Ligand Specificity of the Anaphylatoxin C5L2 Receptor and Its Regulation on Myeloid and Epithelial Cell Lines*

Received for publication, October 16, 2006. Published, JBC Papers in Press, October 26, 2006, DOI 10.1074/jbc.M609734200

Kay Johswich†, Myriam Martin‡, Jessica Thalmann‡, Claudia Rheinheimer§, Peter N. Monk‡, and Andreas Klos‡

From the †Department of Medical Microbiology, Medical School Hannover, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany and the §Department of Neurology, School of Medicine and Biomedical Sciences, Sheffield S10 2RX, United Kingdom

During complement activation the pro-inflammatory anaphylatoxins C3a and C5a are generated, which interact with the C3a receptor and C5a receptor (CD88), respectively. C5a and its degradation product C5a-des-Arg74 also bind to the C5a receptor-like 2 (C5L2). C3a and C3a-des-Arg77, also called acylation-stimulating protein, augment triglyceride synthesis and glucose uptake in adipocytes and skin fibroblasts. Based on data obtained using transfected HEK293 and RBL cells, C5L2 is additionally proposed as a functional receptor for C3a and C3a-des-Arg77. Here we use 125I-ligand binding assays and flow cytometry with fluorescently labeled ligands to demonstrate that neither C3a nor C3a-des-Arg77 binds to C5L2. C5L2 expression and its regulation are investigated on various cell lines by a novel C5L2-restricted binding assay and quantitative real time PCR. Dibutyryl cAMP and interferon-γ stimulate protein kinase C activation that triggers diacylglycerol acyltransferase activity (3). Additionally, C3a-des-Arg77 promotes translocation of glucose transporters GLUT1, GLUT3, and GLUT4 to the plasma membrane, leading to a higher rate of glucose influx into the cells (4). Moreover, adipose tissue serves as producer of complement factor C3, factor B and D (5, 6). In vitro experiments demonstrated enhanced levels of C3a-des-Arg77 in the supernatant of adipocytes stimulated with insulin or chylomicrons (7). However, the exact mechanism of C3a-des-Arg77 formation in adipose tissue is not known.

C5L2 is the third member of the anaphylatoxin receptor family sharing 58% identity to C5aR and 55% identity to C3aR in the transmembrane domains (8). Northern blot data suggest that C5L2 is coexpressed with C5aR on various cells and tissues, such as neutrophils/macrophages, mast cells, immature dendritic cells, as well as in the brain, lung, heart, kidney, liver, ovary, or testis (8–12). However, other data concerning C5L2 expression on neutrophils are conflicting (10, 11). C5L2 binds C5a with nearly the same affinity as the C5aR and C5a-des-Arg74 with a 20-fold higher affinity compared with C5aR (13). C5L2 couples only weakly to G-proteins because of the lack of key sequence motifs for interaction of heptahelical receptors with the corresponding G-proteins. For example, the DRY (DRF in CD88) motif is DLC in C5L2, and the mutation of this sequence to DRC can partially restore signaling ability (10). In cells transfected with wild-type C5L2, no increase in cytosolic calcium levels or activation of the mitogen-activated protein kinase pathway could be observed, and C5L2-transfected RBL cells failed to degranulate upon stimulation with C5a, C3a, or C5a-des-Arg74 (10, 13). C5L2−/− mice show increased influx of neutrophils into the lungs and higher levels of TNF-α and IL-6 when compared with wild-type mice in a model of pulmonary immune complex injury (14). Thus it is likely that C5L2 serves as a nonsignaling decoy receptor for C5a with a negative modulatory influence on C5aR-mediated inflammation. However, C5L2 is also thought to be the specific receptor for C3a-des-
Arg77 (ASP) and C3a, mediating their effects on lipid metabolism and glucose uptake in adipocytes and skin fibroblasts (15, 16). C5L2 might therefore play an important role in obesity and related diseases. However, ligand specificity of C5L2 remains a controversial issue. On the one hand, C5L2 was reported to bind solely C5a and C5a-des-Arg74 and no other anaphylatoxic peptide (10). On the other hand, binding of C3a, C3a-des-Arg77 (ASP), and even C4a has been reported (16). Anaphylatoxin-induced internalization of C5L2 is disputed as well; β-arrestin-mediated internalization in transfected HEK293 cells was observed not only after binding of C5a but also of C3a and C3a-des-Arg77 (15). Conversely, other authors did not find C5L2 internalized after ligand binding (10, 13).

Regulation of C5L2 as decoy receptor could help to fine-tune the C5a-mediated inflammatory response. C5L2 is known to be regulated during sepsis in mice, rats, and humans. It is up-regulated in lung, liver, and heart but not in kidneys of mice in a cecal ligation and puncture model of sepsis (17). However, results differ among different publications (12, 17) for C5L2 up- or down-regulation in neutrophils from cecal ligation and puncture rats. In human patients C5L2 is transiently down-regulated in neutrophils during sepsis (12). Apart from sepsis, nothing is known about the regulation of C5L2, and no regulating factors have been described so far.

Therefore, in this study we wanted to clarify whether C3a and C3a-des-Arg77 (ASP) bind specifically to C5L2 or not, and whether this ligand/receptor system can be directly involved in the regulation of lipid metabolism. An additional objective was to identify C5L2-expressing cells and stimuli that regulate C5L2 expression in naturally expressing cell types.

**MATERIALS AND METHODS**

Recombinant human IL-1β, IFN-γ, and TNF-α were purchased from R & D Systems (Minneapolis, MN), and IL-6 was obtained from Tebu-Bio (Offenbach, Germany). All other materials were obtained from Sigma unless indicated otherwise.

**Cell Lines and Culture Conditions**—The human embryonic kidney cell line HEK293 (ATCC CRL 1573) was cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 10% fetal calf serum; human epithelial (cervix carcinoma) HeLa cells (kindly provided by R. Heilbronn, Berlin, Germany) were cultured in minimum Eagle’s medium supplemented with sodium pyruvate, nonessential amino acids, and 10% fetal calf serum. The rat basophilic leukemia cell line RBL-2H3 (CRL 1593; ATCC, Manassas, VA; a gift from Dr. M. Oppermann, Göttingen, Germany) was cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum; RBL cells stably transduced with C3aR or C5L2 were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 800 μg/ml G-418. All media, fetal calf serum, and additives and G-418 were purchased from PAA (Pasching, Austria).

**Subcloning of C5L2 and C3aR and Stable Transduction of RBL and HEK Cells**—RBL cells and HEK cells were stably transduced with human C3a receptor and C5L2 using a retroviral gene transfer system (RetroX®, Clontech). Human C3a receptor was subcloned into pQCXIN® (Clontech) from genomic DNA using the sense primer 5′-AAAAAGCGGCCCAGccac-gactggctttcttcgc-3′ and the antisense primer 5′-AAAAAG-GATCCtcacagttgattc-3′. The added NotI and BamHI are shown in italics, and the Kozak sequence is underlined.

**Competitive 125I-Labeled Ligand Binding Assays**—Binding assays were carried out in HAG-CM (20 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 0.25% BSA, 0.5 mM glucose) in a total volume of 50 μl per well of a microtiter plate (Greiner, Esen, Germany). Tracer concentrations of 125I-labeled anaphylatoxins (0.12 nM for 125I-C3a and 125I-C3a-des-Arg77 or 0.1 nM for 125I-C5a, respectively) were incubated overnight at 4 °C with increasing concentrations of nonlabeled anaphylatoxins (Calbiochem) and 3 × 105 stably transfected RBL cells or 1.5 × 105 transiently transfected HEK293 cells per well or (because of extremely high receptor expression) 6.25 × 104 transiently transfected HEK293 cells per well, respectively. 40 μl of the mixture was filtered using Multiscreen-HTS® filter plates (Millipore, Billerica, MA) and washed twice with 100 μl of HAG-CM. The filter plates were dried, and 50 μl of MicroScint O (PerkinElmer Life Sciences) per well were added. Bound radioactivity was determined using a TopCount NXT (Canberra-Packard, Dreieich, Germany). For 125I-C3a- and 125I-C3a-des-Arg77-binding assays, the filter plates were pre-treated with 2% protamine sulfate overnight at 4 °C and extensively washed with HAG-CM before use. For selective detection of C5L2, the cells were preincubated with the C5aR inhibitor AcF[OPdChaWR] for 10 min at room temperature prior to adding the cells to the mixture of labeled and unlabeled ligand leading to a final concentration of 1 μM of the antagonist in the reaction mixture.

**Ligand Specificity and Regulation of C5L2**

Transient Transfection of HEK293 Cells—HEK cells were transiently transfected with pEG6-HCMV.neo-C5L2 (13) using Lipofectamine™ reagent (Invitrogen) according to the manufacturer’s protocol.

**RNA Preparation and cDNA Synthesis**—Total RNA was isolated from cell culture using TRizol™ reagent (Invitrogen) according to the manufacturer’s instructions. Five μg of total RNA was used for transcription with SuperScript II RNaseH− reverse transcriptase (Invitrogen) using oligo(dT) primers (Stratagene, La Jolla, CA).

**C5L2 Real Time PCR**—TaqMan real time PCR was performed using a C5L2-specific TaqMan gene expression assay (hC5L2_s3) in combination with the TaqMan universal PCR master mix and the 7000 sequence detection system for analysis (all from Applied Biosystems, Weiterstadt, Germany). For normalization, the housekeeping gene 18S-rRNA (gene expression assay Hs99999901_s1) was used. Standard curves were generated using serially diluted cDNA.
Ligand Specificity and Regulation of C5L2

![Diagram of binding specificity](image)

**FIGURE 1. C5L2 binds only C5a, but not C3a or C3a-des-Arg as determined by competitive binding studies.**  

--0.1 nM 125I-C3a or 125I-C5a was used as tracer and increasing concentrations of nonlabeled C3a, C3a-des-Arg, or C5a as competitor. HEK293 cells were transiently transfected to express C5L2 (large panel), the C3a receptor (upper right panel), or neither of the two receptors (lower right panel). Depicted is one representative binding study of three.

125I-C3a-des-Arg was generated by treatment of 125I-C3a with 0.6 units of carboxypeptidase B (Calbiochem) per pmol for 20 min at 37 °C in 130 mM NaCl, 2.5 mM KCl, 10 mM NaHCO3, 0.5 mM NaHPO4, 1 mM MgCl2, followed by heat inactivation of the enzyme at 70 °C for 10 min. Specificity of the carboxypeptidase was proved by inhibition of the des-arginination reaction of nonlabeled C3a with Plummer’s inhibitor (DL-2-mercaptoethyl-3-guanidinoethylthiopropanic acid; Calbiochem). Heating C3a or 125I-C3a to 70 °C for 10 min did not affect activity of the ligands as tested by binding assays.

At least three independent binding studies were performed for each cell line and receptor. The number of binding sites per cell and the affinity were determined by iterative curve fitting using Ligand (Kell) software (Biosoft, Cambridge, UK). Transiently transfected HEK293 cells showed high expression levels of the C5L2 receptor in the range of 3.3–5 × 10^5 C5L2 receptors per cell and a K_d of 0.1–0.3 nM. C3aR expression was in the range of 2.5–5 × 10^5 receptors per cell with a K_d of 0.7–1.0 nM. On transduced HEK293 cells (HEK-C5L2, HEK-C5aR, and HEK-C3aR), 1.2–2.8 × 10^5 C5L2, 2.5αR, or C3aR per cell was found with a K_d of 1–2 nM for C5L2, 2–6 nM for C5aR, and 3–6 nM for C3aR.

**Fluorescence Labeling of Anaphylatoxins and Flow Cytometry—**C3a, C3a-des-Arg, and C5a were fluorescently labeled using MFP488 N-hydroxysuccimide-ester (MobITec, Göttingen, Germany) in 160 mM NaHCO3 for 1 h at 20 °C. Excess of free MFP488 N-hydroxysuccimide-ester was eliminated using Centri-Spin 10 columns (EMP Biotech, Berlin, Germany). Mass spectrometry indicated 1–4 labels per molecule of anaphylatoxin. For binding assays 5 × 10^6 cells were incubated overnight at 4 °C in a final volume of 25 µl of buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 0.25% BSA, 0.5 mM glucose) with 50 nM of fluorescently labeled anaphylatoxin. Specificity of binding was determined using 20-fold excess of unlabeled anaphylatoxin. Cells were then washed twice in 150 µl of ice-cold buffer and centrifuged at 500 x g. Cells were then fixed using Cellfix (BD Biosciences), and flow cytometry was performed using a FACScalibur cytometer (BD Biosciences).

Fura2 Assay for Determination of Free Cytosolic Ca^{2+}—Cells were loaded with 10 µM Fura2-AM (Calbiochem) in the presence of 0.2% Pluronic F-127 at a density of 1 × 10^7 cells/ml in HBSS (20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 0.5% BSA) for 30 min at 37 °C and then diluted 1:10 with HBSS and again incubated for 30 min at 37 °C. Cells were washed with HBSS and resuspended at a density of 1 × 10^7 cells/ml. Measurements of [Ca^{2+}]_i were carried out in the luminescence spectrometer LS 50B (PerkinElmer Life Sciences) at 37 °C in a total volume of 500 µl. Therefore, the Fura2-AM-loaded cells were diluted 1:10 in HBSS and 25 µl of stimuli were added after recording of the baseline. For C5L2-restricted measurements, the cells were preincubated for 10 min at 37 °C with AcF[OPdChaWR] at a final concentration of 10 nM. [Ca^{2+}]_i was calculated as described by Grynkiewicz et al. (18).

**RESULTS AND DISCUSSION**

C5L2 Is Neither a High nor a Low Affinity Receptor for C3a or C3a-des-Arg—C3a and C3a-des-Arg (ASP) are known to stimulate triglyceride synthesis and glucose uptake in adipocytes and skin fibroblasts. C5L2 is proposed to be the functional receptor for these mediators. This claim has been primarily based on binding studies performed with transfected RBL cells (16). Functional responses, phosphorylation, and internalization were observed in transfected HEK293 cells after stimulation with C3a or C3a-des-Arg (15). However, the binding of C3a and C3a-des-Arg (ASP) to C5L2 is still a controversial matter.

To clarify this discrepancy, competitive 125I-ligand binding assays as well as flow cytometry using fluorescently labeled ligands were performed. HEK293 cells were transiently transfected expressing either C5L2, C5aR, or C3aR. In accordance with Okinaga et al. (10), no specific binding of 125I-C3a or 125I-C3a-des-Arg to C5L2 could be observed in C5L2-transfected HEK cells. Specific binding of C5a demonstrated that high expression levels of this anaphylatoxin receptor had been achieved (Fig. 1, left panel). 125I-C3a-des-Arg was generated by carboxypeptidase B treatment of 125I-labeled C3a. The integrity of the 125I-C3a was demonstrated by its specific binding to C3aR-transfected cells (Fig. 1, upper right panel). The complete des-argination by carboxypeptidase B treatment was confirmed in the same binding study, as C3a-des-Arg generated did not bind to the C3aR (Fig. 1, upper right panel). No binding of 125I-labeled C3a, C3a-des-Arg, or C5a occurred in mock-transfected cells (Fig. 1, lower right panel).
Additionally, RBL cells expressing C5L2 or C3aR were generated using viral gene transfer and expression systems. The transduced RBL cells showed exactly the same binding pattern of $^{125}$I-C3a and $^{125}$I-C3a-des-Arg$^{77}$ (data not shown) as described for transiently transfected HEK293 cells, indicating that the lack of binding of these ligands to C5L2 was independent of the cell line used. Taken together, these data exclude high affinity binding of C3a or C3a-des-Arg$^{77}$ to C5L2 in HEK293 cells or RBL cells.

However, a low affinity interaction with these ligands could not be excluded using competitive binding assays because the $^{125}$I-labeled ligands must be applied in tracer amounts ($\sim 0.1 \text{ nM}$) for this type of assay. To investigate whether C3a and C3a-des-Arg$^{77}$ bind to C5L2 with low or intermediate affinity, flow cytometry using $50 \text{ nM}$ fluorescently labeled C3a, C3a-des-Arg$^{77}$, and C5a was performed (Fig. 2). For this purpose, HEK293 cells highly expressing C5L2, C5aR, or C3aR were generated (HEK-C5L2, HEK-C5aR, and HEK-C3aR) using the above-mentioned retroviral gene transfer and expression system. Exposure of transduced HEK-C5L2 to MFP488-labeled C3a or C3a-des-Arg$^{77}$ did not lead to a shift in fluorescence intensity (Fig. 2a, b) and of fluorescently labeled C5a to C5L2 (b) and of fluorescently labeled C3a to the C3aR (c) could be inhibited (thin lines) almost down to the buffer controls (shaded curves) by a 20-fold excess of the corresponding nonlabeled anaphylatoxins. No binding of fluorescently labeled C3a or C3a-desArg$^{77}$ was observed in mock transduced cells (d). Depicted is one flow cytometric analysis representative of three separate experiments.

![Flow cytometric analysis of transduced HEK293 cells](image)

**FIGURE 2.** Flow cytometric analysis of transduced HEK293 cells highly expressing C5L2 or C3aR demonstrating that C5L2 binds C5a but not C3a or C3a-des-Arg$^{77}$ (ASP). HEK293 cells were incubated with $50 \text{ nM}$ MFP488-labeled C3a or C3a-des-Arg$^{77}$ or C5a as indicated. Incubation of C5L2-transduced HEK293 cells with fluorescently labeled C3a or C3a-des-Arg$^{77}$ did not lead to a shift in fluorescence intensity (a, b). Binding of fluorescently labeled C5a to C5L2 (b) and of fluorescently labeled C3a to the C3aR (c) could be inhibited (thin lines) almost down to the buffer controls (shaded curves) by a 20-fold excess of the corresponding nonlabeled anaphylatoxins. No binding of fluorescently labeled C3a or C3a-desArg$^{77}$ was observed in mock transduced cells (d). Depicted is one flow cytometric analysis representative of three separate experiments.

**FIGURE 3.** Apparently specific binding of $^{125}$I-C3a (upper panel) or $^{125}$I-C3a-des-Arg$^{77}$ (lower panel) to filter plates/plastic in the absence of any cells. The calculated $K_d$ was $25 \text{ nM}$. This phenomenon only occurs with C3a/C3a-des-Arg$^{77}$ (closed symbols) and not with C5a (data not shown) after presaturation with BSA. Pretreatment of the plates with protamine sulfate inhibited this type of C3a/C3a-des-Arg$^{77}$ binding (open symbols). Depicted is one representative binding study from three performed.
applied to verify the integrity of native C3a-des-Arg\(^{77}\) as well as of the MFP488-labeled C3a-des-Arg\(^{77}\), the ligands being used in the competitive binding studies and flow cytometric experiments. As expected, the mass of MFP488-labeled and nonlabeled C3a-des-Arg\(^{77}\) differed from the corresponding form of C3a only by the mass of the enzymatically removed amino acid arginine. Notably, no degradation products of C3a or C3a-des-Arg\(^{77}\) were detectable (data not shown).

Taken together, flow cytometric analysis with fluorescently labeled ligands confirmed the results of the competitive \(^{125}\)I-ligand binding assays and additionally excluded a low affinity interaction of C3a and C3a-des-Arg\(^{77}\) with C5L2.

Nonreceptor Binding of C3a and C3a-des-Arg\(^{77}\) as a Possible Reason for Controversial Results among Previous Reports—Our preliminary binding assays with \(^{125}\)I-labeled C3a and C3a-des-Arg\(^{77}\) suggested an explanation for previous reports of binding to C5L2. There was a remarkably high level of binding of these ligands to the filter membranes/plastics used in binding assays even after pretreatment of the filter membranes with 0.25% BSA solution. This binding was highly suggestive of a specific receptor-ligand interaction and was not shown by \(^{125}\)I-C5a (Fig. 1). In contrast, \(^{125}\)I-labeled C3a and C3a-des-Arg\(^{77}\) bound at very high levels in binding assays even in the absence of any cells. More important, this binding to filter membranes could be competed with nonlabeled C3a or C3a-des-Arg\(^{77}\) with an IC\(_{50}\) of \(~25\) nM (Fig. 3). To overcome this critical and potentially misleading technical problem, we pretreated the filters in all our \(^{125}\)I-C3a and \(^{125}\)I-C3a-des-Arg\(^{77}\) binding assays with the cationic agent protamine sulfate (Figs. 1 and 3). Hence, this unusual and apparently specific binding to filter membranes/plastics might be one explanation for the controversial findings in previous studies. However, it should be noted that binding of C3a-des-Arg\(^{77}\) to filter membranes instead of cells might explain only divergent results of ligand binding assays.

Kalant et al. (15) also demonstrated that addition of C3a-des-Arg\(^{77}\) to C5L2-transfected HEK293 cells increased glucose uptake and triglyceride production. In adipocytes, triglyceride synthesis correlated with surface expression of C5L2 in knockdown experiments using small interfering RNA. Moreover, \(\beta\)-arrestin recruitment and internalization could be visualized in these cells after stimulation with C3a, C3a-des-Arg\(^{77}\), C5a, and C5a-des-Arg\(^{77}\). However, these functional data do not prove at all a direct interaction of C3a or C3a-des-Arg\(^{77}\) with C5L2. Consequently, it is feasible that C5L2 might be indirectly involved in mediating functions of these complement fragments.

It should be noted that other ligand binding assay protocols for the characterization of the C3a receptor applying sucrose gradient centrifugation instead of filter membranes are not prone to this phenomenon (19, 20).
Additionally, one has to address the question of the conditions in which C3a-des-Arg77/ASP has been shown to influence lipid metabolism. The functional assays (15) were performed using relatively high concentrations of C3a or C3a-des-Arg77 in the range of 1 μM and above. This would only be achieved by a cleavage of ~20% of serum C3. It is unlikely that such a degree of complement activation would occur in peripheral blood under physiological conditions. However, it is feasible to assume that lipid metabolism is influenced by C3a-des-Arg77 in pathophysiological situations with a high degree of complement activation, i.e. in diseases like sepsis. Additionally, such high concentrations of this stimulus might also be found locally in the adipose tissue, e.g. if bacteria are present. On the other hand, several enzymes such as secreted procathepsin-L (21), thrombin (22), and others (23–25) can cleave complement factors C3 or C5, respectively, independent of complement activation by the classical, the alternative, or the lectin pathway. Similar inflammation-independent mechanisms might lead to a local increase of C3a-des-Arg77 in adipose tissue.

**Design of a Novel C5L2-restricted Competitive [125]I-C5a Binding Assay**—To easily detect and quantify C5L2 expression on target cells, a modified competitive [125]I-C5a binding assay was designed that restricted specific C5a binding to C5L2. To discriminate C5L2 from C5aR, the binding assay was carried out in the presence of the C5aR-specific cyclic antagonist AcF[OPdChaWR]. 1 μM of the antagonist was sufficient to completely prevent binding of [125]I-C5a to transiently C5aR-transfected HEK293 cells (Fig. 4, left panel), but even 0.1 μM of the antagonist had no effect on C5a binding to C5L2-transfected HEK293 cells (Fig. 4, right panel). To prove the specificity of the antagonist, a structurally related cyclic peptide in which alanine replaces tryptophan (AcF[OPdChaAR]) was applied in the same concentrations and caused no inhibition of [125]I-C5a binding to the C5aR (data not shown). This binding assay was used to determine C5L2 expression on various cell lines.

**Detection of C5L2 and Its Regulation on Myeloid and Epithelial Cell Lines**—C5L2 is apparently broadly expressed, but no cell lines have been shown to express this receptor at high levels, and only few data are available on its regulation. Applying the C5L2-restricted [125]I-C5a binding assay, various cell lines were screened for the expression of this receptor before and after treatment with different stimuli.

Both C3aR and C5aR are known to be up-regulated in myeloblastic cell lines after induction with Bt2cAMP or IFN-γ, respectively (19, 26). Here we wanted to analyze whether C5L2 is also regulated in these cells. Native U937 and HL-60 cells expressed undetectable levels of C5L2 or C5aR, but both receptors were up-regulated after 3 days of treatment with Bt2cAMP, as determined by competitive [125]I-C5a binding in the absence of any C5aR inhibitor (not depicted) and C5L2-restricted competitive [125]I-C5a binding in the presence of the antagonist (HL-60 cells, Kd = 0.5–0.8 nM, 2–2.8 × 10^5 C5L2 receptors per cell, n = 3; U937 cells, Kd = 0.6–0.8 nM, 0.4–1.4 × 10^5 C5L2 receptors per cell, n = 3) (Fig. 5, left panels). However, C5aR is more strongly expressed than C5L2 (~5-fold in U937 and ~9-fold in HL-60 cells; data not shown) (19). In U937 but not in HL-60 cells, a slight increase in C5L2-specific [125]I-C5a binding could be observed after IFN-γ treatment. TaqMan real time PCR revealed that C5L2 transcription was induced by more than 100-fold by Bt2cAMP (1 mM for 3 days) in HL-60 cells. IFN-γ (1,000 units/ml for 3 days) had only a slight effect, and TNF-α (20 ng/ml for 3 days) had no effect on C5L2 transcription in these cells (Fig. 5, upper panel). Induction of U937 cells with Bt2cAMP also led to a drastic increase in C5L2 mRNA (~40-fold). In contrast, IFN-γ and TNF-α caused only a small or no elevation in C5L2 mRNA (~10-fold) in this cell line (Fig. 5, right lower panel).

Epithelial HeLa cells showed specific binding of [125]I-C5a indicating constitutive expression of C5aR and/or C5L2. No difference in binding was observed in the presence or absence of 1 μM AcF[OPdChaWR], indicating that only C5L2 but not C5aR is expressed on these cells (Kd = 0.2–0.73 nM, 9–11 × 10^3 C5L2 per cell, n = 3) (Fig. 6). C5L2 expression levels in HeLa cells decreased drastically during a 3-day period of treatment with either 1,000 units/ml IFN-γ or 20 ng/ml TNF-α as assessed by C5L2-restricted [125]I-C5a binding (Fig. 6, lower panel). In contrast, Bt2cAMP, IL-1β, and IL-6 did not alter C5L2 expression in these cells (data not shown). TaqMan real time PCR confirmed a higher receptor mRNA level in native HeLa cells compared with various other cell lines. The amount of C5L2 mRNA (normalized in relation to 18 S rRNA) was ~20-fold
Depicted are the results of one representative experiment. HL-60 or U937 cells does not lead to any increase in [Ca_{2+]}. This receptor couples to intracellular calcium signaling in cells. In this study we wanted to investigate whether this anaphylatoxin receptor couples to intracellular calcium signaling in cells. Additionally, after binding of C5a, C5L2 shows only a basal level of this activated protein kinase pathway after addition of C5a. Addi-
tionally, after stimulation with up to 100 nM C5a, no increase in cytosolic calcium could be observed in the absence or presence of the C5a receptor inhibitor (data not shown).

Together, our observations support the hypothesis that C5L2 is a scavenger receptor for C5a and C5a-des-Arg^{74}, which does not couple to G-proteins, even in cells endog-
ernously expressing C5L2. C5L2 does not bind C3a or C3a-des-
Arg^{77}/ASP, as demonstrated in the same cell lines (HEK and RBL) that were used previously to demonstrate binding or functional responses (15). These results confirm observations of Okinaga et al. (10) and Gerard et al. partially obtained on knock-out mice (14), which indicate strongly that C5L2 is a decoy receptor for C5a and C5a-des-Arg^{74} but not a receptor for C3a. Previous reports on the specific binding of C3a and C3a-des-Arg^{77} to C5L2-expressing cells might have been mis-
led by the apparently specific binding of these ligands to plastic surfaces and/or filter membranes. Nevertheless, we cannot exclude the possibility that C3a-des-Arg^{77} can affect lipid metabolism if applied in a relatively high concentration and that C5L2 might influence this process indirectly. Additional studies will define the role of C5L2 in this context. Further investigation is also needed to clarify whether high amounts of C3a or C3a-des-Arg^{77} can be found in adipose tissue and to elucidate putative new mechanisms that locally produce these complement split products independent of inflammation.

Acknowledgment—We thank the head of the Department of Medical Microbiology, Medical School Hanover, Prof. Suerbaum, for his support.

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Ligand Specificity and Regulation of C5L2

DECEMBER 22, 2006 • VOLUME 281 • NUMBER 51
JOURNAL OF BIOLOGICAL CHEMISTRY 39095

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