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The C5a Receptor (C5aR) C5L2 Is a Modulator of C5aR-mediated Signal Transduction*

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The complement anaphylatoxin C5a is a proinflammatory component of host defense that functions through two identified receptors, C5a receptor (C5aR) and C5L2. C5aR is a classical G protein-coupled receptor, whereas C5L2 is structurally homologous but deficient in G protein coupling. In human neutrophils, we show C5L2 is predominantly intracellular, whereas C5aR is expressed on the plasma membrane. Confocal analysis shows internalized C5aR following ligand binding is co-localized with both C5L2 and β-arrestin. Antibody blockade of C5L2 results in a dramatic increase in C5a-mediated chemotaxis and ERK1/2 phosphorylation but does not alter C5a-mediated calcium mobilization, supporting its role in modulation of the β-arrestin pathway. Association of C5L2 with β-arrestin is confirmed by cellular co-immunoprecipitation assays. C5L2 blockade also has no effect on ligand uptake or C5aR endocytosis in human polymorphonuclear leukocytes, distinguishing its role from that of a rapid recycling or scavenging receptor in this cell type. This is thus the first example of a naturally occurring seven-transmembrane segment receptor that is both obligately uncoupled from G proteins and a negative modulator of signal transduction through the β-arrestin pathway. Physiologically, these properties provide the possibility for additional fine-tuning of host defense.

The complement anaphylatoxin C5a is one of the most potent inflammatory mediators of the innate immune system, with the ability to activate all classes of myeloid cells as well as cells of many other lineages. Multiple studies using experimental animals deficient in C5 or mice with targeted deletion of the C5a receptor (C5aR)[4] have demonstrated the critical role for this anaphylatoxin in host defense. Generation of C5a has also been associated with disease conditions, including asthma, contact sensitivity reactions, autoimmune arthritis, and sepsis (reviewed in Refs. 1–3). C5a manifests its activities by interaction with two known receptors, C5aR and C5L2. Cellular stimulation of the C5aR through $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_{16}$ results in intracellular calcium mobilization and activation of signaling pathways including phosphatidylinositol 3-kinase, diacylglycerol, MAPK, ERK, and others (4, 5), (reviewed in Ref. 6).

Like the C5aR, C5L2 is a putative seven-transmembrane segment protein that was identified by Ohno *et al.* (7) as a cDNA with homology to the C5aR. It has similar sequence homology with the formyl peptide receptor (FPR) and the chemokine receptor chemR23. C5L2 is expressed on neutrophils, macrophages, and immature dendritic cells in coordination with the C5aR, although its mRNA is present at significantly reduced levels (7, 8). Expression has additionally been reported in adrenal gland, spinal cord, thyroid, liver, lung, spleen, brain, and heart (9, 10).

Studies of the distinct properties of C5L2-utilizing transfection systems demonstrate its ability to bind C5a with affinity similar to that of the C5aR. It binds the metabolite des-Arg C5a with higher avidity (11, 12). In contrast to the C5aR, however, we and others (12) have demonstrated that C5L2 is devoid of the ability to couple to intracellular G proteins due to an amino acid replacement of arginine by leucine in the DRY motif located in the second intracellular domain. Following transfection in L1.2 or rat basophilic leukemia cells, which are permissive for C5a-mediated signal transduction by the C5aR, ligand binding to C5L2 failed to induce calcium mobilization and resulted in minimal receptor phosphorylation relative to the C5aR. As a result, questions have been raised whether C5L2 activates G protein-independent intracellular signaling pathways or serves an alternate, ligand scavenging or other regulatory function similar to the chemokine receptors D6, CXCR7, and DARC (13–15). To this end, a recent study by Scola *et al.* (16), reported ligand-independent internalization of C5L2 in transfected rat basophilic leukemia cells, which resulted in intracellular accumulation and degradation of C5a and des-Arg C5a.

Information gleaned from studies of mice with targeted deletion of C5L2 reveals the anti-inflammatory role of the receptor. $C5L2^{-/-}$ animals exhibit significantly increased inflammation in a model of pulmonary immune complex injury compared with wild type animals (16, 17). In a rat model of sepsis induced by cecal ligation and puncture, antibody blockade of C5L2 was

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[4] The abbreviations used are: C5aR, C5a receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; FPR, formyl peptide receptor; fMLP, formylmethionylleucylphenylalanine; mAb, monoclonal antibody; DAPI, 4′,6-diamidino-2-phenylindole; PMN, polymorphonuclear leukocyte.

associated with a dramatic increase in circulatory interleukin-6 (10). An independently generated line of C5L2-deficient mice yielded contrasting results, that C5L2 is not only a positive regulator of the C5aR, but that it is also critical for signaling by both C5a and C3a (8). Among the signaling pathways affected were MAPK, ERK, and protein kinase B/Akt. Yet another study showed that genetic deficiency- or antibody-mediated blockade of C5L2 provided modest protection from cecal ligation and puncture-mediated sepsis (18). Here, protection from "high grade" sepsis, which resulted in 100% lethality, was afforded only by the combined inhibition of C5L2 and the C5aR. This report additionally showed that signaling through C5L2 but not the C5aR, led to release of the inflammatory protein, high mobility group box 1 (HMGB1) (18). The latter data supported a proinflammatory role for C5L2.

Because of these apparently conflicting observations, as well as complexities presented by the several mouse models described and limitations based on the availability of reagents, we turned to the human system as an independent approach to understanding the role of C5L2. Human PMNs, in particular, provide the advantage that, as both C5L2 and the C5aR are expressed endogenously, the possibility for cooperative interactions between the two receptors may be evaluated. Here, we describe our findings based on utilization of monoclonal antibodies that selectively recognize and block C5a binding to human C5L2 or the C5aR but do not cross-react. Our findings reveal C5L2 functions as an intracellular receptor, which is activated as a consequence of C5aR activation. Activation of C5L2 results in inhibition of C5aR-β-arrestin-mediated ERK1/2 activation, with no apparent alteration of G protein-mediated functions. Thus, in human PMNs, C5L2 serves as a negative modulator of C5a-C5aR-mediated ERK1/2 signal transduction.

EXPERIMENTAL PROCEDURES

Cells and Transfections—Mouse pre-B lymphoma L1.2 cells were maintained in RPMI 1640 supplemented with 10% bovine calf serum. Stably transfected cells expressing C5L2 or the C5aR were maintained under selection with 0.8 mg/ml G418 as described previously (12).

Hybridoma Production and Antibody Screening—C57BL/6 mice were immunized by intraperitoneal injection of 1×10^7 C5L2-transfected L1.2 cells six times at 2-week intervals followed by a single intravenous injection. Four days after the final injection, animals were sacrificed, and splenocytes were isolated and fused with SP2/0 cells using established procedures (2). Hybridomas were grown in Dulbecco's modified Eagle's medium containing 10% Fetalclone (HyClone) and antibodies purified by protein A or G chromatography. Antibody specificity was assessed by indirect immunofluorescence using L1.2 transfectants or parent cells (FACSCalibur with CellQuest software) (12, 17). Generation and characterization of blocking antibodies selective for the C5aR were described previously (2). Receptor blocking ability was assessed as competition for ligand binding.

Receptor Binding—L1.2 cells expressing human C5L2 or C5aR were incubated in binding buffer (20 mM HEPES, pH 7.5, 0.125 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM sucrose, and 0.2% bovine serum albumin) with 0.05 nM ^{125}I-C5a (PerkinElmer Life Sciences) and increasing concentrations of anti-C5L2 antibodies, isotype-matched IgG, unlabeled C5a, or a non-peptide C5aR-selective antagonist, NDT 2001 (generous gift of Neurogen, Branford, CT), as described previously (12). Results are expressed as mean ± S.E. of duplicate or triplicate determinations, and at least three independent experiments were performed. Data were analyzed using PRISM software (GraphPad).

GeneChip Analysis—The gene expression profiles for C5L2 and the C5aR were analyzed using GeneChip arrays according to the manufacturer's protocols (Affymetrix).

Flow Cytometry—Ficoll-purified human neutrophils were suspended in phosphate-buffered saline containing 4% goat serum or permeabilized with saponin (Cytofix/Cytoperm, BD Biosciences) and stained by indirect immunofluorescence using mouse monoclonal anti-human C5L2 antibody 4C8 or 1D9, anti-human C5aR antibody 3C5, rabbit anti-human C5L2 antibody (Imgenex), or rabbit anti-human antibody C5aR (BD Biosciences), followed by the indicated Alexa-labeled anti-mouse or anti-rabbit IgG (Invitrogen). Cells were analyzed by 1- or 2-color flow cytometry (MoFlo XDP with Summit software, Beckman Coulter).

Chemotaxis—Neutrophil chemotactic responses were assessed using modified Boyden chambers (Neuroprobe) as described previously (17).

Calcium Mobilization—Calcium mobilization in response to C5a was assessed as described previously (12).

MAPK Activation—Purified human PMNs were preincubated for 20 min at 37 °C with 5 μg/ml anti-C5L2 antibody 1D9 or 4C8, anti-C5aR antibody 3C5, the C5aR antagonist NDT2001, or isotype control IgG. Cells were activated with 1 nM C5a for the times indicated, and cell lysates were analyzed for ERK1/2 phosphorylation by Western blotting. Blots were stripped and re-probed for total ERK to ensure equal loading of samples. ERK1/2 phosphorylation was quantitated by densitometry using ImageJ software (NIH), and the band intensity expressed as the mean percent (± S.E.) of isotype control IgG in 9 independent experiments.

Ligand Uptake and Internalization—C5a binding and internalization in the presence of receptor recycling were assessed as described (19). PMNs were preincubated with antibodies in binding buffer as above. ^{125}I-C5a was added at 2 nM, and duplicate samples were removed at the times indicated and washed with the same buffer to determine total uptake, or with 0.2 M acetic acid and 0.5 M NaCl to determine internalized ligand. Free ligand was eliminated by filtration on 1% polyethyleneimine-soaked glass fiber filters, and cell-associated C5a was determined by gamma counting. Results are expressed as the mean ± S.E., and at least four independent experiments were performed. To evaluate ligand internalization in the absence of receptor recycling, neutrophils were incubated in the presence of antibodies as above with 2 nM ^{125}I-C5a at 0 °C for 45 min. Cells were washed to remove unbound ligand and transferred to 37 °C for the times indicated. Internalized ligand was determined by washing at low pH as above. Results are expressed as the mean ± S.E. of duplicate determinations, and at least three independent experiments were performed.

Confocal Analyses—Human PMNs adherent to fibronectin-coated glass coverslips were incubated in binding buffer with 0 or 100 nM C5a at 37 °C as indicated. Cells were fixed, permeabilized (Cytofix/Cytoperm, BD Biosciences), and immunostained for C5L2 and/or C5aR using the antibodies described above, rabbit anti-C5L2 (Imgenex), anti-C5aR (BD Pharmingen), and the appropriate control antibodies. Alternatively, cells were reacted with anti-receptor antibodies prior to permeabilization and immunostaining. Permeabilized cells were also stained with anti-human β-arrestin-1 (Abcam), or control, in the combinations indicated, followed by Alexa-labeled anti-mouse, anti-rabbit or anti-goat IgG (Invitrogen). Nuclei were visualized with DAPI (ProLong Gold, Invitrogen), and cells were imaged by confocal microscopy (LSM510 META 2-Photon confocal microscope, Zeiss).

Cellular Co-immunoprecipitation Assays—Purified human PMNs were incubated in binding buffer (4×10^7/ml) with 0 or 100 nM C5a for 10 min at 37 °C. Reactions were quenched by the addition of 2 mM dithiobis(succinimidyl proprionate) (Pierce), mixing gently for 30 min at 22 °C. Cells were pelleted and lysed by sonication in 20 mM HEPES, pH 7.5, containing 137.5 mM NaCl, 1% Triton X-100 0.5% deoxycholate, 5 mM EDTA, a protease inhibitor mixture (Halt, Thermo), and a phosphatase inhibitor mixture (Sigma Aldrich). Lysates were incubated overnight at 4 °C with goat-anti-β-arrestin-1 (R&D Systems) or an irrelevant goat IgG and protein G magnetic beads (Invitrogen). Immunoprecipitates were washed exhaustively and eluted with PAGE sample buffer. Proteins were separated under reducing conditions on 10% Bis/Tris polyacrylamide gels in Mops buffer and transferred to polyvinylidene difluoride for immunoblotting. Chemiluminescence detection was performed using ECL Plus (Amersham Biosciences).

Statistical Analyses—Data were analyzed using PRISM software (GraphPad), and results were expressed as mean ± S.E. of duplicate or triplicate determinations. Student's unpaired *t* test was utilized to evaluate the significance of differences and was considered significant for $p \leq 0.05$.

RESULTS

Human Cell Types Expressing C5L2—To begin to elucidate the mechanism of action of C5L2 in the context of a naturally expressing human cell that also expresses the C5aR, we sought to identify a cell type with endogenous expression and function of both receptors. This approach has the advantage that it provides for the possibility of evaluating potential cooperative interactions between the two molecules. Gene chip profiling indicates mRNA expression of C5L2 is roughly parallel to that of the C5aR, although the levels are consistently lower (Fig. 1*A*). Neutrophils are the most abundant source of C5L2 (several unrelated samples are shown), followed by eosinophils and central memory T cells. Based on this result, we focused subsequent analyses of C5L2 function on C5a-mediated responses of human neutrophils.

C5L2-blocking Antibodies—Crucial to the utility of this approach is the ability to selectively block the function of each of the C5a receptors. To accomplish this, we generated C5L2-specific monoclonal antibodies using human C5L2-transfected murine pre-B L1.2 cells as the immunogen (12). We identified several clones with specificity for C5L2 and no detectable reactivity with either C5aR-transfected or untransfected L1.2 cells. Two such clones, 1D9 and 4C8, also exhibit competitive inhibition of C5a binding to C5L2-transfected L1.2 cells with an EC_{50} of 0.55 nM (Fig. 1, *B* and *C*). These antibodies were used interchangeably for the studies described here. Experiments involving blockade of C5a interactions with the C5aR were performed using the C5aR-specific monoclonal antibodies, 3C5 or 7F3, which exhibit no detectable interaction with C5L2 (2).

Cellular Localization of C5L2—In our initial studies of human C5L2 expressed either transiently or stably in transfected cells, plasma membrane expression was routinely evident (Fig. 1, *B* and *C*) (12, 17). In characterization of the ligand-binding properties of human neutrophil C5L2, contributions of the C5aR were eliminated by performing experiments in the presence of either the C5aR-selective blocking monoclonal antibody 3C5 or the small molecule antagonist NDT2001. As shown in Fig. 2*A*, antibody blockade of the C5aR eliminates the majority of radiolabeled C5a binding. Inhibition with NDT2001 had a similar effect (Fig. 2*A*, *inset*). In the presence of the C5L2-selective blocking antibody 1D9, which is completely effective for inhibition of C5a binding to C5L2-transfected cells (Fig. 1*C*), ^{125}I-C5a binding to neutrophils was not significantly altered. This result suggested that little or no C5L2 is available to interact with C5a on the plasma membrane.

As shown in Fig. 2*B*, flow cytometric analysis was consistent with this result. Staining with the C5L2-specific monoclonal antibody 4C8 revealed negligible expression of this receptor relative to the C5aR in 12 of 13 independent experiments using cells from multiple donors. C5L2 expression in cells from the single positive donor was not consistent. However, when cells were permeabilized with saponin prior to immunostaining, abundant intracellular C5L2 was reproducibly revealed (Fig. 2*B*). Immunostaining for the C5aR on permeabilized PMNs was similar to that observed on cells with intact membrane, reflecting its predominant expression on the cell surface.

The FPR is also expressed predominantly in intracellular compartments in resting neutrophils, and when cells are activated with phorbol 12-myristate 13-acetate, surface expression of this receptor is significantly increased (20). When we treated PMNs with phorbol 12-myristate 13-acetate, the distribution of C5L2 was not altered, but surface expression of the FPR was increased by ~100% (data not shown). Additional treatments directed toward inducing the surface expression of C5L2 on neutrophils, including activation with CXCL1, formylmethionylleucylphenylalanine, and C5a; induction of apoptosis; or adherence to fibronectin, were similarly unproductive.

Confocal Localization of C5L2—The G protein-independent chemokine receptors CXCR7 and D6 are also expressed as predominantly intracellular proteins. In the case of CXCR7, studies have shown it resides just below the plasma membrane surface in human T lymphocytes and is believed to modulate the functions of CXCR4 (21). D6 also exhibits minimal (<5%) plasma membrane expression but undergoes rapid constitutive recycling to bind extracellular ligand and internalize it for degradation in the absence of signaling (22). Because the data of Fig. 2*B* indicate abundant intracellular C5L2, we used immunostaining and confocal microscopy of membrane-intact and sap-

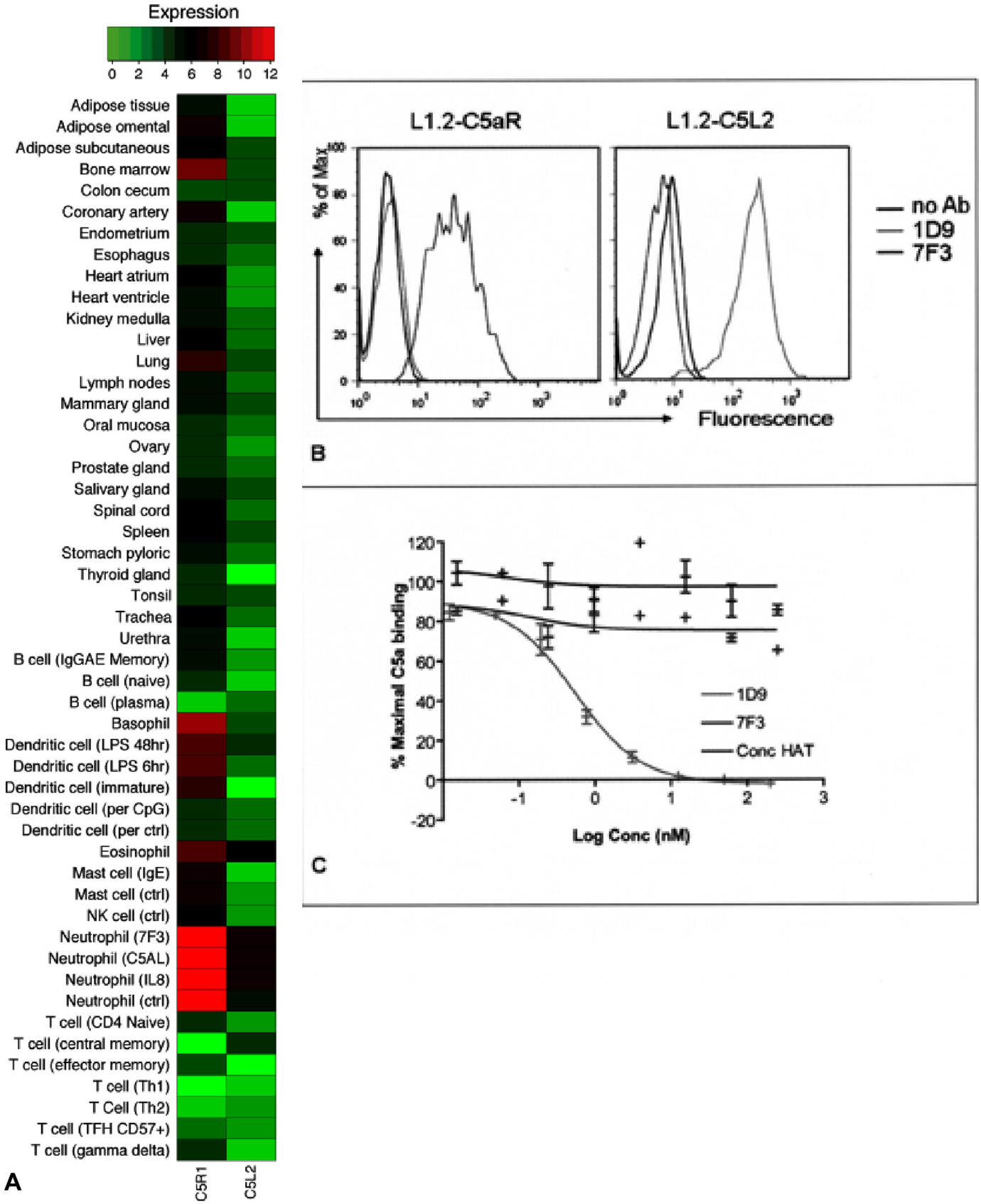


FIGURE 1. **Expression analysis of human C5L2 and C5aR.** *A*, the relative mRNA abundance for C5L2 (*right*), relative to the C5aR (*left*) in the human cells and tissues shown, is indicated on a scale of 0–12, where 0 (*pale green*) is at the low extreme and 12 (*red*) is most abundant. *B*, flow cytometric staining of murine pre-B L1.2 cells transfected with human C5aR (*left*) or human C5L2 (*right*), using anti-human C5L2 mAb, 1D9, anti-human C5aR, 7F3, or isotype control mAbs. *C*, inhibition of ^{125}I-C5a binding to human C5L2-L1.2 transfectants by anti-C5L2, 1D9 (○), or anti-C5aR, 7F3 (■). Hybridoma medium (*HAT*) was used as a negative control (+). Data are expressed as the mean ± S.E. of triplicate determinations and representative of three independent experiments.

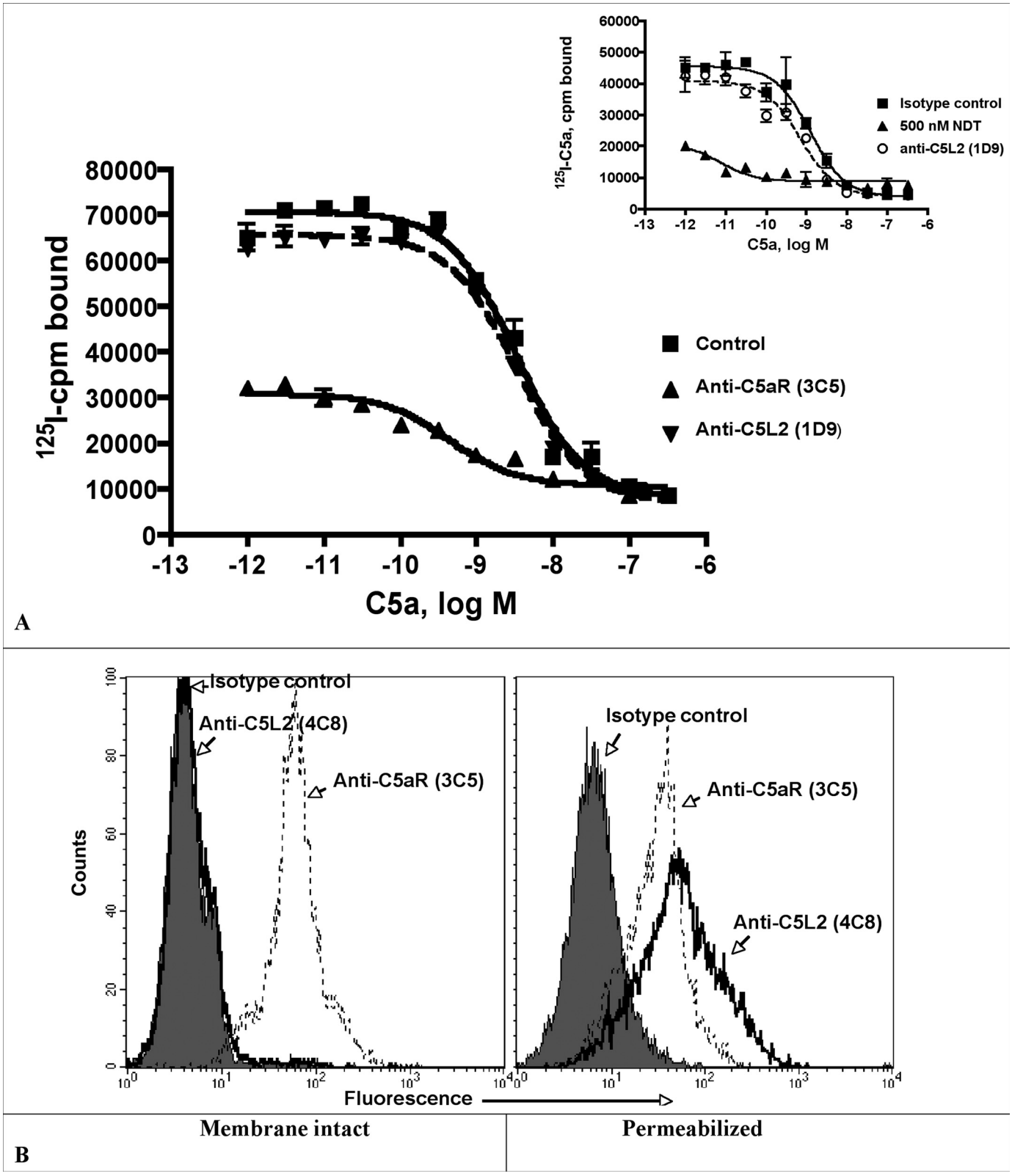


FIGURE 2. **C5L2 is an intracellular receptor in human PMNs.** *A*, the anti-C5L2 blocking mAb 1D9 does not block binding of ^{125}I-C5a to human PMNs. Purified human PMNs were incubated with 0.05 nM ^{125}I-C5a and increasing concentrations of unlabeled C5a in the presence of 1 μg/ml anti-C5L2, 1D9, anti-C5aR, 3C5, or isotype-matched control. After 60 min at 22 °C, unbound ligand was separated, and the cell-associated ligand was determined by gamma counting. Data are the mean ± S.E. of duplicate determinations, representative of at least four independent experiments. *Inset*, shown is an example of human PMN binding of ^{125}I-C5a under the same conditions but using the C5aR antagonist, NDT2001 (*NDT*, 500 nM) to block interactions with the C5aR. *B*, flow cytometric analysis of C5L2 (*solid line*) and the C5aR (*dashed line*) on membrane intact (*left*) and saponin-permeabilized (*right*) human PMNs. The histograms for isotype-matched control mouse IgG are shaded in *gray*. Data are representative of 12 independent experiments.

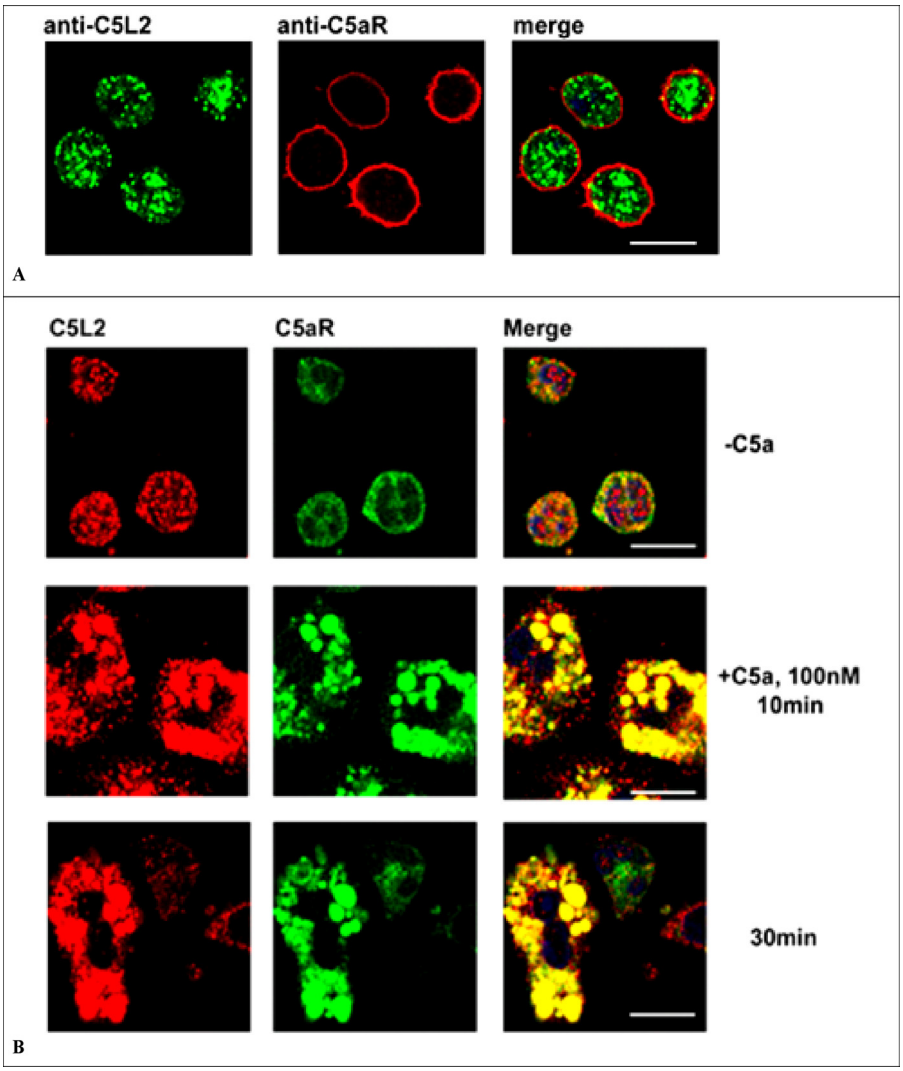


FIGURE 3. **Ligand-dependent co-localization of the C5a receptors in human PMNs.** *A*, confocal microscopic localization of C5L2 in human PMNs was determined using human neutrophils adherent to fibronectin-coated coverslips. Cells were loaded with anti-C5L2 and anti-C5aR at 4 °C for 30 min then warmed to 37 °C for 10 min to allow endocytosis. Cells were then fixed, permeabilized, and immunostained with Alexa488 anti-mouse IgG (*green*) and Alexa555 anti-rabbit IgG (*red*). Nuclei were visualized with DAPI. *Bar*, 10 μm. *B*, purified human PMNs adherent to fibronectin-coated coverslips were incubated with 0 or 100 nM C5a at 37 °C for the times indicated. Cells were then fixed and permeabilized with saponin and immunostained with mouse anti-human C5L2, 1D9, and rabbit anti-human C5aR, followed by Alexa555 anti-mouse IgG (*red*) and Alexa488 anti-rabbit IgG (*green*). Nuclei were visualized with DAPI. *Bar*, 10 μm.

onin-permeabilized cells to determine the subcellular distribution of C5L2. As shown in Fig. 3*A*, when cells were reacted with primary antibody prior to permeabilization and then saponin-treated and stained with Alexa-tagged secondary antibodies, virtually no C5L2 is evident on the plasma membrane, consistent with the results of flow cytometry. Cells permeabilized prior to immunostaining similarly reveal C5L2 distributed diffusely throughout the cytoplasm in resting cells, with the C5aR expressed abundantly at the cell surface (Fig. 3*B*). Following ligation of C5a, the cells exhibit the enlarged and irregular morphology consistent with cellular activation (23). As previous studies have demonstrated, interaction with C5a also initiates endocytosis of the ligand-receptor complex (24). Simultaneously, C5L2 appears to coalesce to more discrete regions of the cytoplasm and becomes co-localized with the C5aR. This co-localization is maintained for prolonged periods of time (>30 min) in the presence of ligand.

C5L2 Is Devoid of C5a-scavenging Activity—Expression of C5L2 results in reduction of C5a-dependent inflammation, but the receptor is predominantly an intracellular protein, both in resting cells and following stimulation. We therefore questioned the possibility that C5L2 functions in a manner similar to D6, with a small amount recycling rapidly to the plasma membrane to bind C5a and facilitate its intracellular clearance in the absence of signal transduction (13, 22). We anticipated that such a scavenging function for C5L2 would be manifested as an alteration in the rate of ligand uptake and/or internalization in the presence of C5L2 blocking antibodies.

To first confirm the susceptibility of C5L2 to antibody blockade, we incubated membrane intact cells with anti-C5L2 antibodies at 37 °C, under conditions that permit fluid phase endocytosis (25). The cells were then fixed and permeabilized, the intracellular antibody was immunostained with fluorochrome-labeled secondary antibody and visualized by confocal microscopy (Fig. 3*A*). Under these conditions, staining of C5L2 reveals granular distribution throughout the cytoplasm. Antibody staining of the C5aR reveals only the receptor population discretely present on the plasma membrane surface. In contrast, cells permeabilized prior to staining with primary antibody reveal an identical pattern for CL2 (Fig. 3*B*). Immunostaining of the C5aR on permeabilized cells also reveals receptor more deeply embedded in the membrane and small amounts in intracellular compartments.

PMNs incubated with ^{125}I-C5a under conditions that allow detection of receptor recycling, *i.e.* in the presence of excess ligand (2 nM) (19), demonstrate rapid binding and internalization of ligand, which reaches steady state within ~10 min (Fig. 4*A*). Internalized C5a (*i.e.* ligand resistant to stripping at low pH) comprises ~80% of the total ligand bound. Cells pretreated for up to 60 min with a saturating concentration of C5L2-blocking antibody (1D9) exhibit no alteration in either the binding or internalization of C5a relative to isotype-matched control. In contrast, antibody blockade of the C5aR reduces both uptake and internalization of C5a to baseline levels.

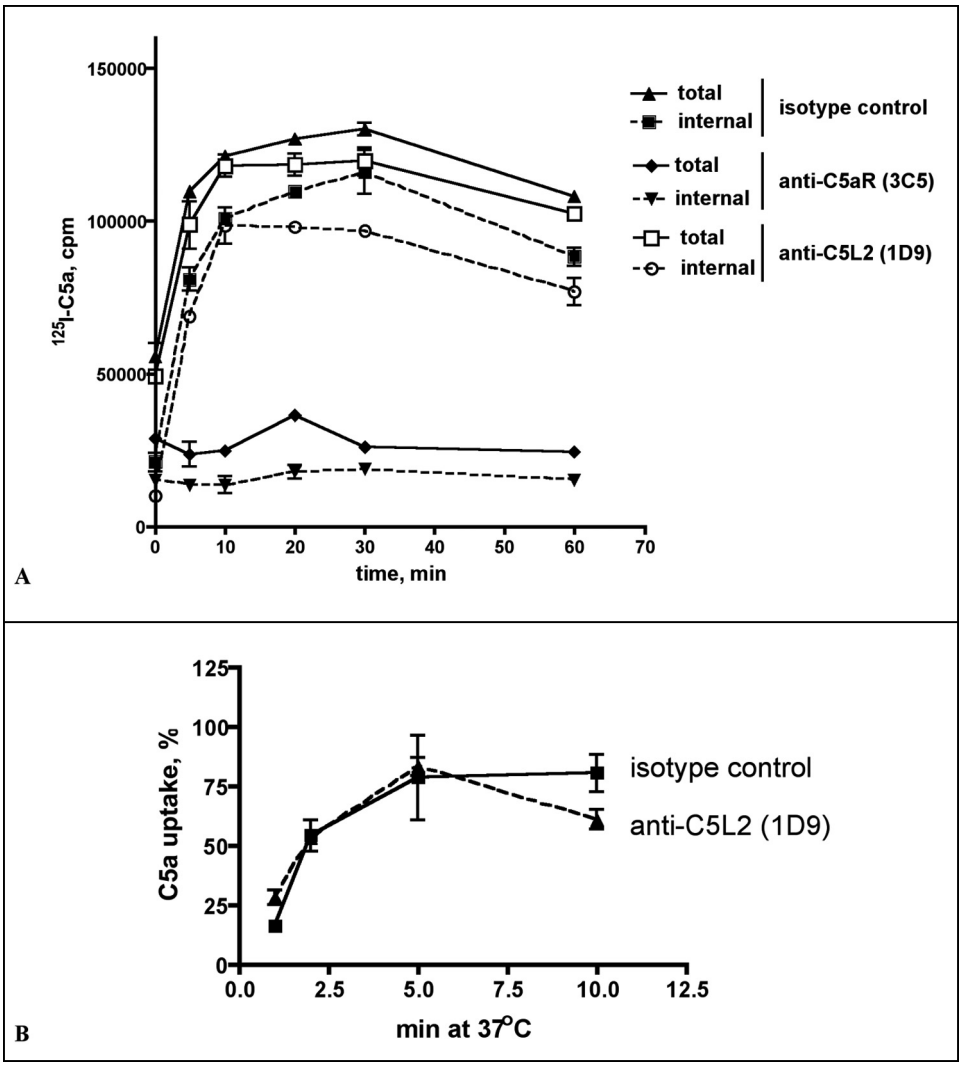
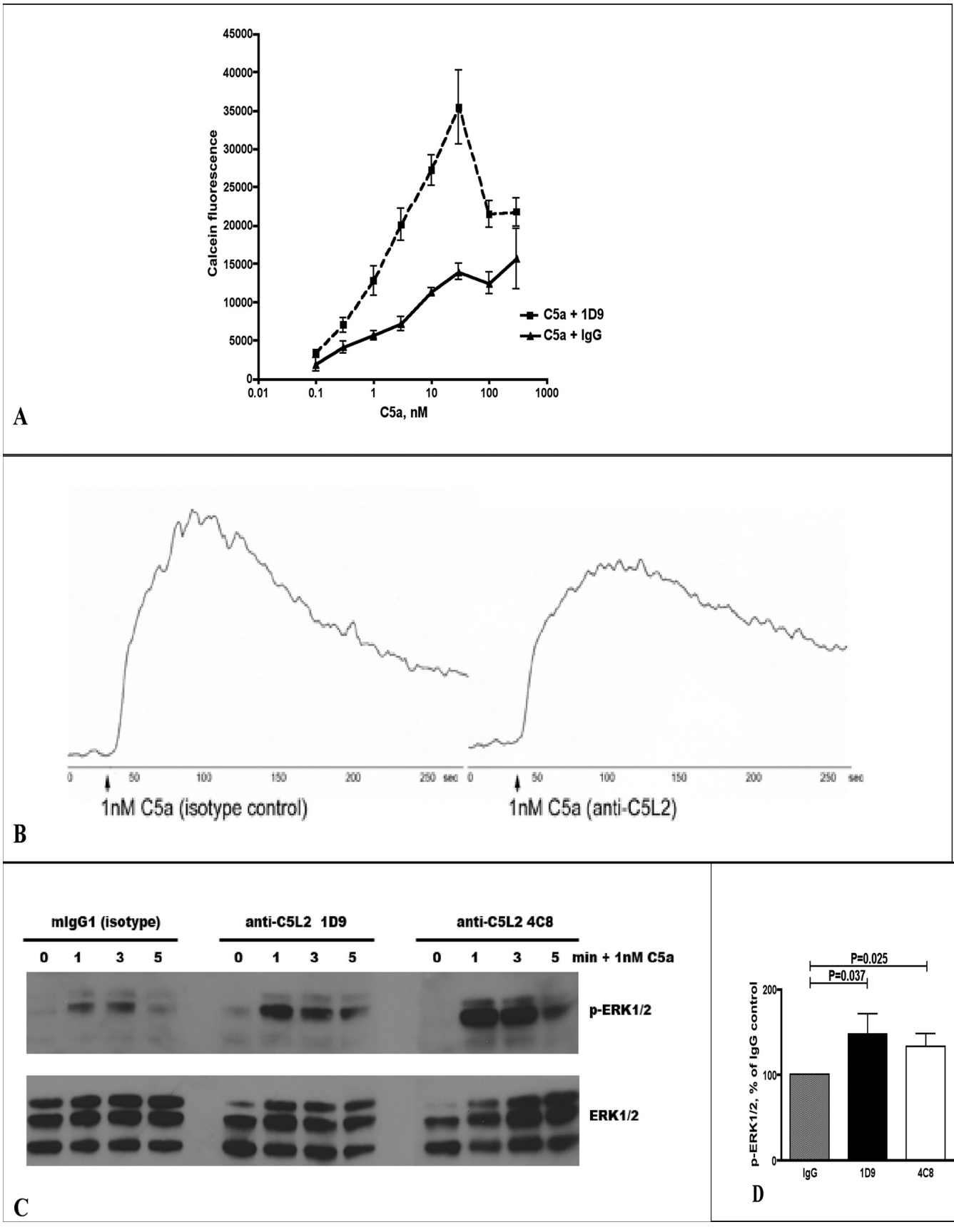


FIGURE 4. **Antibody blockade of C5L2 does not alter ligand uptake or internalization.** *A*, C5a binding and internalization under conditions of receptor recycling were assessed using human PMNs incubated with 2 nM ^{125}I-C5a in the presence of 5 μg/ml anti-C5L2, 4C8, anti-C5aR, 3C5, or isotype control. Total and internalized (resistant to stripping at low pH) ligand (*cpm*) were determined as a function of time at 37 °C. Data represent the mean ± S.E. of duplicate determinations from five independent experiments. *B*, to evaluate ligand internalization under conditions of single pass endocytosis, PMNs were preincubated with antibodies as in *A* and 2 nM ^{125}I-C5a for 60 min at 0 °C. Cells were then washed and transferred to 37 °C, and internalized ligand was determined as a function of time by washing at low pH as above. Data represent the mean ± S.E. of duplicate determinations from three independent experiments.

Alterations in ligand uptake by C5L2 blockade were also assessed in PMNs incubated with ^{125}I-C5a under conditions designed to evaluate single pass endocytosis. In this case, cells are loaded with ligand at 4 °C and then washed and shifted to 37 °C to initiate internalization. This condition also showed no difference in the presence of C5L2 blockade (Fig. 4*B*). These results suggest the C5aR regulates the rate-limiting steps of ligand binding and internalization with no detectable contribution by C5L2. Further, flow cytometric assessment of the rate of C5aR endocytosis following addition of C5a also revealed no change in the presence of anti-C5L2 monoclonal antibody (mAb) (data not shown). Thus, C5L2 functions by a mechanism distinct from scavenging C5a, perhaps entirely as an intracellular receptor.

C5L2 Suppresses C5aR-mediated Chemotaxis by Inhibiting ERK1/2 Activation—We demonstrated previously that isolated bone marrow cells from C5L2-deficient mice exhibit enhanced chemotactic responses to C5a both *in vivo* and *in vitro* (17). To further characterize this function of C5L2 in human PMNs, we first evaluated their chemotactic responsiveness to C5a in the presence and absence of the C5L2-blocking antibody 1D9. As shown in Fig. 5*A*, antibody inhibition of C5L2 results in a dramatic enhancement in C5a-mediated chemotaxis relative to isotype control antibody. In contrast, no change was observed in the integrated signal for calcium mobilization, indicating the impact of C5L2 on C5a-mediated signal transduction is distinct from alteration of G protein-dependent pathways (Fig. 5*B*). By analogy, with the angiotensin AT1a ligand receptor system, modulation of chemotaxis but not calcium mobilization has been shown to function through activation of ERK1/2 via the β-arrestin pathway independently of G proteins (26).

This result suggests the modulation of C5aR-mediated cellular activation by C5L2 may also be specific to the β-arrestin pathway and independent of G proteins, although in contrast to the AT1aR, the result is suppression of downstream events and not activation. To test this possibility, we assessed C5a-mediated ERK1/2 phosphorylation in the presence and absence of C5L2-blocking antibodies by Western blot (Fig. 5*C*). Cells treated with either anti-C5L2 blocking antibody 4C8 or 1D9 exhibit an increase in C5a-mediated ERK1/2 phosphorylation relative to isotype control. Densitometric quantitation of the phospho-ERK1/2 signals at 1–3 min after addition of C5a demonstrates an increase of ~60% in the presence of C5L2 blockade over that observed in the presence of isotype-matched control mAb (Fig. 5*D*). Blots were stripped and reprobed for total ERK1/2 to ensure equal sample loading. PMNs treated with the blocking antibody against the C5aR were completely inhibited for ERK1/2 activation (data not shown). Thus, C5L2 functions to suppress C5a-mediated chemotactic activity in human PMNs at least in part by reducing the C5aR-mediated activation of ERK1/2. Similarly, genetic deletion of C5L2 in the mouse results in enhanced C5a-C5aR-mediated chemotactic



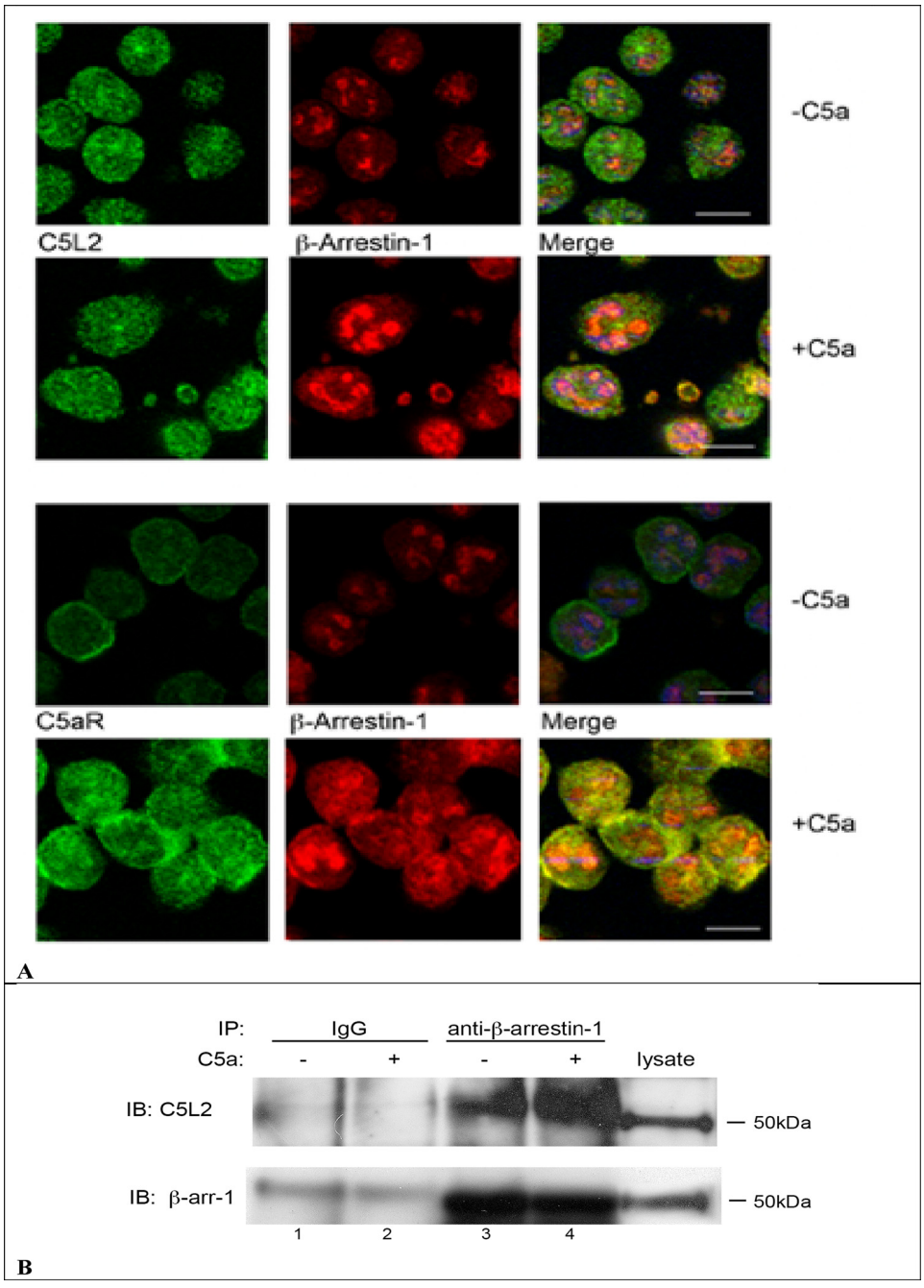


FIGURE 6. **Co-localization of C5L2 with β-arrestin in human PMNs is increased following C5a stimulation.** *A*, purified human PMNs adherent to fibronectin-coated coverslips were saponin treated fixed and immunostained with mouse anti-C5L2 or anti-C5aR and rabbit anti-β-arrestin-1, followed by Alexa488 anti-mouse IgG (*green*) and Alexa555 anti-rabbit IgG (*red*) in the absence of C5a or 10 min after addition of 100 nM C5a. Nuclei were visualized with DAPI. *Bar*, 10 μm. *B*, PMNs incubated with 0 (−) or 100 nM (+) C5a for 10 min at 37 °C were cross-linked with dithiobis(succinimidyl proprionate), detergent-solubilized, and immunoprecipitated (*IP*) with goat anti-β-arrestin (*lanes 3* and *4*) or an irrelevant goat IgG (*lanes 1* and *2*). Immunoprecipitates were separated by SDS-PAGE and immunoblotted (*IB*) for C5L2 or β-arrestin-1. Molecular mass markers (kDa) are indicated on *right*. Data are representative of three independent experiments.

responses in bone marrow cells relative to cells from wild type animals (17).

C5L2 Is Co-localized with β-Arrestin—G protein-independent activation of ERK1/2 by G protein-coupled receptors has been shown to depend on the association of ligand bound receptor with β-arrestin (24, 27). Mechanistically, phosphorylation of receptor by G protein receptor kinases promotes their association with β-arrestin and targeting to endosomes by way of clathrin-coated pits (28). The endosomal G protein-coupled receptor-β-arrestin complex then triggers the activation of ERK1/2 and downstream signaling reactions (29–31).

To determine whether C5L2 participates in this scenario, we used confocal microscopic analysis to evaluate potential interactions between C5L2 and β-arrestin. The micrographs shown in Fig. 6*A* reveal limited co-localization of β-arrestin with C5L2 in resting cells and essentially none with the C5aR. Following stimulation with C5a, the C5L2-β-arrestin co-localization becomes markedly pronounced, as indicated by the intense *yellow* of the merged images, and is commensurate with an association between the C5aR and β-arrestin. Cellular co-immunoprecipitation assays confirm this result, revealing association of C5L2 with β-arrestin in both resting and C5a-stimulated cells (Fig. 6*B*). Stimulation of PMNs with C5a also induces co-localization of the C5aR with C5L2 (Fig. 3). These data suggest the formation of a molecular complex containing β-arrestin and both of the C5a receptors. Such a complex could facilitate a modulatory function of the C5L2-β-arrestin complex on C5aR-β-arrestin-

FIGURE 5. **C5L2 suppresses C5a-mediated chemotaxis and ERK1/2 activation in PMNs.** *A*, calcein-loaded human PMNs were preincubated with anti-C5L2, 1D9, or isotype control IgG, and chemotactic responses to C5a were assessed in modified Boyden chambers. Data are the mean ± S.E. of triplicate determinations, representative of five independent experiments. *B*, C5a-mediated mobilization of intracellular calcium in cells preincubated with 5 μg/ml anti-C5L2, 1D9, or isotype control IgG. Data are representative of four to five replicates in two independent experiments. *C*, representative Western blot analysis of the phospho-ERK1/2 signal generated in human PMNs in the presence of the anti-human C5L2 antibodies, 1D9 or 4C8, or isotype control at 5 μg/ml, as a function of time after addition of 1 nM C5a. Blots were stripped and reprobed for total ERK1/2 to ensure equivalent loading of samples. *D*, densitometric quantitation of the relative phospho-ERK1/2 signal generated by 1–3 min of incubation with 1 nM C5a in cells treated with anti-human C5L2 antibodies, 1D9 or 4C8, normalized to isotype control IgG. Data represent the mean ± S.E. of 9 independent experiments; the significance of differences between anti-C5L2 treatment and isotype control are indicated.

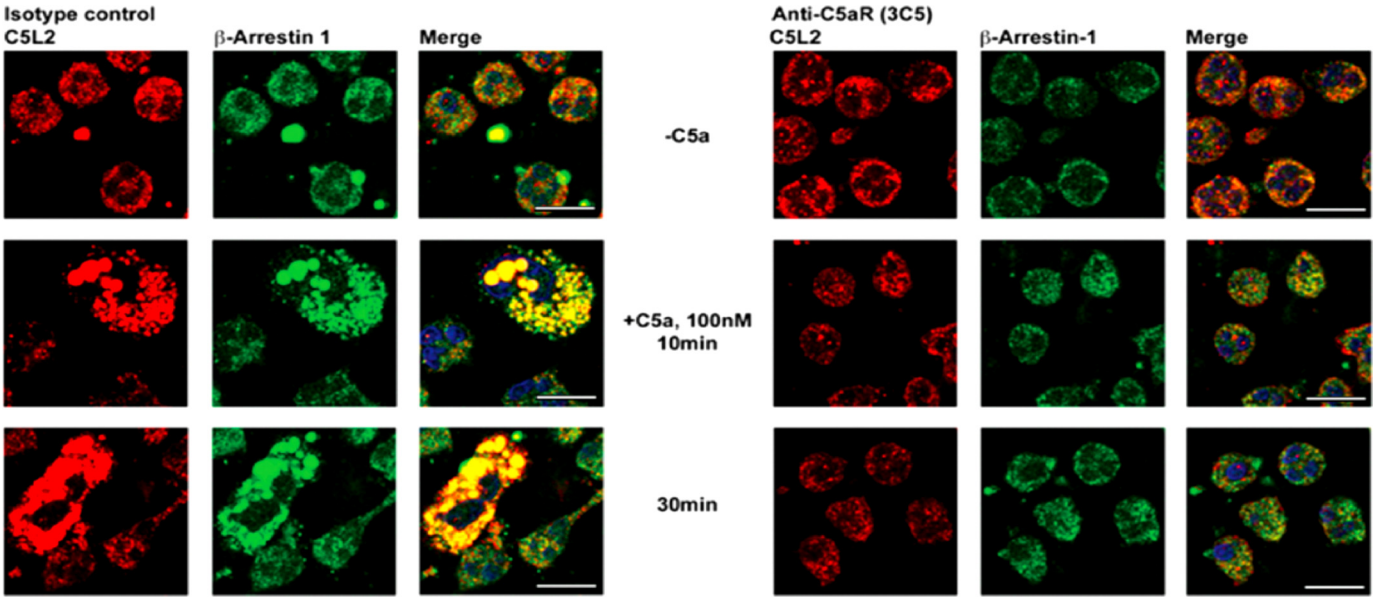


FIGURE 7. **C5L2 activation is a consequence of activation of the C5aR.** Human PMNs were incubated with C5a in the presence of 5 μg/ml isotype control IgG or anti-C5aR antibody 3C5, as indicated, and then permeabilized and stained with rabbit anti-C5L2 and goat anti-β-arrestin-1 antibodies, followed by Alexa555-labeled anti-rabbit IgG and Alexa488-labeled anti-goat IgG. Nuclei were visualized with DAPI. *Bars*, 10 μm.

mediated activation of ERK1/2, potentially by binding in a competitive and/or nonproductive manner.

Activation of C5L2 Is a Consequence of C5aR Activation—The apparent lack of plasma membrane expression for C5L2 coupled with the absence of change in ligand uptake and internalization in the presence of C5L2 blockade, suggests that C5L2 activation may occur only as a consequence of activation of the C5aR. To test this possibility, we utilized the C5a-mediated translocation of C5L2 to β-arrestin as an indicator of activation in the presence and absence of blocking antibody against the C5aR. C5a stimulation of cells in which the C5aR is blocked with the antibody 3C5 resulted in no translocation of C5L2 to β-arrestin as occurs in the presence of activated C5aR (Fig. 7). A similar result was obtained in the presence of the small molecule C5aR antagonist NDT2001 (data not shown).

DISCUSSION

The studies reported here represent the first characterization of a seven-transmembrane segment receptor that is both obligately uncoupled from intracellular G proteins and a negative modulator of β-arrestin signaling pathways in human neutrophils. Since it was initially described in 2000, elucidation of the molecular mechanism of C5L2 has been relatively enigmatic. In our initial studies, we demonstrated that C5L2 transfected into murine pre-B L1.2 cells binds both C5a and des-Arg with nM affinity, but the interaction does not induce calcium mobilization or activation of the MAPK pathway (12). In contrast, L1.2 cells transfected with the C5aR exhibit robust signaling. We further showed that the deficiency of C5L2 in coupling to heterotrimeric G proteins is due to an amino acid replacement in the highly conserved DRY sequence at the end of the third transmembrane domain (12). Similar results were reported for the behavior of C5L2 transfected in rat basophilic leukemia cells (11).

Data based on *in vivo* studies using mice with a targeted deletion of C5L2 revealed an anti-inflammatory role for this C5a receptor. In models of immune complex alveolitis, autoimmune arthritis, and contact sensitivity reactions, deficiency of C5L2 results in exacerbation of the injury, where deficiency of the C5aR is protective (17, 33–35). Isolated C5L2$^{-/-}$ mouse bone marrow cells exhibit greater chemotactic responses to C5a compared with cells from wild type animals, supporting a direct involvement of C5L2 on inflammatory cells (17). Based on these findings, coupled with observations of coordinate expression of the two receptors, we hypothesized that C5L2 serves a modulatory role on C5a-C5aR-mediated activities.

Confounding our investigations, however, a report of the phenotype of an independently generated line of C5L2-deficient mice contained divergent, and in some cases, completely opposing data (8, 12, 17). Further, additional studies with transfected cells indicated the ability of C5L2 to bind and transduce signals in response to C3a, C4a, and their des-arginine derivatives (9, 11, 36, 37). Some of these discrepancies have subsequently been resolved (6, 38).

As a result of these conflicting reports, and in efforts to provide a more in-depth analysis of the molecular mechanism of C5L2, we thus sought an independent approach using human cells to identify the molecular mechanisms for the actions of C5L2. We further sought to avoid the use of transfection systems because of the potential for mismatches in components of the signaling pathways to yield misleading results.

Prior to the present report, most of the cell biological studies of C5L2 were conducted using transfected cells, and plasma membrane expression was not limiting. In neutrophils from both humans and mice, however, ligand binding and flow cytometric analyses consistently reveal negligible C5L2 surface expression (Fig. 2, *A* and *B*).[5] In contrast, the C5aR is abundantly expressed on the cell surface. When PMNs are perme-

[5] C. E. Bamberg, S. Craig, and N. P. Gerard, unpublished observations.

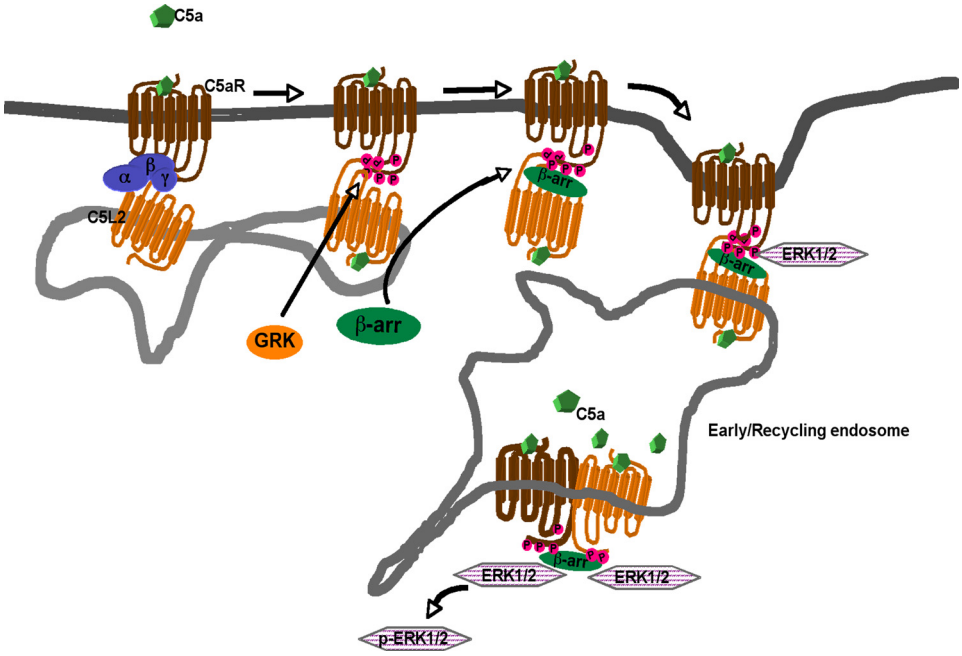


FIGURE 8. **Schematic representation of the mechanism by which C5L2 negatively modulates C5a-C5aR-mediated ERK1/2 activation in human neutrophils.** C5L2 functions as an intracellular receptor, becoming activated only after ligand binding to the C5aR. Receptor activation induces phosphorylation by G protein receptor kinases (*GRK*), facilitating their association with β-arrestin. The C5aR-β-arrestin complex activates ERK1/2, whereas the C5L2-β-arrestin complex inhibits ERK1/2, and the net signal is a result of the balance of the two.

abilized prior to immunostaining, abundant C5L2 is apparent, demonstrating the majority of this receptor is expressed in intracellular compartment(s) (Figs. 2*B* and 3*A*).

Similar intracellular expression has been reported for several other seven-transmembrane segment receptors, including the FPR and the non-G protein-coupled chemokine receptors D6 and CXCR7 (13, 20, 21). In the case of the FPR, cellular activation by phorbol ester is a significant mechanism for inducing surface expression (20); however, it does not change the distribution of C5L2. The chemokine-scavenging receptor D6 recycles to the plasma membrane where it binds ligands and promotes their clearance in the absence of signaling (22). Scola *et al.* (16), recently reported a ligand-scavenging activity for C5L2 in transfected rat basophilic leukemia cells; however, these cells also exhibit plasma membrane expression of the receptor and little or no endogenous C5aR. In neutrophils, our data indicate a similar mechanism does not appear significant since inhibition of C5L2 by blocking mAbs does not alter either the uptake or internalization of C5a (Fig. 4, *A* and *B*). While such a scavenging mechanism is formally possible in endogenously expressing cells under circumstances in which C5L2 is exposed to the extracellular milieu, the present work indicates it is at most minimal in human PMNs.

CXCR7 has been shown to lie just below the plasma membrane in human T lymphocytes and serves to modify CXCR4-mediated expression of adhesion molecules (21). In resting human PMNs, C5L2 appears in granular structures throughout the cytoplasm (Fig. 3). Like CXCR7, blocking antibodies against C5L2 are able to access the receptor, despite negligible surface expression. When neutrophils are activated with C5a, both C5L2 and C5aR appear to be translocated to the same compartments. One consequence of C5L2 activation is a dramatic suppression of C5a-mediated chemotaxis, with no concomitant change in mobilization of intracellular calcium (Fig. 5, *A* and *B*). Analysis of the signal transduction pathway reveals enhanced C5a-mediated ERK1/2 activation in the presence of C5L2 blockade compared with the response elicited in cells in which both receptors are functional (Fig. 5, *C* and *D*). Similarly, bone marrow cells from C5L2-deficient mice exhibit significantly greater ERK1/2 activation following C5a stimulation than cells from wild type animals.[5] Although the mechanism of CXCR7-mediated activation is not yet entirely clear, our studies indicate that CXCL12 activates this receptor exclusively through the β-arrestin pathway (39).

In resting neutrophils, confocal analysis reveals C5L2 distributed throughout the cytoplasm co-localized with β-arrestin, particularly in regions juxtaposed to the nucleus (Fig. 6*A*). Co-immunoprecipitation of C5L2 with β-arrestin is consistent with molecular association of the two proteins (Fig. 6*B*). In resting cells, the presence of C5aR is evident at the plasma membrane. Following addition of C5a, association of both C5L2 and C5aR with β-arrestin is greatly increased. Further, the increased co-localization of C5L2 with β-arrestin that occurs following addition of C5a is blocked in cells treated with anti-C5aR antibodies (Fig. 7), a finding additionally supportive of the function of C5L2 as an intracellular receptor.

While this manuscript was under review, two literature reports appeared demonstrating C5a-dependent redistribution of β-arrestin-GFP in cells co-transfected with C5L2 (40, 41). Although the functional significance to C5aR-mediated responses was not shown, these studies confirm our findings in human PMNs. One of the reports additionally corroborated their result with a β-galactosidase fragment complementation assay, in which the receptor was labeled with a mutated peptide of β-galactosidase and β-arrestin was labeled with a corresponding deletion mutant of the enzyme (41). Following cellular activation generation of a chemiluminescent signal indicated receptor-mediated association with β-arrestin. Importantly, while these investigators demonstrated recruitment of β-arrestin to C5L2 following C5a stimulation, they also showed an absence of ERK1/2 activation in these cells, a result also consistent with our findings in PMNs because we find ERK1/2 activation results from activation of the C5aR.

Taken together, our data support a model depicted schematically in Fig. 8 in which C5L2 functions as an intracellular receptor, becoming activated only after ligand binding to the C5aR.

Activation of the receptors induces their association with β-arrestin, which, in the case of the C5aR, results in activation of ERK1/2. The C5L2-β-arrestin complex inhibits ERK1/2, and the net signal is a result of the balance of the two. By analogy, β_2-adrenergic and AT1a receptors bearing mutated DRY sequences have been shown to induce distinct conformations for β-arrestin compared with their wild type counterparts (32). These mutated receptors are completely uncoupled from G proteins but maintain their ability to activate ERK1/2. Our previous studies demonstrate the mutation in the DRY sequence determines the lack of coupling to G proteins. The ERK1/2 inhibitory property of the C5L2-β-arrestin complex represents a novel regulatory feature of C5a-mediated neutrophil activation. Understanding the molecular mechanism of this suppression provides another clue to the complex regulation of innate immunity.

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