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J Immunol 1999; 162:453-459; ; http://www.jimmunol.org/content/162/1/453

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Regulation of IL-6 Synthesis in Human Peripheral Blood Mononuclear Cells by C3a and C3a\textsubscript{desArg} 1

Wolfgang H. Fischer,\textsuperscript{2,3} Mark A. Jagels,\textsuperscript{2} and Tony E. Hugli\textsuperscript{4}

The anaphylatoxin C3a has been reported to have immunomodulatory effects on a number of different cell types. In this study we investigated the effects of C3a and C3a\textsubscript{desArg} on gene expression and protein secretion of IL-6 in human PBMCs, either alone or in combination with LPS or IL-1\textbeta. C3a or C3a\textsubscript{desArg} alone exhibited no effect on the expression or secretion of IL-6. However, when PBMC were stimulated with LPS or IL-1\textbeta, both C3a and C3a\textsubscript{desArg} were found to enhance IL-6 release by PBMC in a dose-dependent manner. Since C3a has been shown to induce PGE\textsubscript{2} production by monocytes, and PGE\textsubscript{2} has been shown to influence cytokine production, we investigated the potential role of PGE\textsubscript{2} in C3a-mediated enhancement of LPS- and IL-1\textbeta-induced IL-6 production. Indomethacin blocked PGE\textsubscript{2} release, but had no influence on the observed effects of C3a, suggesting that the effects of C3a on IL-6 production are independent of PGE\textsubscript{2} formation by monocytes. Northern blot analysis showed that C3a as well as C3a\textsubscript{desArg} enhanced LPS-induced mRNA levels for IL-6. Pretreatment of PBMCs with pertussis toxin blocked the immunomodulatory effect of C3a (as well as C3a\textsubscript{desArg}) on human immune function by modulating IL-6, which possesses potent pleiotropic functions.

1 Address correspondence and reprint requests to Dr. Tony E. Hugli, Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037.
Materials and Methods

Reagents

All chemicals were obtained from Sigma (St. Louis, MO) unless indicated otherwise. Ficoll was purchased from Pharmacia (Piscataway, NJ). RPMI medium was obtained from BioWhittaker (Walkersville, MD). IL-1β was purchased from R&D Systems (Minneapolis, MN). Capture and detection Abs for ELISA were obtained from Pharmingen (San Diego, CA). The synthetic C3a analogue peptide 71/53 (WWGKKYRASKGLAR) was synthesized, and both purity and sequence were confirmed in the protein/nucleotide core facilities of The Scripps Research Institute. The human IL-6 cDNA probe used in these experiments was provided by Dr. Edward Morgan (The La Jolla Cancer Institute, La Jolla, CA).

Preparation of human C3a

Human C3a was purified according to the method described previously (27). The concentration of C3a was determined by amino acid analysis. Based on tracer experiments with [125I]C5a, contamination of the C3a preparation with C5a was <0.0017%. By using the Limulus amebocyte lysate assay (BioWhittaker), LPS contamination of C3a, C3adesArg, or the C3a analogue 71/53 was not detectable. C3adesArg was obtained by treating the highly purified C3a with 1% (w/v) carboxypeptidase B for 30 min at 37°C in ammonium bicarbonate buffer at pH 8.0. The conversion of C3a to C3adesArg was confirmed by mass spectrometry, demonstrating homogenous peaks of 9093 and 8933 mass units, respectively, for C3a and C3adesArg.

Isolation of human PBMCs

PBMCs were isolated from peripheral blood of healthy human donors as described previously (26). Briefly, blood was drawn into EDTA-containing syringes to achieve a final EDTA concentration of 10 mM. Thirty-five milliliters of whole blood was layered over 15 ml of Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The mononuclear cell layer was recovered and washed twice in Earle’s balanced salt solution containing 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin (Bio-Whittaker, Walkersville, MD).

Cell culture condition

For induction of IL-6 protein, triplicate cultures were established in 96-well microculture plates (Corning, NY); each well contained 5 × 10^3 PBMCs. LPS from Escherichia coli (strain EC055B5; Sigma) was added to a final concentration of 500 ng/ml in the presence or the absence of C3a or C3adesArg. The plates were then incubated at 37°C in a humidified atmosphere containing 5% CO₂. Culture supernatants were collected after 18 h and were used at −20°C until assayed for IL-6. For isolation of total RNA and extracts of nuclear proteins, 5 × 10^6 cells were plated in 1 ml of culture medium in six-well plates and incubated for various times under the conditions described above.

RNA isolation and Northern blotting

Total RNA was extracted with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Eight micrograms of total RNA was subjected to electrophoresis in agarose gels containing 0.22 M formaldehyde and transferred to the Hybond-N Plus membranes (Ammersham, Arlington Heights, IL) by diffusion. The probe for human β-actin was generated by RT-PCR, using human β-actin specific primers (Stratagene, La Jolla, CA). To confirm that the amplified product reflects the human β-actin mRNA, it was eluted from the gel and cloned in the TA cloning vector pCR/TM2.1. (Invitrogen, La Jolla, CA) following the manufacturer’s instructions, and the nucleotide sequence was determined. Sequencing was performed in the protein/nucleotide core facilities of The Scripps Research Institute. The sequence obtained was identical with those published. The human IL-6 cDNA probe used in these experiments was provided by Dr. Edward Morgan. For hybridization, the fragments were labeled with [α-32P]dCTP to a sp. act. of >2 × 10⁶ cpm/μg DNA using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Membranes were prehybridized in QuikHyb solution (Stratagene, La Jolla, CA) at 68°C for 20 min and further hybridized with the labeled probes (1 × 10⁶ cpm/ml) for 2 h at the same temperature. Filters were washed twice with 2× SSC and 0.1% SDS at room temperature for 15 min followed by another wash with 0.1× SSC and 0.1% SDS at 60°C for 30 min. The membranes were exposed to a PhosphorImage screen and analyzed by a PhosphorImaging system (Molecular Dynamics, Sunnyvale, CA).

Preparation of nuclear extracts

Nuclear extracts were prepared by a modified method of Dinign et al. (28). Aliquots of PBMC (5 × 10^6/sample) were stimulated with different agent as indicated in the text, after which the cells were washed three times with ice-cold PBS, harvested, and resuspended in 0.4 ml of buffer A (10 mM HEPS (pH 7.9), 10 mM KCl, 1 mM DTT, 0.1 mM each of EDTA and EGTA, and 1 mM PMSF). After 10 min, 23 μl of 10% Nonidet P-40 was added and mixed for 2 s. Nuclei were separated from cytosol by centrifugation at 13,000 × g for 20 s and were resuspended in 50 μl of buffer B (10 mM HEPS (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF). After 30 min at 4°C, lysates were separated by centrifugation at 13,000 × g for 40 s, and the supernatants containing nuclear proteins were transferred into new vials. Protein concentrations were measured using a protein dye reagent (Bio-Rad, Richmond, CA) with BSA as standard, and samples were diluted to equal concentrations in buffer B. Samples were stored at −70°C.

Electrophoretic mobility shift assay (EMSA)

EMSA were performed essentially as previously described (29). Briefly, 5 μg of nuclear extracts were added to 12 μl of binding buffer (5 mM HEPS (pH 7.9), 5 mM MgCl₂, 50 mM KCl, 0.5 mM DTT, 0.4 mg/ml poly(I-lc), 0.1 mg/ml sonicated salmon sperm DNA, and 10% glycerol) and incubated for 15 min at room temperature. Approximately 40 fmol of 32P-labeled oligonucleotide probe (Stratagene) was then added, and the reaction was continued at room temperature for 15 min. For reactions involving competitor oligonucleotides, the unlabeled and labeled probes were added simultaneously to the reaction mixture. The samples were analyzed on 5% acrylamide gel, made with buffers containing 50 mM Tris borate and 1 mM EDTA. After pre-electrophoresis for 1 h at 8 V/cm, samples were applied, and electrophoresis was conducted at the same voltage for 1.5–2 h. The gels were transferred to Whatman paper (Clifton, NJ), dried, exposed to a PhosphorImager screen, and analyzed by a PhosphorImaging system (Molecular Dynamics).

Measurement of cytokine production by human PBMC

IL-6 production in supernatants from human PBMC cultures was measured by sandwich ELISA using paired mAb (PharMingen), following the instructions of the manufacturer.

Results

C3a and C3adesArg enhance LPS- and IL-1β- induced IL-6 release from human PBMCs

Recently, we and others have demonstrated immunomodulatory effects of C3a and C3adesArg on cytokine synthesis in human PBMCs and human tonsil-derived B lymphocytes (15, 17). These studies prompted us to investigate whether C3a and C3adesArg can modulate the production of a related cytokine, i.e., IL-6, under various experimental conditions.

Unstimulated human PBMCs or PBMCs stimulated with 1 μM C3a or C3adesArg alone did not release detectable levels of IL-6 (Fig. 1). However, at concentrations from 1 μM to 10 nM, both C3a and C3adesArg enhanced LPS-induced IL-6 release from human PBMCs in a dose-dependent manner. At a concentration of 1 μM, C3a and C3adesArg significantly increased IL-6 levels in culture supernatants of PBMCs stimulated with LPS (100 ng/ml) by 400 ± 30 and 328 ± 19%, respectively (Fig. 1, A and B). At a concentration of or below 10 nM, neither C3a nor C3adesArg induced a significant increase in IL-6. We next asked whether the observed increase in IL-6 production by C3a and C3adesArg is specific for LPS stimulation or whether activation of cells by other stimuli (i.e., IL-1β (25 ng/ml)) in combination with C3a or C3adesArg would also result in enhanced IL-6 release. As shown in Fig. 1 (C and D), both C3a and C3adesArg enhanced IL-1β-induced IL-6 release in PBMCs. At the highest concentrations of C3a and C3adesArg (1 μM) the increases in IL-1β-induced IL-6 release were

5 Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; PTX, pertussis toxin; AP-1, activating protein-1.
310 ± 40 and 293 ± 38% respectively. Surprisingly, but in agreement with former studies, C3a desArg was almost as effective as C3a itself (15, 17). However, at a concentration of 500 nM an apparent difference was observed between C3a and C3a desArg that was not observed at other concentrations.

To prove that the observed increases in IL-6 production were specific effects of C3a and C3a desArg and not due to contaminants in the C3a preparations used in our experiments, we tested the synthetic C3a analogue peptide 71/53 (peptide sequence WWGKKYRASKLGLAR). Previous studies have shown that this peptide exhibits the same biological activities as native C3a (13, 30). PBMCs were stimulated with LPS (100 ng/ml) and different concentrations of C3a analogue peptide 71/53. As shown in Fig. 2, the peptide analogue was also found to increase LPS-induced IL-6 production in a dose-dependent manner. The concentration of C3a analogue peptide required to produce approximately the same enhancement was 5–10 times higher than that of native human C3a. This is in agreement with former studies of this peptide (30). Taken together, these results indicate that the observed enhancing effects of C3a on IL-6 production in PBMCs are specific effects of the molecule.

C3a and C3a desArg enhance LPS-induced IL-6 mRNA levels in human PBMCs

To investigate whether the effects of C3a and C3a desArg on IL-6 are accompanied by increased IL-6 mRNA levels, PBMCs were stimulated with LPS (100 ng/ml) plus 1 μM C3a or C3a desArg and RNA was prepared for Northern blotting. A time course from 0–24 h demonstrated that neither C3a nor C3a desArg alone can induce IL-6 mRNA in PBMCs (data not shown). However, when cells were stimulated with both LPS plus C3a or C3a desArg, we found an increase in IL-6 mRNA at 3 h compared with that in cells stimulated with LPS alone (Fig. 3). Standardization of IL-6 mRNA levels to β-actin mRNA levels revealed that IL-6 mRNA increased by 60 and 63%, respectively, for C3a and C3a desArg costimulated with LPS.

The effect of indomethacin on C3a- and C3a desArg-mediated increases in IL-6 production in LPS- or IL-1β-stimulated PBMCs

A previous study demonstrated that C3a stimulates PGE2 production in human macrophages (14). Furthermore, it has been shown
that PGE₂ leads to increased intracellular cAMP levels (31). Since both PGE₂ and cAMP are involved in the regulation of proinflammatory cytokines, we addressed the question of whether endogenous PGE₂ contributes to and/or influences these effects of C3a and C3a desArg on IL-6 regulation in LPS- or IL-1β-stimulated PBMCs. Indomethacin at a concentration of 1 μg/ml totally inhibited the production of PGE₂ in PBMC cultures stimulated with LPS (100 ng/ml), C3a (1 μM), or LPS in combination with C3a (data not shown). Furthermore, indomethacin had no effect on IL-6 release in human PBMCs stimulated with LPS or IL-1β alone or in combination with C3a or C3a desArg. Supernatants were harvested after 18 h of culture. Levels of IL-6 were determined by cytokine-specific ELISA. Differences in IL-6 levels were analyzed by Student’s t test (the asterisk indicates p < 0.01). The data are expressed as the mean ± SD of four independent experiments using different donors.

**FIGURE 3.** Effects of C3a and C3a desArg on IL-6 gene expression in human PBMCs. Northern blot with total RNA (8 μg/lane) isolated from PBMCs (5 × 10⁷/ml) treated as indicated below. 1a, Unstimulated cells; cells stimulated with C3a (1 μM) or C3a desArg (1 μM); 1b, PBMCs stimulated with LPS (100 ng/ml) alone or in combination with C3a (1 μM); PBMCs stimulated with LPS (100 ng/ml) alone or in the presence of C3a desArg (1 μM); 1d–1f, β-actin controls. These results are representative of three independent experiments using different donors.

**FIGURE 4.** Influence of indomethacin on the effects of C3a and C3a desArg on IL-6 production by human PBMCs. Cells were cultured at 5 × 10⁵ cells/ml, prestimulated with indomethacin (1 μg/ml) for 0.5 h (open bars), and stimulated with LPS (100 ng/ml; A) or IL-1β (B) in the absence or the presence of C3a or C3a desArg. Supernatants were harvested after 18 h of culture. Levels of IL-6 were determined by cytokine-specific ELISA. The data are expressed as the mean ± SD of four independent experiments using different donors.

**FIGURE 5.** Influence of PTX on the effects of C3a and C3a desArg on IL-6 release by human PBMCs. Cells were cultured at 5 × 10⁵ cells/ml, pretreated with PTX (500 ng/ml) for 3 h (open bars), and stimulated with LPS (100 ng/ml; A) or IL-1β (B) in the absence or the presence of C3a or C3a desArg. Supernatants were harvested after 18 h of culture. Levels of IL-6 were determined by cytokine-specific ELISA. Differences in IL-6 levels were analyzed by Student’s t test (the asterisk indicates p < 0.01). The data are expressed as the mean ± SD of four independent experiments using different donors.
Induction of the transcription factors NF-κB and activating protein-1 (AP-1) by C3a and C3adesArg

Previous reports indicated that the NF-κB binding site located between positions −72 and −63 on the IL-6 gene is important for the induction of IL-6 (34, 35). Another important transcription factor involved in the regulation of IL-6 transcription is AP-1, with a consensus binding sequence found at position −283 to −277 in the IL-6 promoter (36). To examine whether the observed enhanced responses seen after costimulation of PBMCs with LPS and C3a were related to a different induction of the transcription factors NF-κB and AP-1, we measured the DNA binding activity of these factors using EMSAs. As expected, treatment of PBMCs with LPS (100 ng/ml) alone caused induction of NF-κB and AP-1 binding activities. However, costimulation of PBMCs with LPS (100 ng/ml) and C3a (1 μM) resulted in an additional increase in the binding activities of NF-κB and AP-1 (Fig. 7). Surprisingly, nuclear extracts from PBMCs stimulated with C3a (1 μM) alone showed increased DNA binding activity compared with the activity in untreated cells (Fig. 8). To verify that these DNA-protein interactions were specific, we performed competition experiments with unlabeled oligonucleotides. NF-κB and AP-1 binding activities in nuclear extracts from PBMCs stimulated with LPS (100 ng/ml) plus C3a (1 μM) were completely abrogated in the presence of a 100-fold molar excess of unlabeled oligonucleotides, but remained intact in the presence of a 100-fold molar excess of Oct-1 oligonucleotide (Fig. 9). These results indicate that the enhancing effects of C3a are associated with an increased binding activity of the transcription factors NF-κB and AP-1.

Discussion

Involvement of anaphylatoxins in the processes of inflammation and immunoregulation is supported by well-established phenomena (8, 9). The purpose of the present study was to investigate the molecular basis for the biological effects of C3a and C3adesArg on human PBMCs. The effects of C3a on cytokine synthesis are poorly defined. A previous report demonstrated that C3a stimulated human PBMCs to produce IL-1 (37). However, another study failed to show IL-1 synthesis in PBMCs in response to C3a (38). Regarding C3a as an inducer of cytokine synthesis, this latter finding is in agreement with our results as well as other published data (15). Neither C3a nor C3adesArg induced IL-6 specific mRNA or release of IL-6 in human PBMCs. Although rapid expression of cytokine mRNA in monocytes can be induced by adherence without subsequent translation, C3a or C3adesArg alone did not induce or modify IL-6 mRNA synthesis in our in vitro model. However, here we demonstrate that C3a as well as C3adesArg increased IL-6 specific mRNA and protein synthesis by human PBMCs after stimulation with LPS or IL-1β. The C3a analogue peptide 71/53 also enhanced IL-6 release, suggesting that the observed effects of C3a are specific. Recently, Takabayashi et al. reported that C3a and C3adesArg modulate LPS-induced mRNA and protein synthesis of TNF-α and IL-1β in human PBMCs (15). In their study they showed that synthesis of these cytokines was up-regulated in adherent PBMCs, whereas they were down-regulated in nonadherent PBMCs. Very recently, these studies were extended to demonstrate the same effects on IL-6 production (39). In our study we did not observe a difference between adherent and nonadherent PBMCs, independent of the concentration of LPS employed (data...
not shown). It is currently unclear what differences in our experimental systems are likely to account to the discrepancies in these studies.

In a recent study we demonstrated C3a-induced down-regulation of IL-6 and TNF-α release in human tonsil-derived B lymphocytes (40). Taken together, these findings suggest that C3a can modulate cytokine synthesis in a cell-specific manner. Similar effects have been reported for other mediators, such as IL-4, which is known to inhibit IL-6 production in a tissue-specific manner (41).

C3a is known to induce PGE₂ production in human monocytes, leading to increased cAMP levels by stimulation of adenylate cyclase activity. These signals are known to regulate cytokine production. In our experiments indomethacin, an inhibitor of cyclooxygenase, totally suppressed PGE₂ production, but did not affect C3a- and C3a₇desArg-mediated increases in IL-6 expression or release in human PBMCs. This suggests that the effects of C3a and C3a₇desArg on IL-6 production in LPS- or IL-1β-stimulated PBMCs are independent of endogenous PGE₂ synthesis. These data are in agreement with those published by Takabayashi et al. (15, 39).

Recently, the receptor for human C3a was cloned and was found to belong to the family of seven-transmembrane receptors (32, 33, 42). Moreover, several biological responses of C3a can be blocked by PTX, suggesting that the C3a receptor acts as a G protein-coupled receptor (16). In the present study we show that the effects of both C3a and C3a₇desArg on IL-6 synthesis were totally blocked by PTX, suggesting that the C3a receptor acts as a G protein-coupled receptor (16). In the present study we show that the effects of both C3a and C3a₇desArg on IL-6 synthesis were totally blocked by PTX, indicating that these molecules act through a G protein-dependent pathway, possibly involving the C3a receptor. However, the biological activity of C₇desArg as well as the concentrations of C3a and C3a₇desArg required to elicit enhancement of IL-6 production are inconsistent with the currently known properties of the receptor on other cell types (33, 50). Definitive assignment of a receptor-mediated mechanism currently awaits the generation of specific receptor antagonists or blocking Abs to the C3a receptor.

A number of previous studies have revealed that NF-κB and AP-1 are important transcription factors involved in the control and regulation of IL-6 transcription. The major form of NF-κB is a heterodimer composed of p50 and p65 subunits that exist in the cytoplasm in an inactive form due to association with the specific inhibitor protein IκB (43, 44). Phosphorylation of IκB by candidate kinases leads to the release and subsequent nuclear translocation of NF-κB, which then binds to a decameric DNA sequence originally identified in the κ light chain enhancer of Ig (45). The transcription factor AP-1 is a heterodimer formed by a product of the fos and jun families (46). In the present study we show that the enhancing activities of C3a on IL-6 expression in LPS-stimulated PBMCs are associated with increased binding of NF-κB and AP-1 to their consensus sequences. Unexpectedly, nuclear extracts from PBMCs stimulated with C3a alone also showed elevated DNA binding activities of NF-κB and AP-1 compared with those from untreated cells. Despite this observation, C3a was not able to induce IL-6 expression. Furthermore, in the studies by Takabayashi et al., C3a alone did not induce IL-1β, TNF-α, or IL-6 (15, 39). A possible explanation could be that other transcription factors shown to be important for IL-6 expression (e.g., NF-IL-6 and SP-1) are not inducible by C3a (47). A study by Matsuoka et al. (48) showed that in the absence of NF-IL-6, NF-κB (p50/p65), or any other combination of p50 and p65, IL-6 expression was not inducible in a cotransfection system. These results indicate that a cooperative effect exists among these transcription factors (48). Recently, LeClair et al. (49) found that p50 and NF-IL-6 directly associate with each other via the b-Zip domain and the Rel homology domain. These data raise the possibility that C3a alone might be competent to induce the transcription of yet uninvestigated genes by inducing binding activity of NF-κB and AP-1. To our knowledge, this is the first report showing that the anaphylatoxin C3a is capable of inducing activation of transcription factors, namely NF-κB and AP-1, which have been shown to be important regulators of many immune response and acute phase response genes, including IL-8, TNF-α, IL-1β, granulocyte CSF, Ig κ light chain, and serum amyloid A-1. Therefore, it is possible that C3a is involved in the regulation of a number of immunological functions in vivo.

Surprisingly, C3a₇desArg shared the ability to enhance IL-6 production with C3a. C3a₇desArg, which lacks the C-terminal arginine, has generally been regarded to be biologically inactive (13, 14, 50, 51). C3a₇desArg is not chemotactic for eosinophils (13), does not bind to specific receptors on guinea pig platelets (50) or on a human mast cell line (52), and does not induce calcium mobilization in either human mast cells (52) or human monocytes (53), all properties that have been demonstrated by the intact molecule. Because the C-terminal arginine is rapidly cleaved by serum carboxypeptidase N to convert C3a to C3a₇desArg, this enzymatic process has been considered a major mechanism for controlling C3a function in vivo (51). However, recent studies have reported biological activities for C3a₇desArg including inhibition of cytotoxicity by human NK cells (54), induction of histamine release from rat peritoneal mast cells (55), regulation of TNF-α and IL-1 in human PBMC (15), and regulation of B cell function (17). Both C3a and C3a₇desArg are highly cationic molecules that can bind to anionic components on the cell membrane, leading to nonspecific cell activation (55). This nonspecific effect, which depends on the net charge of the molecules, could explain why C3a₇desArg and C3a share some biological activities. There is also evidence that C3a (and possibly C3a₇desArg) binds to the β-chain of the Fcε receptor on mast cells (56). Similar Fc interactions on monocytes could influence the signaling induced by LPS. However, the question remains as to why C3a₇desArg activity is observed in some, but not all, cell types responding to C3a. A possible explanation would be differences in the cell surface of these cell types. An even more speculative possibility would be that there is a second receptor for C3a and/or C3a₇desArg. Two groups who have cloned the C3aR found a second band in their Northern blots supporting this possibility (33, 42). Our findings that a synthetic peptide analogue of C3a mimics the effects of C3a and C3a₇desArg and that both the effects of C3a and C3a₇desArg are inhibited by PTX support a receptor-mediated signaling pathway. Nevertheless, more studies are necessary to identify receptor vs nonreceptor mechanisms of cellular activation by C3a. Regardless of the mechanism, the enhancing effect of C3a/C3a₇desArg on IL-6 production in LPS- and IL-1β-stimulated PBMCs is readily demonstrated.

In conclusion, by influencing IL-6 production in B cells (17) and PBMCs, C3a and C3a₇desArg may contribute to the regulation of both immune responses and inflammation. To our knowledge, this is the first report showing a biological function of C3a or C3a₇desArg that is associated with increased binding activities of the transcription factors NF-κB and AP-1.

Acknowledgments

We thank Philippe Pfeifer for purification of the C3a, and Alicia Palestini for her help in preparing this manuscript.

References
