Dendritic Cell Function in Allostimulation Is Modulated by C5aR Signaling

Qi Peng, Ke Li, Naiyin Wang, Qijun Li, Elham Asgari, Bao Lu, Trent M. Woodruff, Steven H. Sacks and Wuding Zhou

_J Immunol_ 2009; 183:6058-6068; Prepublished online 28 October 2009; doi: 10.4049/jimmunol.0804186

http://www.jimmunol.org/content/183/10/6058

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/10/28/jimmunol.0804186.DC1

References

This article cites 39 articles, 23 of which you can access for free at:

http://www.jimmunol.org/content/183/10/6058.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
Dendritic Cell Function in Allostimulation Is Modulated by C5aR Signaling

Qi Peng,* Ke Li,* Naiyin Wang,* Qijun Li,* Elham Asgari,* Bao Lu,† Trent M. Woodruff,‡ Steven H. Sacks,* and Wuding Zhou2*"2

Regulation of T cell immunity by C5a has been suggested from recent studies. However, the underlying mechanisms, particularly the involved cells and biochemical basis, are not well defined. In this study, the direct modulation of dendritic cell (DC) activation and its function in T cell stimulation by C5a-C5aR interaction and the involved signaling pathways were investigated. We show that DCs from C5aR−/− mice and normal DCs treated with C5aR antagonist have less-activated phenotype characterized with increased IL-10 and decreased IL-12p70 production in response to LPS stimulation, lowered surface expression of MHC class II, B7.2, and consequently have reduced capacity to stimulate allospecific T cells. Conversely, C5a stimulation up-regulates DC activation and its function in allostimulation. Furthermore, stimulation of C5aR mediates the inhibition of cAMP production and protein kinase A activity and is involved in activation of PI3K/AKT and NF-κB signaling in DCs. These results demonstrate that C5a acts directly on C5aR expressed on DCs resulting in cell activation and subsequently enhances its capacity for allospecific T cell stimulation. It also suggests that NF-κB signaling induced by down-regulation of cAMP/protein kinase A pathway and up-regulation of PI3K/AKT pathway following C5a stimulation may contribute to up-regulation of DC function. The Journal of Immunology, 2009, 183: 6058–6068.

The complement system is an important component of innate immunity. Complement activation in response to pathogens, immune complexes, and injured tissues, generates a set of effector molecules with diverse biological functions. The large fragments C3b/C4b opsonize pathogens and immune complexes, allowing them to be up-taken more efficiently by phagocytes, and the terminal product C5b-9 mediates direct killing of pathogens or injured cells. The released small fragments (e.g., C5a, C3a), namely anaphylatoxins, are mediators of local inflammatory process. They can act on specific receptors on various types of cells (e.g., phagocytes, endothelial cells, mast cells) resulting in contraction of smooth muscle, increased vascular permeability, and histamine release from mast cells and basophilic leukocytes. All of these functions are vital for host defense and waste clearance.

In addition to its role in innate immunity, the complement system also participates in regulating adaptive immunity. A great deal of research has shown that complement can influence specific T cell responses. C3 deficiency or C3-depletion is associated with reduced T cell responses in several disease models including viral infection, autoimmune disease, and transplant rejection, while C5 deficiency or C5-depletion is associated with increased T cell responses. C5aR deficient mice or treated with C5aR antagonist, have similar reduced T cell responses in response to LPS stimulation, lower surface expression of MHC class II, B7.2, and consequently have reduced capacity to stimulate allospecific T cells. Conversely, C5a stimulation up-regulates DC activation and its function in allostimulation. Furthermore, stimulation of C5aR mediates the inhibition of cAMP production and protein kinase A activity and is involved in activation of PI3K/AKT and NF-κB signaling in DCs. These results demonstrate that C5a acts directly on C5aR expressed on DCs resulting in cell activation and subsequently enhances its capacity for allospecific T cell stimulation. It also suggests that NF-κB signaling induced by down-regulation of cAMP/protein kinase A pathway and up-regulation of PI3K/AKT pathway following C5a stimulation may contribute to up-regulation of DC function.

Received for publication December 15, 2008. Accepted for publication August 27, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Medical Research Council of the U.K.
2 Address correspondence and reprint requests to: Dr. Wuding Zhou, Complement Laboratory, MRC Centre for Transplantation, King’s College London, 5th Floor Tower Wing, Guy’s Hospital, Great Maze Pond, London, U.K.
3 Reagents

Rat recombinant murine GM-CSF was from R&D Systems. CD11c microbead and FITC-conjugated anti-mouse CD11c Ab were purchased from Miltenyi Biotech.

Materials and Methods

Reagents

Recombinant murine GM-CSF was from R&D Systems. CD11c microbead and FITC-conjugated anti-mouse CD11c Ab were purchased from Miltenyi Biotech.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/S2.00
Biotec. CsAR antagonists (Cs5aRα) (W54011) was purchased from Merck Biosciences and PMX53 (15) was provided by Dr. T. Woodruff. Recombinant human Cs5a was purchased from Sigma-Aldrich. Ab for Cs5AR (CD88, H1001) was purchased from Santa Cruz Biotechnology. Spin-Sep Enrichment Cocktail Kit used for CD4 T cell purification was purchased from Stem Cell Technologies. General molecular biology reagents were purchased from Promega. Ab reagents used in flow cytometry (i.e., PE-conjugated rat anti-mouse MHC class II (I-A/E, M5/114.15.2), PE-conjugated rat anti-mouse CD40 (3/23), PE-conjugated rat anti-mouse B7.2 (CD86, GL1)), ELISA kits used for measuring IFN-γ, IL-12p70, TNF-α, and IL-10, and ELISA Abs for Cs5a were purchased from BD Biosciences.

**Analysis of immune response in vivo**

Allogeneic mice (BALB/c) received an i.p. injection of 5 × 10⁵ irradiated donor DCs (C57BL/6). After 10 days, mice were killed. Splenocytes and CD4 T cells were prepared from each mouse and used for measuring allospecific T cell reactivity ex vivo by MLR. In brief, splenocytes (2 × 10⁶/well) from immunized mice were cocultured with irradiated splenocytes (2 × 10⁵/well) from donor strain (C57BL/6) in T cell culture medium for up to 5 days. T cell responses were assessed by measuring IFN-γ production and [3H]thymidine uptake.

**[3H]thymidine uptake assay**

The cocultures were set up in 96-well round-bottom plates for 72 h. [3H]thymidine (1 µCi/well) was added during the last 8 h. The amount of [3H]thymidine incorporation was measured by liquid scintillation (1205 BS Betaplate). Controls (i.e., stimulator alone and responder cells alone) were included in each experiment and gave consistently low backgrounds.

**Flow cytometry**

For MHC and costimulatory molecules, cells were stained with PE-conjugated Ab or the appropriate isotype control Ab. Ab reagents used were rat anti-mouse MHC class II, rat anti-mouse CD40, and rat anti-mouse B7.2. For Cs5AR, indirect immunofluorescence staining was performed using rat monoclonal anti-Cs5AR and PE-conjugated goat anti-rat IgG. FITC-conjugated Ab against CD11c was used to gate the CD11c⁺ cells. The stained cells were then fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry (FACScan, BD Biosciences).

**ELISA**

Sandwich ELISA was performed using OptEIA ELISA set for mouse IFN-γ, IL-12p70, TNF-α, and IL-10, and a pair of mAbs for Cs5a, according to the manufacturer’s instructions. The recombinant Cs5a was used as standard control in Cs5a ELISA.

**Measurement of intracellular cAMP**

The 6-day purified DCs (i.e., WT DCs, Cs5ARα−/− DCs, Cs5aRa-treated DCs) (2 × 10⁶ per well) were incubated with forskolin (10 nM) for 30 min. In some experiments, following forskolin treatment, DCs were further incubated in the presence or absence of LPS (200 ng/ml). The treated cells were then washed twice with cold PBS and underwent lysis in 250 µl cell lysis buffer by freezing and thawing three times. The cell lysate were used for measuring cAMP using the Parameter cAMP assay kit according to the manufacturer’s instruction (R&D Systems).

**Statistical analysis**

ELISA was performed in four to six replicate wells of the culture or coculture per sample. T cell proliferation assays were performed in six to eight replicate wells of the coculture per sample. Results were expressed as a mean ± SEM and subjected to statistical analysis. Student’s t test or ANOVA was used where appropriate to determine significant differences between samples.

**Results**

DCs from Cs5AR-deficient mice have less activated phenotype and reduced capacity to stimulate allospecific T cells in vitro

To test the hypothesis that Cs5AR signal is critical for DC activation and function, we cultured DCs from Cs5AR−/− and C57BL/6 WT mice. The purified DCs at day 6 were further cultured for 24 h in the presence of LPS. The cells and supernatants were then used for analyzing the cell activation phenotype and their capacity to stimulate allogeneic T cells. We found that Cs5AR−/− DCs produced significantly lower proinflammatory cytokine IL-12p70 but more anti-inflammatory cytokine IL-10 in response to LPS stimulation, compared with WT DCs (Fig. 1A). In addition, Cs5AR−/− DCs had reduced surface expression of MHC class II and B7.2, though not CD40 (Fig. 1B) and lowered frequency of MHC class II-high population, compared with WT DCs. Cs5AR−/− DCs, when cocultured with naive allogeneic T cells, caused less T cell proliferation and IFN-γ production than WT DCs (Fig. 1C). As the above experiments were performed in the LPS-stimulated DCs, to assess the effect of Cs5AR signaling on DC activation under steady state, we

---

4 The online version of this article contains supplementary material.
analyzed surface expression of MHC class II and B7.2 in the DCs without LPS stimulation. The purified DCs from the 6-day culture were further cultured 24 h in the presence of LPS. A, The supernatants were used for measuring IL-12 and IL-10 by ELISA. B, The cells were used for analyzing expression of MHC class II, B7.2, and CD40 by flow cytometry, the upper two panels showing the representative histograms of those molecules expression in WT and C5aR−/− DCs and the lower panel showing the geometric mean of fluorescence intensity (Geo MFI) of each molecules from six independent assays. C, Irradiated DCs were cocultured with naive alloreactive CD4+ T cells. T cell proliferation was assessed by thymidine uptake at 72 h and IFN-γ production was measured at day 5 by ELISA. Data were analyzed by Student’s t test. p values are for comparisons between WT DCs and C5aR−/− DCs. **, p < 0.01, ns no significant difference. A representative of at least three independent experiments is shown.

**FIGURE 1.** DCs from C5aR−/− mice have less activated phenotype and reduced capacity to stimulate allospecific T cells in vitro. The purified DCs from the 6-day culture were further cultured 24 h in the presence of LPS. A, The supernatants were used for measuring IL-12 and IL-10 by ELISA. B, The cells were used for analyzing expression of MHC class II, B7.2, and CD40 by flow cytometry, the upper two panels showing the representative histograms of those molecules expression in WT and C5aR−/− DCs and the lower panel showing the geometric mean of fluorescence intensity (Geo MFI) of each molecules from six independent assays. C, Irradiated DCs were cocultured with naive alloreactive CD4+ T cells. T cell proliferation was assessed by thymidine uptake at 72 h and IFN-γ production was measured at day 5 by ELISA. Data were analyzed by Student’s t test. p values are for comparisons between WT DCs and C5aR−/− DCs. **, p < 0.01, ns no significant difference. A representative of at least three independent experiments is shown.

Detection of C5/C5a in BM cell culture and C5aR expression in DCs

Next, we assessed the possibility that locally generated C5a interacts with C5aR expressed on DCs in our culture system. We examined synthesis of C5 and generation of C5a in BM cell cultures...
as well as expression of C5aR in DCs. The supernatants collected from the BM cell cultures were used for measuring C5a by ELISA. The purified C5a was used as the standard. B, The concentrated supernatants collected from WT and C5aR−/− BM cell cultures at day 6 were used for detection of C5a by Western blot, the purified C5a as a positive control is shown alongside the gel. C, BM cells cultured for 6 days with or without LPS stimulation for 24 h were used for detection of C5 mRNA by RT-PCR, the agarose gel showing the sizes of C5 and 18S (as an internal control). The liver tissue as a positive control is shown alongside the gel. D, The purified DCs from the 4-, 6-, and 7-day cultures were used for detection of C5aR mRNA by RT-PCR, the agarose gel showing the sizes of C5 and GAPDH (as an internal control). E, The purified DCs from the 6-day culture were used for detection of C5aR protein by Western blot. F, The purified DCs from the 6-day culture were used for detection of C5aR protein expression in CD11c+ cells by flow cytometry. G, The purified DCs from the 6-day culture with or without LPS or C5a stimulation were used for analyzing C5aR mRNA by real time quantitative RT-PCR. Data in G were analyzed by Student’s t test. **, p < 0.05. A representative of three independent experiments is shown for all results.

as well as expression of C5aR in DCs. The supernatants collected from the BM cell cultures were used for measuring C5a by ELISA and Western blot, while the BM cells were used for detection of C5 mRNA by RT-PCR. The purified DCs were used for detection of C5aR by RT-PCR and Western blot. Our results showed that C5a was detected by ELISA in the supernatants collected from WT and C5aR−/− BM cell cultures at day 2, 4, and 6 (Fig. 2A). C5a was also detected by Western blot in supernatants collected from WT and C5aR−/− BM cell cultures at day 6 (Fig. 2B). C5 mRNA was clearly detected in BM cells cultured for 6 days by RT-PCR; its level was increased following LPS stimulation (Fig. 2C). C5aR were detected in the purified DCs (cultured for 4, 6, and 7 days) by RT-PCR (Fig. 2D), by Western blot (Fig. 2E) and by flow cytometry (Fig. 2F). The expression of C5aR transcripts was up-regulated following LPS or C5a stimulation (Fig. 2G). These data show that C5 and C5a are produced in BM cell culture and C5aR is expressed in DCs, suggesting that local C5a-C5aR interaction could occur in murine BM DCs in our model and may be potentiated by up-regulation of C5aR expression and C5 production following LPS stimulation.

**Blockade of C5aR lowers DC activation phenotype and allostimulatory capacity in vitro and in vivo**

To further confirm the importance of C5a-C5aR interaction for DC activation and functional development observed in C5aR−/− DC experiment, we exploited another approach and examined whether C5aRa treatment could alter the DC’s cytokine profile and down-regulate DC’s allostimulatory capacity. C5aRa (W54011) at different concentrations (1–100 nM) was added in the cell culture medium from the day 2 of BM cell culture. The purified DCs at day 6 were further cultured for 24 h in the presence of LPS. The cells and supernatants were then used for analyzing the cytokine production and the
surface expression of MHC class II and costimulatory molecules as well as the capacity to stimulate alloreactive T cells in vitro. We found that, compared with untreated DCs, the C5aRa-treated DCs produced significantly less IL-12p70 but more IL-10 (Fig. 3A), had reduced surface expression of MHC class II, B7.2, and CD40 and lowered frequency of MHC class II-high population (Fig. 3B), and when cocultured with allogeneic T cells, the C5a-treated DCs elicited lower T cell responses measured by thymidine uptake and IFN-γ production, in a C5aRa dose dependent manner (Fig. 3C). In addition to in vitro studies, we also assessed the effect of C5aRa on DC’s allostimulatory capacity in vivo. The purified DCs at day 7 were injected into allogeneic mice peritoneally. After 10 days, the allospecific T cell reactivity of the recipient mice was assessed ex vivo by MLR. The cell proliferation and IFN-γ production were measured by thymidine uptake and ELISA, respectively. E, The purified DCs from the 6-day culture in the absence or presence of C5aRa (W54011 or PMX53) were further cultured 24 h in the presence of LPS. The supernatants were used for measuring IL-12 by ELISA and cells were used for assessing DC’s allostimulatory capacity in vitro as in C. Data were analyzed by Student’s t test. p values are for comparisons between untreated DCs and C5aRa-treated DCs. *p < 0.05; **p < 0.01; ***p < 0.005. A representative of at least three independent experiments is shown.

FIGURE 3. Blockade of C5aR lowers DC activation phenotype and allostimulatory capacity in vitro and in vivo. A–C, DCs cultured in the presence of different doses of C5aRa were purified at day 6, and then were further cultured for 24 h in the presence of LPS. A, The supernatants were used for measuring IL-12p70 and IL-10 by ELISA. B, The cells were used for analyzing expression of MHC class II, B7.2, and CD40 by flow cytometry, the upper two panels showing the representative histograms of those molecules’ expression in untreated and treated WT DCs and the lower panel showing the geometric mean of fluorescence intensity (Geo MFI) of each molecule from five independent assays. C, Irradiated DCs were cocultured with naive alloreactive CD4⁺ T cells. T cell proliferation was assessed by thymidine uptake at 72 h and IFN-γ production was measured at day 5 by ELISA. D, The purified DCs (5 × 10⁵) from the 7-day culture in the presence or absence of C5aRa were administrated in allogeneic mice (BALB/c) by i.p injection. After 10 days, the allospecific T cell reactivity of the recipient mice was assessed ex vivo by MLR. The cell proliferation and IFN-γ production were measured by thymidine uptake and ELISA, respectively. E, The purified DCs from the 6-day culture in the absence or presence of C5aRa (W54011 or PMX53) were further cultured 24 h in the presence of LPS. The supernatants were used for measuring IL-12 by ELISA and cells were used for assessing DC’s allostimulatory capacity in vitro as in C. Data were analyzed by Student’s t test. p values are for comparisons between untreated DCs and C5aRa-treated DCs. *p < 0.05; **p < 0.01; ***p < 0.005. A representative of at least three independent experiments is shown.
C5aRa (W54011) with another C5aRa (PMX53), which is known working in mice, in the context of their effects on DC functional modulation. Our results showed that the two antagonists have the similar effects on DC functional modulation (i.e., down-regulating LPS induced IL-12 production and allostimulatory capacity) (Fig. 3E), thus further confirming the effectiveness of W54011 in our model.

C5a stimulation enhances DC activation phenotype and allostimulatory capacity
Following studies of C5aR−/− DCs and C5aRa-treated DCs, we examined the effect of C5a on DC’s activation phenotype and allostimulatory capacity. Like the C5aRa treatment, C5a (1 and 10 nM) was added in the cell culture medium from the day 2 of BM cell culture. The purified DCs at day 6 were further cultured for 24 h in the presence of LPS. The cells and supernatants were then used for analyzing the cytokine production and the surface expression of MHC class II and costimulatory molecules as well as the capacity to stimulate alloreactive T cells. In contrast to the DCs treated with C5aRa, the C5a-treated DCs produced significantly higher IL-12p70 and TNF-α and lower IL-10 (Fig. 4A), had increased surface expression of MHC class II, B7.2, and CD40 and higher frequency of MHC class II-high population, compared with the untreated DCs (Fig. 4B). When cocultured with allogeneic T cells, the C5a-treated DCs elicited higher T cell responses measured by thymidine uptake and IFN-γ production in a C5a dose-dependent manner (Fig. 4C). These findings suggest a positive impact of C5a on DC activation and its function in allostimulation, which is in accordance with the observations in DCs from C5aR−/− mice and C5aRa-treated DCs.

To examine the relation between down-regulation of IL-10 and up-regulation of IL-12 production by C5a stimulation, we performed an additional experiment. The purified DCs at day 6 were treated with C5a or/and IL-10R blocking Ab for 24 h in the presence of LPS. The supernatants were then used for analyzing IL-12p70 production. We found that the treatment with IL-10R blocking Ab or C5a alone increased IL-12 production; the combined treatment further increased IL-12 production (Fig. 4D). These findings suggest that C5a mediated down-regulation of IL-10 production may contribute to the increased IL-12 production through other mechanisms.

Engagement of C5aR on DCs mediates down-regulation of the cAMP/PKA pathway
The next question we asked was how C5a-C5aR interaction could regulate DC activation and function. C5aR signaling has been studied in several types of cells (e.g., macrophages, neutrophils, endothelial cells, astrocytes) in which C5aR couples to G protein αi and βγ subunits and initiates several downstream signaling pathways (e.g., PI3K, NF-κB, MAPK, cAMP) (20–23). However, the C5aR signaling in DCs is unknown. cAMP pathway is a key signaling pathway for G protein coupled receptors (24). cAMP is a cyclic nucleotide that functions as an intracellular second messenger (25), which regulates a wide range of important cellular processes. It has been shown that cAMP has a negative impact on DC activation and function (26) and the increased intracellular cAMP level is associated with generation of naturally occurring T regulatory cells (27). Furthermore, we have shown in a recent study that activation of C3aR on DCs induces inhibition of cAMP production, consequently lifting the restraint of cAMP on DC activation and thereby up-regulating DC activation and function (10). As C3a and C5a have many similarities (e.g., structure, function), we hypothesized that the C5aR/cAMP signaling pathway is involved in modulation of DC function. To test this hypothesis, we first examined the relationship between the intracellular cAMP level and the C5a-C5aR axis in DCs. We compared the levels of intracellular cAMP between WT DCs and C5aR−/− DCs or the untreated WT DCs and the C5aRa (or C5a) treated WT DCs. We found that the cAMP level in C5aR−/− DCs was higher than that in WT DCs (Fig. 5A). The cAMP level in WT DCs increased following the C5aRa treatment (Fig. 5B). Conversely, the cAMP level in WT DCs decreased following C5a stimulation, in a dose dependent manner (0.1–100 nM) (Fig. 5C). Taken together, these data provide evidence that C5a-C5aR interaction is associated with a change in intracellular cAMP level in DCs; stimulation of C5aR decreases cAMP level, while blocking the receptor increases cAMP level, suggesting cAMP pathway is a key signaling pathway for C5aR.

The downstream signaling of cAMP is mediated by its interaction with effector molecules such as cAMP dependent-protein kinase A (PKA), which has been shown to negatively modulate cytokine production (28). To further confirm our observations on the impact of C5a-C5aR axis on the intracellular cAMP level, we investigated the effect of C5a on PKA activation in DCs. Our results showed that phosphorylated PKA (p-PKA) was clearly detectable in unstimulated DCs, suggesting its constitutive activities in DCs. As we expected, C5a stimulation resulted in reduction of p-PKA in DCs, in a time- (15–120 min, 10 nM) and dose- (0.1 to 10 nM, 15 min) dependent manner (Fig. 5, D and E). This result is in agreement with the decrease of intracellular cAMP following C5a stimulation, suggesting that C5a-mediated inhibition of cAMP production and PKA activity could be an important mechanism by which C5a-C5aR interaction up-regulate DC activation and function.

C5a stimulation activates PI3K/AKT signaling in DCs
In addition to the adenylyl cyclase pathway/cAMP production, G protein β and γ subunits of G protein-coupled receptor can also mediate PI3K/AKT signaling pathway. We have shown in a recent study that stimulation of C3aR on DCs can induce the activation of PI3K pathway (10), raising the possibility that stimulation of C5aR could have a similar effect on this pathway in DCs. To test this, we examined the phosphorylation of AKT (a downstream effector of PI3K) following C5a stimulation and the effect of C5a mediated PI3K/AKT signaling on inflammatory cytokine production in DCs by using the pathway specific inhibitor (wortmannin). Our results showed that phosphorylated AKT was detectable in unstimulated DCs and clearly increased following C5a stimulation (0.1–100 nM) (Fig. 6A). Wortmannin treatment significantly inhibited the C5a-induced up-regulation of IL-12 and TNF-α production in DCs (Fig. 6B). These results indicate that stimulation of C5aR can induce the activation of PI3K/AKT pathway and subsequently up-regulate proinflammatory cytokine production in DCs.

C5a stimulation activates NF-κB signaling in DCs
NF-κB is a key transcription factor responsible for regulation of numerous genes during inflammation and immune responses. A variety of extracellular signals can activate NF-κB through activating the enzyme IκB kinase and subsequent phosphorylation of the IκBα protein, which then is dissociated from the IκB/NF-κB complex to release NF-κB. Previous studies in human monocytes and endothelial cells have suggested a link of cAMP level and NF-κB activity (29). Furthermore, it has been shown that NF-κB is a target of AKT signaling in human fibroblasts (30, 31). In this study we have demonstrated that C5a-C5aR interaction altered the cAMP level and AKT activity. Therefore, we next studied the effect of C5a on NF-κB activation in DCs. We examined phosphorylation of IκBα, a key biomarker for NF-κB activation, in
response to C5a stimulation. As shown in Fig. 6C, the phosphorylated 1κB was detectable in both C5a stimulated and unstimulated DCs, with a striking increase following C5a stimulation (10 nM, a pretitrated dose), which was more profound with C5a stimulation for 15 and 30 min. When combined with LPS, C5a stimulation resulted in a further increase in phosphorylated 1κB (Fig. 6D).

These results clearly demonstrate the effect of C5a stimulation in activating NF-κB signaling in DCs and also suggest that C5a and LPS have a synergistic effect on NF-κB activation in DCs, which are in agreement with the previous findings that C5a alone, or C5a cooperating with LPS, activates NF-κB signaling in macrophages (20, 32).

**Discussion**

Regulation of T cell immunity by C5a-C5aR axis has been shown by recent studies. Understanding the underlying mechanisms
In contrast to in the virus model, Kohl et al. (34) found that in an important role in generation of Ag specific T cell responses. Enza type A virus, suggesting that C5a-C5aR interaction plays C5aR antagonist have impaired CD8 T cell response to influ-
cells (33). Later, Kim et al. (12) observed that mice treated with on human T cells suggesting that C5a exerts direct effects on T
immunity. would provide essential background information for new therapeu-
tic approaches to modulate T cell responses, as the reagents (tar-
geting C5a generation or C5a-C5aR interaction or cAMP or PI3K)
suitable for clinical investigation are already available. Crucial to
this is to identify the cell type(s) which are affected and the sig-
naling pathways which are involved by C5a-C5aR interaction. In the
present study, using several approaches (i.e., use of C5aR de-
cient DCs, C5aR antagonist, and C5aR agonist) we investigated the influence of C5a-C5aR interaction on DC activation and its function in the transplant rejection-related allospecific T cell responses.

The small complement fragments C3a and C5a have been docu-
mented to produce inflammatory responses. Of the three, C5a has
the highest specific biological activity. C5a can act on its receptors on basophilies and mast cells and cause release of histamine and
TNF-α, directly increase the activity of the phagocytes, activate
endothelial cells resulting in increased vascular permeability and
phagocyte binding, and activate epithelial cells regulating cytokine
secretion. All of these biological functions induced by C5a signifi-
cantly contribute to local inflammation. In addition to its tradi-
tional role in inducing innate tissue inflammation, C5a has also
been shown to play an important role in regulation of T cell
immunity.

Early studies on T cells have found the expression of C5aR on human T cells suggesting that C5a exerts direct effects on T
cells (33). Later, Kim et al. (12) observed that mice treated with
C5aR antagonist have impaired CD8 T cell response to influ-
enza type A virus, suggesting that C5a-C5aR interaction plays an
important role in generation of Ag specific T cell responses. In
contrast to in the virus model, Kohl et al. (34) found that in
the asthma model, blocking C5aR resulted in induction of
marked enhancement of Th2 immune response, suggesting that
C5a-C5aR interaction down-regulates Th2 immune response
which is thought to play a critical role in asthma pathogenesis.
Recent studies reported that when APC and T cell were encoun-
tered, C5a interaction with C5aR on both cells can provide co-
stimulatory and survival signals to naive CD4 T cells, thus
up-regulate Ag-specific T cell responses (14). These studies
provide evidence that Ag-specific T cell responses are influ-
enced by C5a, and T cell expression of C5aR is critical for the
proliferation and survival of T cells.

The present study is distinct in specifying the influence of
C5a on DC activation and its functional development. Using
different approaches, we demonstrate that, before encountering
T cells, C5a-C5aR interaction can up-regulate DC activation
which is evident by a distinct DC’s cytokine profile (higher
IL-12 and lower IL-10) and the increased surface expression of
MHC class II and costimulatory molecules in DCs. Lacking the
interaction of C5a-C5aR by using either C5aR-deficient DCs or
by blocking the C5aR on DCs with C5aRaα, leads to down-
regulation of DC activation. Based on the facts such as direct
allostimulation being largely dependent on MHC and costimu-
ulatory molecules expressed on donor APCs, IL-12 being a T cell
stimulating factor, and IL-10 being not only capable of inhibiting
synthesis of proinflammatory cytokines by APC and T
cells but also displaying potent abilities to suppress the Ag
presentation capacity of APC, C5a mediated modulation of
these molecules would confer the enhanced allostimulatory ca-
pacity of DCs. Indeed, our results of DC functional assays
clearly show that C5a-mediated DC activation is correlated
to DC’s capacity to stimulate alloreactive T cells in vitro and in
vivo. Therefore, our study provides evidence for the direct mod-
ulation of DC function by C5a-C5aR interaction and it also
suggests that modulation of APC function is an important
mechanism by which C5a participates in the regulation of T cell
response.

Another important finding in this study is the identification of
the intracellular signaling pathways by which C5a regulates DC
function. Results showing that C5a stimulation decreases intracel-
lular cAMP level and subsequent PKA activity suggest that
C5aR/cAMP/PKA signaling pathway is critical for modulating DC
activation and function. Increase or decrease in cAMP depends on
the subunit of G proteins: Gαi protein-coupled receptors mediate the
activation and Gαo protein-coupled receptors mediate inhibition. It
is thought that binding of ligands to Gαi protein-coupled receptor
causes increase in cAMP, while binding of ligands to Gαo results
in decrease in cAMP (24). Previous studies have found that pros-
taglandins (I2 and E2) with anti-inflammatory properties, when
binding to Gαo protein-coupled receptors (PGI2 receptor and PGE2
receptor), increase intracellular cAMP levels and subsequently in-
hbit proinflammatory cytokine production in DCs (35–37). In con-
trast, C3a, as a potent inflammatory peptide, when stimulating DCs
and astrocytes, decreases the level of intracellular cAMP (10, 23).
Consistent with the observation on C3a, in this study we found that
the intracellular cAMP level in DCs decreased following C5a stimu-
lation, suggesting that C5a may also exert its function through the
Gαo protein-coupled C5aR and lead to decreased levels of
intracellular cAMP.

It is thought that further effects of PKA could be through direct
phosphorylation of other proteins (e.g., I-κB/NF-κB) and activat-
ing the CREB protein, subsequently regulating gene expression/
protein synthesis. In addition to the changes in intracellular CAMP
and PKA activity, our study also observed that NF-κB activity

![Figure 5](http://www.jimmunol.org/)
increased in DCs following C5a stimulation, suggesting that inhibition of cAMP production and PKA activation could mediate the increased activity of NF-κB. This was supported by previous observations in human monocytes and endothelial cells that elevated cAMP/PKA inhibited NF-κB activity and NF-κB activity mediated transcription of certain genes associated with inflammatory responses (e.g., TNF-α, E-selectin, and VCAM-1) (29). We have attempted to demonstrate the positive association between the cAMP level/PKA activity and the activation of CREB; however, we found that the decreased cAMP/PKA was not associated with the reduction of phosphorylated CREB in DCs following C5a stimulation (data not shown). This could be explained by the recent finding that the CREB phosphorylation alone may not be a reliable predictor of target gene activation, as additional promoter-bound factors (e.g., the coactivator CREB-binding protein) are required for inducing cellular gene expression in response to cAMP (38). In addition to the cAMP/PKA/NF-κB pathway, our data suggests that C5aR also involves PI3K signaling pathway. Given that NF-κB can be a target of PI3K/AKT pathway (30, 31), C5aR-mediated activation of AKT may also contribute to the regulation of NF-κB dependent gene expression.

In this study, we assessed the possibility that locally generated C5a could interact with C5aR on BM DCs. Our results show that C5a was clearly detected in BM cell cultures by ELISA and Western blot, and C5 mRNA was detected in the cultured BM cells, which suggest that C5 can be produced by certain populations of BM cells (e.g., stromal cell, monocytes/macrophages) and subsequently be cleaved into C5b and C5a. Although BM DC itself may not produce C5 (9), it expresses C5aR as shown in this study and in the previous study (9), thus C5a-mediated DC modulation could occur in the BM culture. C5a-mediated up-regulation of C5aR expression could enhance DC’s response to C5a stimulation. Beside the in vitro effect of C5a on DC activation in the BM culture, locally generated C5a could also participate in DC modulation in vivo. Previous publication and our own study have shown that C5 was detected in human monocytes derived DCs and other types of DCs generated in vitro, including myeloid DCs (data not shown). It is possible that the murine BM DCs have gradually lost their ability to produce C5 in culture. In addition, a variety of organs/tissues and cells are able to synthesize complement proteins including C5, either constitutively or in response to various stimuli. C5 can be activated as downstream event of C3 activation or independent of C3, by the coagulation pathway component (thrombin) (39). Therefore, locally generated C5a, particularly under pathological conditions, is likely to act on C5aR expressed on DCs, thus modulating the cell activation and contributing to adaptive immune responses.

Functional modulation of DCs by C5a-C5aR interaction may have implication in organ transplantation. The allospecific T cell response is thought to be the major mechanism that causes cellular
rejection in transplantation and which is elicited by both donor and recipient APCs, mainly DCs. We have shown that C5a-C5aR interaction has significant impact on the allostimulatory function of DCs, which leads to the enhanced allo-specific T cell responses in vitro and ex vivo. This raises question whether C5a-C5aR interaction in donor or recipient tissue, which contains DCs and other APCs, could have an effect on allograft rejection, which is needed to be addressed in the future studies. Strategies to block this signaling pathway in DCs (e.g., by administrating anti-C5a or anti-C5aR Abs or C5aR-specific antagonist, and targeting downstream signaling pathways) may reduce allo-specific T cell responses and prolong allograft survival.

In conclusion, our study demonstrates that C5a-C5aR interaction provides a positive regulation on DC activation and its function in allostimulation, which involves the regulation of DC’s inflammatory cytokine profile and surface expression of MHC and costimulatory molecules, suggesting that C5a-mediated DC modulation is an important mechanism by which complement activation participates in the regulation of T cell responses. Regarding the signaling pathway for C5aR in DCs, our data suggest that down-regulation of AMP/PKA pathway and up-regulation of PI3K pathway could activate NF-κB signaling, consequently promoting the NF-κB-dependent gene expression (e.g., proinflammatory cytokines, MHC, and costimulatory molecules), which may contribute to up-regulation of DC function in allostimulation (Fig. 7).

Disclosures
The authors have no financial conflict of interest.

References


