejection found that 90% of T cells responded to directly presented donor MHC peptide, whereas 10% of T cells recognized allopeptide indirectly presented by recipient APCs (43). Thus, donor APCs appear to play an important role in acute rejection. DCs are the main APCs involved in initiation of the T cell response that mediates acute rejection (44). However, DCs also play a critical role in induction/maintenance of peripheral T cell tolerance, possible through the Th2-driven response and the development of regulatory T cells. In this study, we showed that all three of these DC properties—CD4<sup>+</sup> T cell stimulation, Th2 polarization, and regulatory T cell-driving capacity, are modulated by donor-derived C3. Additionally, we demonstrated the functional consequences of defective T cell priming with C3<sup>-/-</sup> DCs, in terms of the tempo of skin allograft rejection. Together these findings provide compelling evidence that the C3 status of DCs can regulate the alloimmune response. This offers an
DC SYNTHESIS OF C3 IS REQUIRED FOR T CELL ACTIVATION

FIGURE 10. C3−/− DCs have no hyporesponsiveness to LPS stimulation. A and B, WT and C3−/− DCs were cultured for 8 days were used for RNA extraction and cDNA synthesis, and subsequently for RT-qPCR. A. A typical agarose gel showing the 326-bp TLR4 band and the 453-bp GAPDH (internal control) band. The 100-bp DNA markers (M) are shown along the gel. B. The results of RT-qPCR. C: Nonirradiated DCs (2 × 10^5) from WT or C3−/− mice, after purification at day 6, were further cultured in 0.2 ml of medium for 48 h, in the presence or absence of LPS. Supernatants of DC culture were then analyzed for TNF-α by ELISA. Data in B and C were analyzed by Student’s t test. **, p < 0.001. Values of p are for comparisons between C3+/+ and C3−/− DCs. All results are representative of three independent experiments.

explanation, at least in part, for our earlier observation that C3−/− renal allografts result in much weaker graft rejection (24).

The mechanism by which the C3 status of donor DCs affects DC function in alloreactive T cell responses needs further elucidation. However, one of the possible explanations is that, in the presence of other relevant components and factors, locally produced C3 could lead to complement activation and generate complement effector molecules. Released soluble effectors such as C3a, binding to the G-protein-coupled receptor on DCs, could lead to enhanced DC activation and migration (45). A sublethal dose of C5b-9 deposited on the DC surface may also activate DCs causing cytokine release. Such an effect has been shown on macrophages (46). In addition, C3 could regulate DC function through an intracellular mechanism. Previous studies using B cells as APCs, have revealed that Ag presented covalently to C3b is protected from excessive proteolytic degradation in the endosomal/lysosomal compartment of the MHC class II pathway, suggesting that activated C3 fragment functions as a “chaperone” in the MHC class II pathway of APCs, which is essential for maintaining the normal function of APCs (47, 48). Our data showing that the impact of DC synthesis of C3 was mainly on CD4+ T cells, and the finding of reduced MHC class II expression on C3−/− DCs, are consistent with an effect of C3 on the MHC class II pathway of Ag presentation. These findings also agree with the notion that C3 produced by donor DCs could prevent allogeneic MHC molecules from excessive degradation in intracellular organelles, thus enhancing allo-MHC molecule stability and consequently promoting DC activation.

In conclusion, our data provide evidence of a new and fundamental characteristic of DCs that appears to influence the development of the Th1 response and emergence of the regulatory T cell phenotype. Lack of C3 defines a shift in the immunoregulatory function of DCs, with a swing toward a Th2 regulatory phenotype and a delay of graft rejection. These findings provide further insight into the mechanism of T cell hyporesponsiveness in C3−/− mice, and offer an explanation, at least in part, for the amelioration of graft rejection with C3−/− donor kidney. Finally, our findings may provide an additional basis for investigating possible therapeutic uses of DCs in organ transplantation and other immunological conditions.

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Disclosures
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References


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