Immunology 2004 **111** 435–443 doi: 10.1111/j.1365-2567.2004.01829.x

Human monocyte-derived dendritic cells are chemoattracted to C3a after up-regulation of the C3a receptor with interferons

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SUMMARY

The anaphylatoxin C3a is an important inflammatory mediator in the innate and adaptive immune systems. Recent reports in various animal models have fostered the role of C3a in mediating allergic reactions such as pulmonary allergies. However, data in humans are limited and the cellular targets for C3a are not fully understood. We sought to explore human dendritic cells as a new target for C3a, because C3a receptor (C3aR) expression has been described on myeloid cells, and dendritic cells are likely make contact with C3a at sites of inflammatory reactions. In this study, we demonstrated the expression of the C3aR on human monocyte-derived dendritic cells (MoDC) and its up-regulation by interferon (IFN)-α, IFN-γ and prostaglandin E_2 (PGE_2). The strongest up-regulation was yielded by the combination of IFN-α + IFN-γ. Tumour necrosis factor-α (TNF-α) down-regulated the C3aR. After up-regulation of the C3aR by IFN-α + IFN-γ, C3a significantly up-regulated the surface expression of CD54, CD83 and CD86, but not of CD40, CD80 or human leucocyte antigen (HLA)-DR. C3a had no effect on the production of interleukin (IL)-10 or IL-12p70, or on the capacity of MoDC to stimulate autologous T-cell proliferation. However, C3a had a direct migratory effect on MoDC, as indicated by the induction of F-actin polymerization and migration in Boyden chamber experiments, which was pronounced after up-regulation of the C3aR with IFN-α + IFN-γ. Therefore, dendritic cells represent another group of target cells that might be recruited by C3a to areas of inflammation, in particular under conditions where IFNs are increased in the surrounding environment.

INTRODUCTION

The anaphylatoxin C3a is an important inflammatory mediator that is able to affect both innate and adaptive immunity. C3a can be released from its precursor C3 by activation of complement or of enzymes such as human mast cell tryptase.[1]

Increased levels of C3a in the circulation have been found in diseases such as the adult respiratory distress syndrome,[2] rheumatoid arthritis,[3] psoriasis and atopic dermatitis.[4] C3a is also found intralesionally in inflammatory diseases, e.g. psoriasis,[5] eczema[5–7] and asthma.[5,8,9]

Received 16 November 2003; revised 8 January 2004; accepted 9 January 2004.

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The effects of C3a are mediated via the C3a receptor (C3aR), a G-protein coupled seven-transmembrane receptor. Studies in animal models with deficient C3aR demonstrate protection of C3aR knockout mice from endotoxin shock,[5,10] as well as the reduction of bronchial hyperreactivity in mice and guinea-pigs lacking a functional C3aR.[8,11,12] However, mechanisms and target cells of C3a in such complex reactions are less clear.

C3aR expression has been described on a variety of cell types, including cells of the innate and adaptive immune system, endothelial cells[13] and astrocytes.[14] Most detailed studies on C3aR expression and function have been performed in cells of the innate immune system, such as neutrophils[15,16] and eosinophilic and basophilic granulocytes.[15] The key effect of C3a on granulocytes is their recruitment to sites of inflammation, infection or trauma, and a direct chemotactic effect of C3a has been shown for eosinophils[17] and mast cells.[18–20] In this respect, C3a has also been shown to increase adhesion of rolling eosinophils to postcapillary venoles, as well as transendothelial migration,[21] and to modulate the adhesion molecule expression

on neutrophils.[22] Furthermore, C3a can activate granulocytes, e.g. by increase of histamine release from mast cells and basophils,[23] or by activation of reactive oxygen radical species in human eosinophils[24] and neutrophils.[25]

In contrast to cells of the innate immune system, there are only few reports of C3aR expression and function on other cell types.[13] Expression of the C3aR has been described on some cells of the adaptive immune response, such as monocytes,[15,16,26,27] activated $CD8^+$ and $CD4^+$ T cells,[28] B cells,[29] macrophages[30,31] and Langerhans' cells in the skin.[32] However, very little is known about the regulation and function of the C3aR on cells of the adaptive immune system.

Dendritic cells (DCs) are key cells in eliciting and maintaining adaptive immune responses and probably make contact with C3a at sites of inflammation. In a previous study we demonstrated expression of the C3aR on human skin- and blood-derived DCs, which was down-regulated by tumour necrosis factor-α (TNF-α).[32] Here, we investigated the expression and regulation of the C3aR on human monocyte-derived dendritic cells (MoDC) and the effects of C3a on human MoDC. We showed that human MoDC express the C3aR, which is up-regulated by interferon (IFN)-α and IFN-γ, and down-regulated by TNF-α. C3a exerted chemotactic effects on MoDC, in particular after up-regulation of the C3aR. In contrast, we were unable to detect effects of C3a on interleukin (IL)-10 or IL-12p70 production and on the T-cell stimulatory capacities of MoDC. Therefore, C3a might contribute to adaptive inflammatory reactions by attracting DCs, rather than by influencing their immunomodulatory capacity.

MATERIALS AND METHODS

Preparation of MoDC

MoDC were differentiated from peripheral blood mononuclear cells (PBMC) as previously described.[33] In brief, PBMC were isolated by Lymphoprep density-gradient centrifugation of heparinized leucocyte-enriched buffy-coats. After 2 hr of plastic adherence, non-adherent cells were removed by five vigorous washes with phosphate-buffered saline (PBS) and the remaining adherent cell fraction was cultured in Iscove's modified Dulbecco's medium supplemented with 4% heat-inactivated human serum, 250 U/ml IL-4 (R & D Systems, Wiesbaden, Germany) and 1000 U/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) (Essex Pharma, Munich, Germany). These cells were fed with fresh medium and cytokines on day 2 of culture. Non-adherent cells, thereafter called MoDCs, were harvested on day 7. Before initiation of further experiments, cells were analysed by two-colour flow cytometry for contaminating $CD3^+$ T cells, $CD20^+$ B cells, $CD56^+$ natural killer (NK) cells and $CD16^+$ granulocytes, as described below. Only preparations with $<5\%$ contaminating T cells, B cells, NK cells or granulocytes were used in subsequent experiments [for reverse transcription–polymerase chain reaction (RT–PCR) experiments the contamination was required to be $<2\%$].

Stimulation of MoDC and FACS analysis

Cells were either unstimulated or stimulated for various periods of time with recombinant C3a or C3a(desArg) (6 μg/ml, prepared as described previously[28]), IFN-γ (200 U/ml; R & D Systems), IFN-α2a (200 U/ml; Roche, Grenzach, Germany), TNF-α (200 U/ml; R & D Systems), prostaglandin E_2 (PGE_2) (1 μg/ml; Sigma, Deisenhofen, Germany), lipopolysaccharide (LPS) (500 ng/ml; Sigma) and CD40 ligand (CD40L) (2 μg/ml; Alexis, Grünberg, Germany). LPS concentrations, as determined by the Limulus assay (Coatest Endotoxin; Pharmacia, Freiburg, Germany), were <20 pg/μg of C3a or C3a(desArg). In IFN-α, IFN-γ, TNF-α and PGE_2 solutions, LPS was not detected.

Then, cells ($1–2 \times 10^5$) were washed and resuspended in phosphate-buffered saline (PBS) containing 0·2% gelatine, 20 mM sodium azide and 10 μg/ml heat-aggregated human immunoglobulin G (IgG) (Sigma) for 15 min. Subsequently, cells were incubated with the following fluorescent-labelled antibodies, on ice for 30 min: CD3–fluorescein isothiocyanate (FITC), CD20–FITC, CD54–FITC, CD56–FITC, CD80–FITC, CD4–phycoerythrin (PE), CD16–PE, CD40–PE, CD86–PE, CD83–PE, or isotype-matched controls (Immunotech, Hamburg, Germany); human leucocyte antigen (HLA)-DR–FITC (Becton-Dickinson, Heidelberg, Germany). For detection of the C3aR, an unlabelled mouse anti-human monoclonal antibody (mAb) against the third extracellular loop of the C3aR was used, as described previously.[15,28,32] In a second step, cells were incubated for another 30 min on ice with an FITC-labelled goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany). Stained cells were washed three times, fixed in PBS with 1% paraformaldehyde and analysed by flow cytometry (FACScan; Becton Dickinson).

mRNA isolation, RT and LightCycler PCR

mRNA was isolated from 10^5 MoDC using the High Pure mRNA isolation kit (Roche Molecular Biochemicals), according to the supplier's instructions. For RT–PCR analysis, RNA was subjected to first-strand cDNA synthesis using $Oligo(dT)_{15}$ for full-length cDNA synthesis. The RT reaction mixture contained final concentrations of 50 U Expand-RT (Roche), 10 mM dithiothreitol (DTT), 1 × first-strand RT buffer for Expand-RT, 0·5 mM of each dNTP (Roche), RNAse inhibitor (Invitrogen, Karlsruhe, Germany) and 80 pMol $Oligo(dT)_{15}$ (Roche). To control for genomic DNA contamination, cDNA synthesis was performed in the absence of reverse transcriptase. First-strand cDNA was stored at -20°.

C3aR amplification was performed using a LightCycler-Primer Set from LC Research (Heidelberg, Germany). For β-actin amplification, the following primers were used: sense 5′-AAGGCCAACCGCGAGAAGATG, and anti-sense 5′-GGAAGAGTGCCTCAGGGCAGCG.[33]

Real-time fluorescence PCR was performed using the LightCycler™ (Roche), as described previously.[33] PCR was performed by rapid-cycling in a reaction volume of 20 μl with 0·5 μM of each primer and 4 μl of cDNA. As reaction buffer, the LightCycler DNA Master SYBR Green I [containing reaction buffer, *Taq* DNA polymerase, dNTPs (with dUTP instead of dTTP), $MgCl_2$ and a calibrated amount of SYBR Green I dye (Roche)] was used. After an initial denaturation step at 95° for 30 seconds, amplification was performed using 35 cycles of denaturation (95° for 10 seconds), annealing (68° for 10 seconds) and extension (72° for 16 seconds). Fluorescence

was measured at the end of the annealing period of each cycle to monitor amplification.

After amplification had been completed, a final melting curve was recorded by cooling the samples to 65° at 20° per second and then increasing the temperature to 95° at 0·2° per second. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the PCR product. The fluorescence signal was plotted in real time against the temperature to produce melting curves of each sample. Melting curves were then converted to melting peaks by plotting the first negative derivative of the fluorescence, with respect to temperature, against temperature (–dF ÷ dT versus T). Thus, each specific PCR product generates a specific signal and therefore a product-specific melting peak.

After completion of LightCycler analysis, PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, visualized and photographed under ultraviolet illumination.

Real-time monitoring of amplification after each annealing period allows quantification of the samples during the log-linear phase of the PCR reaction. For quantitative analyses, the 'second derivative maximum method' with an efficiency-adjusted relative quantification of PCR amplicons,[34] was performed. Standard curves for the C3aR and the housekeeping gene, β-actin, were created, covering a range of six orders of magnitude by dilution series (dilutions from these standard curves were used as calibrators in each PCR run). These standard curves, describing the PCR efficiencies of the target (C3aR) and the reference gene (β-actin), allow an efficiency-corrected quantification using the Relative Quantification Software (Roche). The calibrator-normalized relative quantification results in a target concentration, which is expressed relative to the concentration of the reference gene in the same sample material.

Cytokine assessment

MoDC were stimulated as indicated, and the supernatants were harvested and analysed for IL-10 and IL-12p70 concentration using a commercially available enzyme-linked immunosorbent assay (ELISA) system (Ready-set-go ELISA; Biocharta, Hamburg, Germany).

Autologous proliferation assay

MoDC were either not stimulated or prestimulated for 48 hr with C3a (6 μg/ml), IFN-α + IFN-γ (each 200 U/ml), C3a plus IFN-α + IFN-γ, or TNF-α (200 U/ml) plus PGE_2 (1 μg/ml) as a positive control. $CD4^+$ autologous T cells were separated from non-adherent cells of the buffy-coat using the $CD4^+$ cells MACS isolation kit, according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of $CD4^+$ T cells was at least 95%, as determined by flow cytometry.

A total of 10^5 autologous T cells was added to 10^4 prestimulated and washed MoDC per well in 96-well culture plates. Tetanus toxoid (TT) (10 μg/ml), as antigen, was also added. Co-cultures without TT, T cells alone, T cells plus TT, and MoDC alone served as negative controls.

T cells and MoDC were co-cultured for 5 days, then radioactive thymidine (1 μCurie/well) was added for 16 hr. Afterwards, cells were harvested (PHD cell harvester; Cambridge Technology, Watertown, MA) and the radioactivity was counted in a liquid scintillation counter (Wallac 1409; Perkin Elmer Wallac, Freiburg, Germany).

Assessment of F-actin polymerization

Nitrobenzoxadiazole (NBD)-phallacidin (Molecular Probes, Eugene, OR) staining of MoDC was carried out by modification of the method described by Howard & Meyer.[35,36] Briefly, cells were resuspended at a concentration of 2×10^6 cells/ml in PBS lacking Ca^{2+} and stimulated with the indicated stimulus for different periods of time at room temperature. Following stimulation, cells were fixed using 2% formaldehyde for 60 min. Lysophosphatidylcholine (20 μg/ml, Sigma) and $3{\cdot}7 \times 10^{-7}$ M NBD-phallacidin were added to the sample and incubated for 60 min in the dark. NBD-phallacidin-stained cells were analysed on a Becton Dickinson FACScan with a linear fluorescence channel (FL-1) where the fluorescence is proportional to F-actin content.[36] Relative F-actin content is expressed as the ratio of the mean channel fluorescence (= integrated fluorescence) between stimulated and non-stimulated cells.

Boyden chamber chemotaxis assays

The chemotactic activity of MoDC was determined using a modified Boyden chamber technique, as described previously.[25,37] In brief, Boyden chambers (Nucleopore, Tübingen, Germany) were filled with stimuli [6 μg/ml C3a, 6 μg/ml C3a(desArg) or medium] and covered with polycarbonate filters (pore size 8 μm; Nucleopore). A 100-μl volume of a MoDC suspension, at a concentration of 1×10^6 cells/ml, was added to each chamber. Some MoDC were pretreated for 48 hr with IFN-α + IFN-γ (each 200 U/ml) to up-regulate the C3aR, or with TNF-α (200 U/ml) to down-regulate the C3aR. After incubation for 90 min at 37°, migrated cells in the lower part of the Boyden chambers were lysed by adding 0·1% Triton-X-100, and β-glucoronidase activity in the lysates was determined photometrically using *p*-nitrophenyl β-D-glucuronide as a substrate (all from Sigma). Readings were performed using the EAR400AT reader from SLT/TECAN Labinstruments (Crailsheim, Germany). For calculation of the number of migrated cells, equivalent to β-glucuronidase activity determined in the lower parts of the Boyden chambers, values were calculated from a standard curve using known numbers of MoDC. Chemotactic activity was expressed as follows:

Chemotactic index = quotient of the number of migrated cells in the presence of stimulus ÷ migrated cells in the presence of medium.

Statistical analysis

Statistical analyses were performed using the paired *t*-test.

RESULTS

Expression and regulation of the C3aR on MoDC

C3aR expression on MoDC was investigated at both protein and mRNA levels (Fig. 1). In a subset of 15–20% of immature MoDC, a low-level C3aR expression was detected by flow

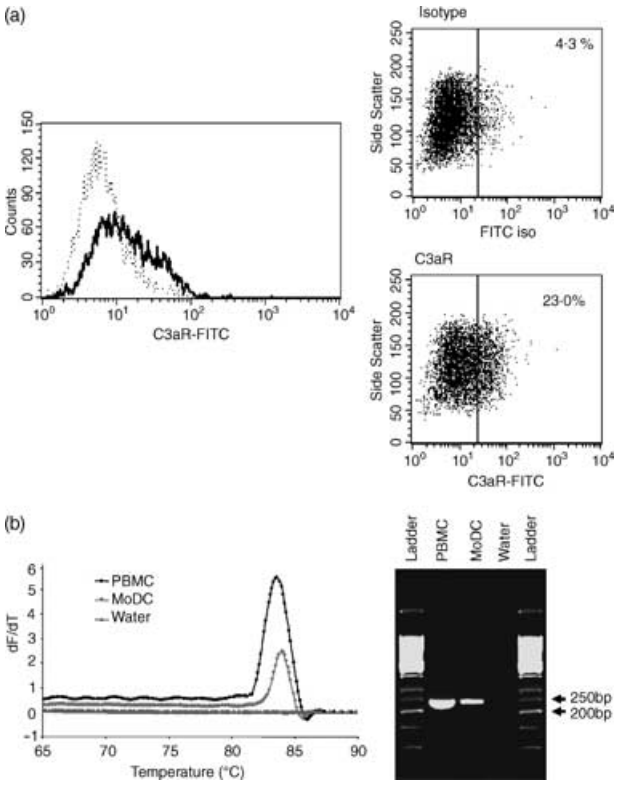

Figure 1. Expression of the C3a receptor (C3aR) on immature monocyte-derived dendritic cells (MoDC). C3aR expression was detected on immature MoDC by flow cytometry at the protein level (a) or by real-time LightCycler reverse transcription–polymerase chain reaction (RT–PCR) at the mRNA level (b). (a) A total of 15–20% of immature MoDC showed low-level C3aR expression. (b) Melting curve analysis of PCR reactions from immature MoDC showed a clear melting point that was identical to the melting point of peripheral blood mononuclear cells (PMBC) used as a positive control. Agarose-gel electrophoresis demonstrated one sharp band of the same size in both MoDC and PBMC. FITC, fluorescein isothiocyanate.

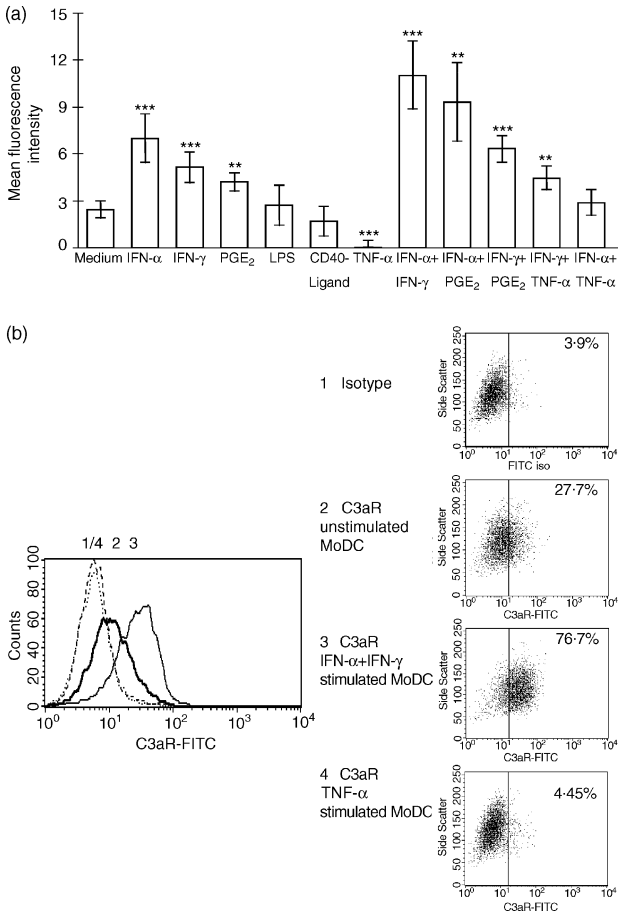

Figure 2. Regulation of the C3a receptor (C3aR) on monocyte-derived dendritic cells (MoDC) at the protein level. MoDC were incubated for 48 hr with various stimuli and then analysed for C3aR expression by flow cytometry. Expression of the C3aR was significantly up-regulated by interferon (IFN)-α, IFN-γ and prostaglandin E_2 (PGE_2), whereas lipopolysaccharide (LPS) and CD40 ligand (CD40L) had no significant effect, and tumour necrosis factor-α (TNF-α) strongly down-regulated the C3aR. The combination of IFN-γ + IFN-α was more effective than combinations of IFN with PGE_2 on up-regulation of the C3aR. (a) Mean fluorescence intensities (mean ± standard error of the mean) of 11 independent experiments; (b) shows one representative experiment. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, compared with the unstimulated control. FITC, fluorescein isothiocyanate.

cytometry (Fig. 1a). For the detection of C3aR mRNA, a real-time LightCycler PCR assay was developed. PCR reactions showed a clear melting point when analysed by melting curve analysis and a sharp band when analysed by agarose-gel electrophoresis (Fig. 1b).

Stimulation with IFN-γ, IFN-α and PGE_2 resulted in a significant up-regulation of C3aR expression in MoDC, as demonstrated by flow cytometry (Fig. 2). The combination of IFN-γ + IFN-α was more effective in up-regulating the C3aR than combinations with PGE_2 (Fig. 2a) and yielded a homogenous up-regulation of the C3aR on the majority of MoDC (Fig. 2b). This up-regulation of the C3aR could also be demonstrated at the mRNA level by efficiency-controlled real-time quantitative LightCycler PCR (Fig. 3).

In contrast, LPS or CD40L had no significant effect on C3aR expression (Fig. 2a), whereas TNF-α significantly down-regulated C3aR expression (Fig. 2a,2b).

This regulatory pattern of the C3aR was used in further experiments to enhance possible C3a effects (by up-regulation of the C3aR with IFN-γ + IFN-α) and to diminish possible C3a effects (by down-regulation of the receptor with TNF-α).

Effects of C3a on surface molecule expression and cytokine production of MoDC

MoDC were stimulated for 24 hr with C3a, and either not prestimulated or prestimulated with IFN-γ + IFN-α for 24 hr to up-regulate C3aR expression. Then, expression of a variety of cell-surface markers was determined by flow cytometry (Fig. 4) and cell-culture supernatants were analysed for expression of cytokines, in particular IL-10 and IL-12p70 (Fig. 5).

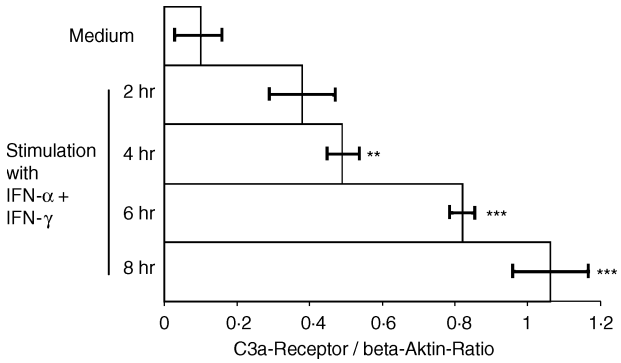

Figure 3. Regulation of the C3a receptor (C3aR) on monocyte-derived dendritic cells (MoDC) at the mRNA level. Quantitative real-time LightCycler polymerase chain reaction (PCR) was employed, as described in the Materials and methods, to determine the regulation of C3aR expression at the mRNA level. mRNA for the C3aR was significantly up-regulated after stimulation with interferon (IFN)-γ + IFN-α for 2–8 hr. The mean value ± standard error of the mean (SEM) of four independent experiments is shown. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, compared with the unstimulated control.

C3a significantly up-regulated the expression of CD83, CD54 and CD86 (Fig. 4). In contrast, the expression of CD40, HLA-DR and CD80 was not affected (data not shown). Although the effects on CD83, CD54 and CD86 expression were consistently observed, they were only slight (10–20% increase compared to controls) and only observed after up-regulation of the C3aR with IFN-γ + IFN-α (Fig. 4).

C3a was unable to alter IL-10 production in human MoDC (Fig. 5a). IFN-γ + IFN-α stimulation resulted in a higher IL-10 production of MoDC, but up-regulation of the C3aR did not gain C3a the ability to influence IL-10 production (Fig. 5a). IL-12p70 was not detectable in cell-culture supernatants of immature MoDC or MoDC stimulated with C3a, IFN-γ + IFN-α, or their combination (data not shown). After incubation of MoDC with polyinosinic-polycytidylic acid (polyI:C, a well known IL-12p70 inducer in human MoDC), MoDC produced detectable amounts of IL-12p70, which was increased 10-fold by pre-incubation with IFN-γ + IFN-α. However, C3a had no significant effect on IL-12p70 production, with or without IFN-γ + IFN-α prestimulation (Fig. 5b).

Effects of C3a-primed MoDC on proliferation of autologous T cells

The effect of C3a-primed MoDC on T-cell proliferation was studied in an autologous system using TT as antigen in donors immunized to TT (Fig. 6). Thymidine incorporation was used as evidence of T-cell proliferation. Preincubation with C3a did not significantly alter the ability of MoDC to stimulate autologous T-cell proliferation (Fig. 6). Incubation of MoDC with C3a after up-regulation of the C3aR with IFN-γ + IFN-α also did not increase their ability to stimulate autologous T-cell proliferation (Fig. 6), whereas incubation of MoDC with the strong maturation stimulus TNF-α + PGE_2 (used as a positive control) yielded an increase in the T-cell stimulatory capacity of MoDC (Fig. 6).

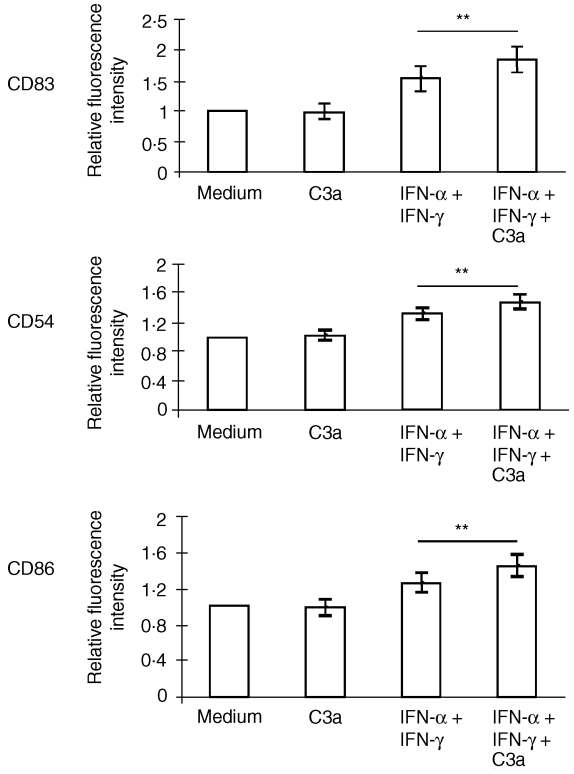

Figure 4. Effect of C3a on the expression of surface molecules on monocyte-derived dendritic cells (MoDC). MoDC were stimulated for 24 hr with C3a, either not prestimulated or prestimulated with interferon (IFN)-γ + IFN-α (200 U/ml each) for 24 hr to up-regulate C3a receptor (C3aR) expression. Mean fluorescence intensity of a variety of surface molecules was determined by flow cytometry. Relative fluorescence intensity was calculated by dividing the mean fluorescence intensity of the stimulated cells by the mean fluorescence intensity of the unstimulated cells (basal). The mean value ± standard error of the mean (SEM) of 11 independent experiments is shown. C3a increased consistently, but only slightly, the expression of CD83, CD54 and CD86 after up-regulation of the C3aR with IFN-γ + IFN-α ($^{**}P < 0{\cdot}01$).

Chemotactic effects of C3a on MoDC

The effect of C3a on the migratory capacity of MoDC was assessed by two different assays. First, by determining F-actin polymerization induced by C3a as a parameter for cytoskeleton reorganization and an indirect marker for cell migration. Second, by determining cell migration in Boyden chamber chemotaxis assays.

C3a induced F-actin polymerization in MoDC. The polymerization was increased after pretreatment of MoDC with IFN-γ + IFN-α to up-regulate the C3aR and decreased after down-regulation of the C3aR with TNF-α (Fig. 7). C3a(desArg) had no effect on F-actin polymerization (Fig. 7).

MoDC migrated towards a C3a gradient in Boyden chamber assays (Fig. 8). This effect was increased after pretreatment of MoDC with IFN-γ + IFN-α and decreased after preincubation of MoDC with TNF-α (Fig. 8). Interestingly, C3a(desArg) also resulted in MoDC migration in Boyden chambers. However, this effect was not significant in the six experiments performed

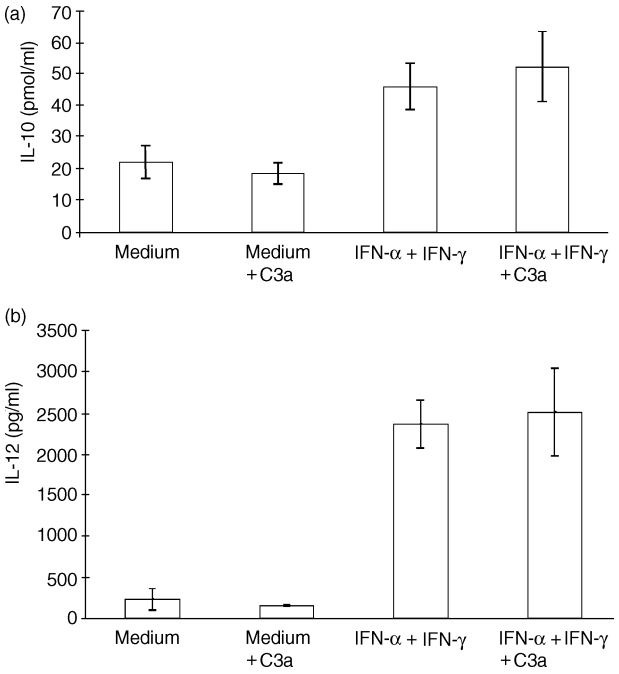

Figure 5. Effects of C3a on the cytokine production of monocyte-derived dendritic cells (MoDC). MoDC were stimulated for 24 hr with C3a, either not prestimulated or prestimulated with interferon (IFN)-γ + IFN-α (200 U/ml each) for 24 hr to up-regulate C3a receptor (C3aR) expression. Cell culture supernatants were analysed for concentrations of interleukin (IL)-10 (a) and IL-12p70 (b) by enzyme-linked immunosorbent assay (ELISA). The mean value ± standard error of the mean (SEM) of four independent experiments is shown. C3a did not significantly alter the IL-10 production of unstimulated or IFN-γ + IFN-α prestimulated MoDC (a). As IL-12p70 production was not observed in unstimulated MoDC, or in MoDC after stimulation with C3a, IFN-γ + IFN-α or their combination (data not shown), 10 μg/ml polyinosinic-polycytidylic acid (polyI:C, a well known IL-12p70 inductor in human MoDC) was added 16 hr before the supernatants were harvested. PolyI:C stimulation resulted in IL-12p70 production, which was markedly induced by IFN-γ + IFN-α pretreatment. However, C3a had no significant effect on IL-12p70 production (b).

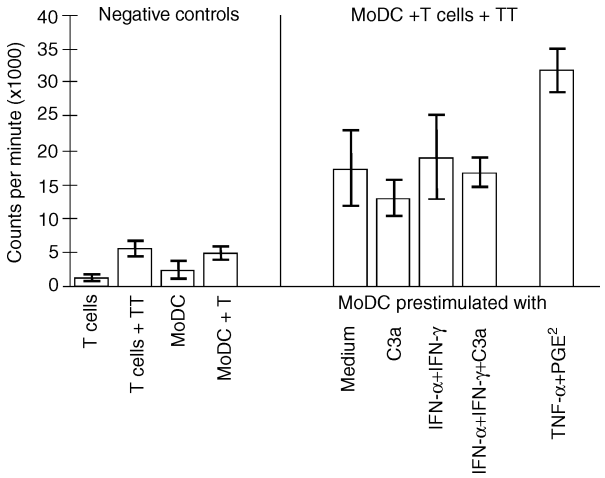

Figure 6. Effects of C3a on the T-cell stimulatory capacity of monocyte-derived dendritic cells (MoDC). MoDC (1×10^4 cells/well) were either not stimulated or stimulated with C3a with or without interferon (IFN)-γ + IFN-α to up-regulate the C3a receptor (C3aR). After 48 hr, MoDC were washed and autologous T cells (1×10^5 cells/well) and tetanus toxoid (TT; 10 μg/ml) were added. After 5 days of co-culture, cell proliferation was determined by the incorporation of radioactive thymidine. MoDC prestimulated with tumour necrosis factor-α (TNF-α) + prostaglandin E_2 (PGE_2) (a known strong maturation stimulus) served as positive control, co-cultures of MoDC and T cells without TT, T cells and MoDC alone and T cells plus TT served as negative controls. The mean value ± standard error of the mean (SEM) from six independent experiments is shown. Prestimulation of MoDC with C3a did not alter their ability to induce T-cell proliferation with or without up-regulation of the C3aR. Maturation of MoDC with TNF-α + PGE_2 resulted in an increase of the T-cell stimulatory capacity of MoDC as compared to the medium control.

and appeared to be independent of regulation of the C3aR by IFN-γ + IFN-α or TNF-α (Fig. 8).

DISCUSSION

Expression of the C3aR has been described on a variety of cells of myeloid origin, such as U937, and other myeloblastic cell lines,[38] monocytes[15,16,26,27] and macrophages.[30,31] In this study we confirm and extend our previous findings of expression of the C3aR on DCs of skin and blood[32] by a detailed analysis of C3aR expression and regulation on human MoDC. TNF-α, but not other maturation stimuli such as LPS or CD40L, was able to down-regulate the C3aR on MoDC; thus, down-regulation of the C3aR seems not to be a general phenomenon during maturation of MoDC, but rather a specific effect of TNF-α stimulation. IFNs and, less efficiently, PGE_2, were able to up-regulate the C3aR, the combination of IFN-α + IFN-γ being the most effective. This reflects the regulation of the C3aR on other cell types. Type I IFNs have been shown to up-regulate the C3aR on T lymphocytes,[28] and the type II IFN, IFN-γ, up-regulated the C3aR on U937 and other myeloblastic cell lines.[38]

The regulation of the C3aR is of consequence for the functional effects of C3a on MoDC. Whereas unstimulated MoDC did respond to C3a with slight (but significant) F-actin polymerization, a significant migration in Boyden chamber assays could not be demonstrated. This is in agreement with recently published results in a murine migration model, where human monocytes and MoDC were injected into the tail vein of severe combined immunodeficiency (SCID) mice and migration was measured towards a gradient of C3a that was injected into the peritoneal cavity.[26] In this model, for human monocytes, but not for (unstimulated) MoDC, a significant migration into the peritoneal cavity was seen. The difference was explained by the down-regulation of the C3aR during the differentiation from monocytes to MoDC in GM-CSF- and IL-4-containing medium, which was caused mainly by IL-4.[26] Also in our Boyden chamber model, C3aR expression on unstimulated MoDC was obviously too low to result in a significant migration of MoDC towards a C3a gradient. However, after up-regulation of the receptor with IFN-α + IFN-γ, a significant migration and a clear increase of F-actin polymerization could be demonstrated. This might have impact for diseases such as psoriasis, where DCs accumulate in a T helper 1 (Th1)-type milieu with

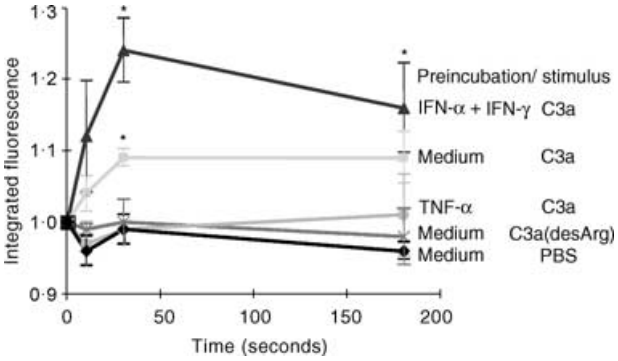

Figure 7. C3a induces F-actin polymerization in monocyte-derived dendritic cells (MoDC). For measurement of F-actin polymerization, MoDC were either not prestimulated or stimulated for 48 hr with interferon (IFN)-γ + IFN-α to up-regulate, or with tumour necrosis factor-α (TNF-α) to down-regulate, the C3a receptor (C3aR). Cells were then stimulated with phosphate-buffered saline (PBS) (as a negative control), C3a or C3a(desArg) for the indicated periods of time. Following stimulation, cells were fixed, stained with nitrobenzoxadiazole (NBD)-phallacidin and FL-1 fluorescence was detected as a measure for F-actin content, as described in the Materials and methods. Relative F-actin content is expressed as the ratio of the mean channel fluorescence (= integrated fluorescence) between stimulated and non-stimulated cells. The mean value ± standard error of the mean (SEM) of six independent experiments is shown. *Significant differences ($P < 0{\cdot}05$) in comparison to the medium control. C3a, but not C3a(desArg), induced a significant F-actin polymerization in MoDC, which was increased by the prestimulation of cells with IFN-γ + IFN-α and completely abolished after the prestimulation of MoDC with TNF-α.

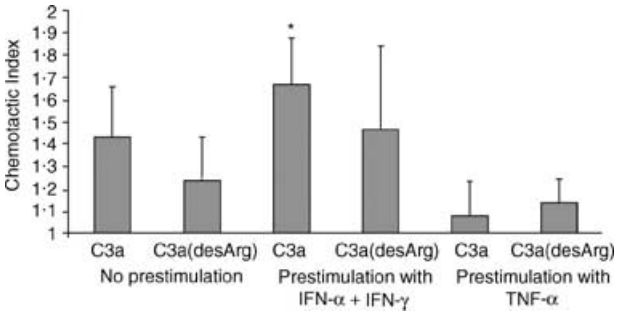

Figure 8. C3a induces the chemotactic migration of monocyte-derived dendritic cells (MoDC) in Boyden chamber assays. For measurement of chemotaxis, MoDC were exposed to a C3a or C3a(desArg) gradient in Boyden chambers for 90 min. Buffer instead of C3a or C3a(desArg) served as the background control. MoDC were either immature or prestimulated with interferon (IFN)-γ + IFN-α to up-regulate the C3a receptor (C3aR) or with tumour necrosis factor-α (TNF-α) to down-regulate the C3aR. Migrated cells in the lower part of the Boyden chambers were lysed and glucoronidase activity in the lysates was determined in a photometrical assay, as described in the Materials and methods. The chemotactic index was calculated as quotient of the number of migrated cells in the presence of stimulus ÷ migrated cells in the presence of medium. The mean value ± standard error of the mean (SEM) of six independent experiments is shown. *Significant differences ($P < 0{\cdot}05$) in comparison with the medium control. C3a induced cell migration of MoDC. Statistical analysis with the paired *t*-test revealed a trend for unstimulated MoDC ($P = 0{\cdot}109$) and a significant migration after up-regulation of the C3aR with IFN-γ + IFN-α ($P = 0{\cdot}024$). Down-regulation of the C3aR with TNF-α diminished MoDC migration. C3a(desArg) also induced some MoDC migration, which was not significant and appeared to be less dependent on the expression of the C3aR.

increased levels of IFNs. Psoriatic skin contains increased levels of C3a.[5] The C3a precursor, C3, can be produced by (psoriatic) keratinocytes,[39,40] and keratinocytes are able to degrade C3 to C3a via the alternative complement activation pathway.[41] Thus, it is tempting to speculate that C3a might be a factor that accounts for the accumulation of DCs in lesional psoriatic skin.

Interestingly, C3a(desArg), a C3a split product, led to a consistent background of chemotactic migration in Boyden chamber assays that appeared to be independent of regulation of the C3aR. Other effects of C3a(desArg) have been described, such as regulation of TNF-α, IL-1β and IL-6 synthesis in LPS-stimulated human PBMC.[42–44] As C3a(desArg) does not bind to or signal through the C3aR,[45,46] it might exert its effects via other receptors such as the recently described alternative anaphylatoxin receptors C5L2/grp77[47,48] or ChemR23.[49] Further investigations are required to clarify possible C3a(desArg) effects on MoDC and other cell types.

Either T helper 2 (Th2)-type immune reactions,[50,51] or immunosuppressive capabilities,[52,53] have been ascribed to C3a. Therefore, we tested the hypothesis that C3a might influence the maturation or T-cell stimulatory capacities of MoDC by investigating surface molecule expression, cytokine production and T-cell proliferation.

C3a had slight, but consistent, and therefore significant effects on CD54, CD83 and CD86 expression after up-regulation of the C3aR with IFN-γ + IFN-α. However, the biological relevance of this up-regulation of CD54, CD83 and CD86 remains uncertain as no effect on the capacity of MoDC to stimulate T-cell proliferation was observed.

In recently published studies, C3a was associated with Th2 reactions in murine models of asthma. Disruption of the genes for either C3 or the C3aR resulted in decreased numbers of eosinophils, IL-4-producing cells and Th2 cytokines in bronchoalveolar lavage fluids compared with wild-type mice.[50,51] IL-12p70 is an important cytokine by which DCs can influence the type of immunological reaction. DCs that run through a maturation process which allows them to produce IL-12p70 will stimulate a Th1-type immune response, DCs that stimulate T cells in the absence of IL-12p70 will result in a Th2-type immune response.[54] Therefore, we investigated a possible effect of C3a on IL-12p70 production or DC surface-molecule expression. However, the IL-12p70 production by polyI:C-stimulated MoDC was unaffected by C3a, even after up-regulation of the C3aR.

A number of anti-inflammatory effects have been assigned to C3a, e.g. suppression of LPS-induced TNF-α, IL-1β, IL-6 secretion from human PBMC[43,44] or suppression of human and murine antibody responses *in vitro*.[29,52,53] Subsequently, it has been suggested that the C3a-induced release of PGE_2 from macrophages could be a major element for the C3a-associated immunosuppression.[55] We investigated a possible induction of IL-10 in MoDC by C3a as another anti-inflammatory mediator. An increase of IL-10 by C3a could not be demonstrated, even

after up-regulation of the C3aR, and it seems unlikely that C3a stimulates the production of other immunosuppressive factors from DCs, because the ability of MoDC to stimulate T-cell proliferation was not altered by prestimulation of MoDC with C3a.

In conclusion, we demonstrate the expression of the C3aR on MoDC. The expression was up-regulated most efficiently by the combination of IFN-α + IFN-γ, whereas TNF-α, but not other maturation stimuli, down-regulated the C3aR. A significant chemotaxis towards a C3a gradient, and a slight increase of surface expression of CD54, CD83 and CD86, could be observed after up-regulation of the C3aR with IFN-α + IFN-γ. However, significant effects of C3a on IL-12p70 and IL-10 production or T-cell stimulatory capacity were not observed. Thus, C3a appears to have, in the appropriate microenvironment, chemotactic rather than immunomodulatory effects on human MoDC.

ACKNOWLEDGMENTS

This work was supported by grant Gu434/4-1 from the Deutsche Forschungsgemeinschaft to R.G. and T.W. The excellent technical assistance of Brigitta Köther is greatly appreciated.

REFERENCES

1 Schwartz LB, Kawahara MS, Hugli TE, Vik D, Fearon DT, Austen KF. Generation of C3a anaphylatoxin from human C3 by human mast cell tryptase. J Immunol 1983; **130:**1891–5.

2 Zilow G, Sturm JA, Rother U, Kirschfink M. Complement activation and the prognostic value of C3a in patients at risk of adult respiratory distress syndrome. Clin Exp Immunol 1990; **79:**151–7.

3 Kapp A, Meske-Brand S, Maly FE, Muller W. Complement activation in patients with chronic polyarthritis, measured by the level of complement fraction C3a in plasma. Z Rheumatol 1984; **43:**103–5.

4 Kapp A, Wokalek H, Schopf E. Involvement of complement in psoriasis and atopic dermatitis – measurement of C3a and C5a, C3, C4 and C1 inactivator. Arch Dermatol Res 1985; **277:**359–61.

5 Takematsu H, Ohkohchi K, Tagami H. Demonstration of anaphylatoxins C3a, C4a and C5a in the scales of psoriasis and inflammatory pustular dermatoses. Br J Dermatol 1986; **114:**1–6.

6 Ring J, Senter T, Cornell RC, Arroyave CM, Tan EM. Complement and immunoglobulin deposits in the skin of patients with atopic dermatitis. Br J Dermatol 1978; **99:**495–501.

7 Secher L, Permin H, Juhl F. Immunofluorescence of the skin in allergic diseases: an investigation of patients with contact dermatitis, allergic vasculitis and atopic dermatitis. Acta Derm Venereol 1978; **58:**117–20.

8 Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, Gerard NP, Gerard C. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. Nature 2000; **406:**998–1001.

9 Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Koehl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. Am J Respir Crit Care Med 2001; **164:**1841–3.

10 Kildsgaard J, Hollmann TJ, Matthews KW, Bian K, Murad F, Wetsel RA. Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. J Immunol 2000; **165:**5406–9.

11 Bautsch W, Hoymann HG, Zhang Q *et al.* Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. J Immunol 2000; **165:**5401–5.

12 Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB Jr, Tack BF, Wetsel RA. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. J Immunol 2001; **166:**2025–32.

13 Monsinjon T, Gasque P, Chan P, Ischenko A, Brady JJ, Fontaine MC. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. FASEB J 2003; **17:**1003–14.

14 Gasque P, Chan P, Fontaine M, Ischenko A, Lamacz M, Gotze O, Morgan BP. Identification and characterization of the complement C5a anaphylatoxin receptor on human astrocytes. J Immunol 1995; **155:**4882–9.

15 Zwirner J, Gotze O, Begemann G, Kapp A, Kirchhoff K, Werfel T. Evaluation of C3a receptor expression on human leucocytes by the use of novel monoclonal antibodies. Immunology 1999; **97:**166–72.

16 Martin U, Bock D, Arseniev L *et al.* The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. J Exp Med 1997; **186:**199–207.

17 Daffern PJ, Pfeifer PH, Ember JA, Hugli TE. C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. J Exp Med 1995; **181:**2119–27.

18 el Lati SG, Dahinden CA, Church MK. Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells. J Invest Dermatol 1994; **102:**803–6.

19 Hartmann K, Henz BM, Kruger-Krasagakes S *et al.* C3a and C5a stimulate chemotaxis of human mast cells. Blood 1997; **89:** 2863–70.

20 Nilsson G, Johnell M, Hammer CH, Tiffany HL, Nilsson K, Metcalfe DD, Siegbahn A, Murphy PM. C3a and C5a are chemotaxins for human mast cells and act through distinct receptors via a pertussis toxin-sensitive signal transduction pathway. J Immunol 1996; **157:**1693–8.

21 DiScipio RG, Daffern PJ, Jagels MA, Broide DH, Sriramarao P. A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration *in vitro* and *in vivo*. J Immunol 1999; **162:**1127–36.

22 Foreman KE, Glovsky MM, Warner RL, Horvath SJ, Ward PA. Comparative effect of C3a and C5a on adhesion molecule expression on neutrophils and endothelial cells. Inflammation 1996; **20:** 1–9.

23 Kretzschmar T, Jeromin A, Gietz C, Bautsch W, Klos A, Kohl J, Rechkemmer G, Bitter-Suermann D. Chronic myelogenous leukemia-derived basophilic granulocytes express a functional active receptor for the anaphylatoxin C3a. Eur J Immunol 1993; **23:**558–61.

24 Elsner J, Oppermann M, Czech W, Dobos G, Schopf E, Norgauer J, Kapp A. C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. Eur J Immunol 1994; **24:**518–22.

25 Elsner J, Oppermann M, Czech W, Kapp A. C3a activates the respiratory burst in human polymorphonuclear neutrophilic leukocytes via pertussis toxin-sensitive G-proteins. Blood 1994; **83:** 3324–31.

26 Soruri A, Kiafard Z, Dettmer C, Riggert J, Kohl J, Zwirner J. IL-4 down-regulates anaphylatoxin receptors in monocytes and dendritic cells and impairs anaphylatoxin-induced migration *in vivo*. J Immunol 2003; **170:**3306–14.

27 Zwirner J, Gotze O, Moser A, Sieber A, Begemann G, Kapp A, Elsner J, Werfel T. Blood- and skin-derived monocytes/macrophages respond to C3a but not to C3a (desArg) with a transient

release of calcium via a pertussis toxin-sensitive signal transduction pathway. Eur J Immunol 1997; **27:**2317–22.
28 Werfel T, Kirchhoff K, Wittmann M, Begemann G, Kapp A, Heidenreich F, Gotze O, Zwirner J. Activated human T lymphocytes express a functional C3a receptor. J Immunol 2000; **165:** 6599–605.
29 Fischer WH, Hugli TE. Regulation of B cell functions by C3a and C3a (desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. J Immunol 1997; **159:**4279–86.
30 Kacani L, Banki Z, Zwirner J, Schennach H, Bajtay Z, Erdei A, Stoiber H, Dierich MP. C5a and C5a (desArg) enhance the susceptibility of monocyte-derived macrophages to HIV infection. J Immunol 2001; **166:**3410–5.
31 Zwirner J, Werfel T, Wilken HC, Theile E, Gotze O. Anaphylatoxin C3a but not C3a (desArg) is a chemotaxin for the mouse macrophage cell line J774. Eur J Immunol 1998; **28:**1570–7.
32 Kirchhoff K, Weinmann O, Zwirner J, Begemann G, Gotze O, Kapp A, Werfel T. Detection of anaphylatoxin receptors on $CD83^+$ dendritic cells derived from human skin. Immunology 2001; **103:**210–7.
33 Gutzmer R, Langer K, Lisewski M, Riekborn D, Kapp A, Werfel T. Expression and function of histamine receptors H1 and H2 on human monocyte derived dendritic cells. J Allergy Clin Immunol 2002; **109:**524–31.
34 Pfafl MW. A new mathematical model for relative quantification in real-time RT PCR. Nucleic Acid Res 2001; **29:**E45.
35 Elsner J, Dichmann S, Dobos GJ, Kapp A. Actin polymerization in human eosinophils, unlike human neutrophils, depends on intracellular calcium mobilization. J Cell Physiol 1996; **167:**548–55.
36 Howard TH, Meyer WH. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. J Cell Biol 1984; **98:**1265–71.
37 Schroder JM, Mrowietz U, Morita E, Christophers E. Purification and partial biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. J Immunol 1987; **139:**3474–83.
38 Burg M, Martin U, Bock D, Rheinheimer C, Kohl J, Bautsch W, Klos A. Differential regulation of the C3a and C5a receptors (CD88) by IFN-gamma and PMA in U937 cells and related myeloblastic cell lines. J Immunol 1996; **157:**5574–81.
39 Basset-Seguin N, Caughman SW, Yancey KB. A-431 cells and human keratinocytes synthesize and secrete the third component of complement. J Invest Dermatol 1990; **95:**621–5.
40 Terui T, Ishii K, Ozawa M, Tabata N, Kato T, Tagami H. C3 production of cultured human epidermal keratinocytes is enhanced by IFNgamma and TNFalpha through different pathways. J Invest Dermatol 1997; **108:**62–7.
41 Terui T, Kato T, Tagami H. Complement activation by stratum corneum through the antibody- independent alternative pathway. Dermatologica 1989; **179:**123.
42 Fischer WH, Jagels MA, Hugli TE. Regulation of IL-6 synthesis in human peripheral blood mononuclear cells by C3a and C3a (desArg). J Immunol 1999; **162:**453–9.
43 Takabayashi T, Vannier E, Clark BD, Margolis NH, Dinarello CA, Burke JF, Gelfand JA. A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis. J Immunol 1996; **156:**3455–60.
44 Takabayashi T, Vannier E, Burke JF, Tompkins RG, Gelfand JA, Clark BD. Both C3a and C3a (desArg) regulate interleukin-6 synthesis in human peripheral blood mononuclear cells. J Infect Dis 1998; **177:**1622–8.
45 Hugli TE. Structure and function of C3a anaphylatoxin. Curr Top Microbiol Immunol 1990; **153:**181–208.
46 Wilken HC, Gotze O, Werfel T, Zwirner J. C3a (desArg) does not bind to and signal through the human C3a receptor. Immunol Lett 1999; **67:**141–5.
47 Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN. The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. J Biol Chem 2003; **278:**11123–9.
48 Ohno M, Hirata T, Enomoto M, Araki T, Ishimaru H, Takahashi TA. A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. Mol Immunol 2000; **37:**407–12.
49 Samson M, Edinger AL, Stordeur P *et al.* ChemR23, a putative chemoattractant receptor, is expressed in monocyte-derived dendritic cells and macrophages and is a coreceptor for SIV and some primary HIV-1 strains. Eur J Immunol 1998; **28:**1689–700.
50 Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. J Immunol 2001; **167:**4141–5.
51 Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. J Immunol 2002; **169:**5926–33.
52 Morgan EL, Weigle WO, Hugli TE. Anaphylatoxin-mediated regulation of the immune response. I. C3a- mediated suppression of human and murine humoral immune responses. J Exp Med 1982; **155:**1412–26.
53 Morgan EL, Thoman ML, Weigle WO, Hugli TE. Human C3a-mediated suppression of the immune response. I. Suppression of murine *in vitro* antibody responses occurs through the generation of nonspecific $Lyt\text{-}2^+$ suppressor T cell. J Immunol 1985; **134:**51–7.
54 Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999; **20:**561–7.
55 Morgan EL. The role of prostaglandins in C3a-mediated suppression of human *in vitro* polyclonal antibody responses. Clin Immunol Immunopathol 1987; **44:**1–11.