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*J Immunol* 2012; 188:4032-4042; Prepublished online 19 March 2012;
doi: 10.4049/jimmunol.1103141
http://www.jimmunol.org/content/188/8/4032

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/03/19/jimmunol.1103141.DC1

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
C5aR Expression in a Novel GFP Reporter Gene Knockin Mouse: Implications for the Mechanism of Action of C5aR Signaling in T Cell Immunity

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C5aR is a G protein-coupled receptor for the anaphylatoxin C5a and mediates many proinflammatory reactions. C5aR signaling also has been shown to regulate T cell immunity, but its sites and mechanism of action in this process remain uncertain. In this study, we created a GFP knockin mouse and used GFP as a surrogate marker to examine C5aR expression. GFP was knocked into the 3' untranslated region of C5ar1 by gene targeting. We show that GFP is expressed highly on Gr-1+CD11b+ cells in the blood, spleen, and bone marrow and moderately on CD11b+F4/80+ circulating leukocytes and elicited peritoneal macrophages. No GFP is detected on resting or activated T lymphocytes or on splenic myeloid or plasmacytoid dendritic cells. In contrast, 5–25% cultured bone marrow-derived dendritic cells expressed GFP. Interestingly, GFP knockin prevented cell surface but not intracellular C5aR expression. We conclude that C5aR is unlikely to play an intrinsic role on murine T cells and primary dendritic cells. Instead, its effect on T cell immunity in vivo may involve CD11b+F4/80+ or other C5aR-expressing leukocytes. Further, our data reveal a surprising role for the 3' untranslated region of C5ar1 mRNA in regulating C5aR protein targeting to the plasma membrane. *The Journal of Immunology, 2012, 188: 4032–4042.

The complement system, comprising a potent and carefully regulated proteolytic cascade in blood, is an important component of the innate immune system, and its activation in response to pathogens and other noxious stimuli is a keystone of innate host defense. Complement activation leads to the clearance of invading pathogens through opsonization, direct targeted lysis, and generation of proinflammatory anaphylatoxins that serve as local mediators of inflammation (1, 2). One of the anaphylatoxins generated in response to complement activation is C5a, a 74-aa split product of the N-terminal region of C5, which has paradigmatic functions as a local inflammatory mediator, including induction of leukocyte chemotaxis and increasing vascular permeability (3).

The functional attributes attributed to C5a result from its interaction with its cognate seven-transmembrane G protein-coupled receptors C5aR and C5L2 of the rhodopsin family of proteins (4). C5L2 binds both C5a and its degradation product C5a desArg, the biological significance of which has been a matter of dispute (5, 6). Some studies have provided evidence that C5L2 is a decoy receptor incapable of signaling, whereas others have suggested a functional role for C5L2 in certain disease settings, possibly in cooperation with C5aR (7, 8). In contrast, the biology of C5aR has been well established. Binding of C5a to the extracellular, N-terminal region of C5aR allows conformational changes in the intracellular, C-terminal regions of the receptor resulting in G protein coupling, intracellular signaling, and a wide range of physiological responses (9, 10). C5a production and C5aR signaling have been implicated in a large number of inflammatory disorders, and C5aR blockade long has been pursued as a method of clinical intervention in autoimmune and inflammatory disorders (4).

It has become increasingly clear that the role of complement in informing the immune system does not stop with innate immunity but rather presents a variety of signals to the adaptive immune system. It is well established that complement can serve as a natural adjuvant for the humoral immune response and other aspects of the B cell response (11). Furthermore, the relevance of complement to the development and maintenance of T cell immunity has emerged in recent years (2, 12, 13). The role of C5aR in T cell immunity in particular has garnered much attention, and works from several laboratories have implicated C5aR in APC and T cell function and in models of T cell-mediated diseases (14–21). The mechanism by which C5a impacts these T cell responses remains to be established. It has been postulated that local complement activation, via the alternative pathway, produces C5a, which can act on both APCs and T cells directly to regulate Ag uptake, costimulatory molecule expression, and T cell expansion and differentiation (15, 22–24). Other studies described a role for C5aR signaling more specifically on APCs, [e.g., on the differentiation and function of primary and bone marrow (BM)-derived dendritic cells (DCs)] (17, 25).

A main barrier to fully understanding the mechanism of action of the C5a:C5aR axis in T cell immunity has been the lack of clarity regarding C5aR expression on T cells and APCs. Elicited peritoneal macrophages as well as primary and BM-derived DCs (BMDCs) have been used as APCs in the study of C5aR signaling in T cell immunity (15, 17, 20, 24–27). Although it is well recognized that
C5aR is expressed on peritoneal macrophages and blood monocytes, reported findings of C5aR expression on T cells and DCs have been varied in the literature (15, 17, 28, 29). In this study, we created a C5ar1/GFP knockin mouse by gene targeting and used GFP as a sensitive surrogate marker to examine C5aR expression. Our studies shed new light on the expression and regulation of C5aR and represent a major step forward in understanding the sites and mechanism of action of C5aR signaling in T cell biology.

**Materials and Methods**

**Mice**

In mice, C5ar1 is encoded by the C5ar1 gene. C5ar1+/− mice on a C57BL/6 background were described previously (14, 30). Wild-type (WT) C57BL/6, OT-I, and OT-II TCR transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I and OT-II TCR transgenic mice were crossed with female C57BL/6 mice (The Jackson Laboratory) to derive OT-I-GFP-KI and OT-II-GFP-KI mice, respectively. Gender- and age-matched WT and transgenic mice were used throughout this study. Mice were housed in a specific pathogen-free facility, and all of the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

**Generation of C5ar1 GFP knockin mice**

GFP-KI mice were generated by gene targeting. A schematic of the targeting strategy is shown in Fig. 1A. We used the pND1 plasmid to construct the targeting vector (31). Our strategy was to insert GFP into the 3′-untranslated region (3′-UTR) and simultaneously fox the coding region of C5ar1 with two loxP sites. If successful, this strategy would have produced a C5ar1 GFP knockin as well as a C5ar1 floxed mouse for conditional knockout studies. The short arm homologous sequence is a 5.1-kb fragment containing a segment of the 3′-UTR and the immediate downstream flanking sequence of the C5ar1 gene. This fragment was amplified by PCR from 129/SvEv mouse genomic DNA using the following primers: 5′-GAT-ATC-GCA-GTG-GCT-AGG-ATA-CA-3′ (upstream, italicized for the EcoRV site), 5′-GAG-AAC-ATG-CTT-GCT-ACC-CA-3′ (downstream). It was cloned into pCR2 (Invitrogen) and then excised by EcoRI restriction digestion and subcloned into pNDA1 at the EcoRI site. The long arm of the homologous sequence contained two segments (5.3 and 1.5 kb) separated by a loxP site. The 5.3-kb segment, corresponding to an intron sequence immediately upstream of exon 2, was amplified by PCR using the following primers: 5′-GCG-GCC-GCT-GCT-GGG-ATA-CGA-GTT-ATG-CA-3′ (upstream, italicized for the NotI site), 5′-TCT-AGA-AAG-CCC-AGG-ATG-ACA-TGA-ATA-C-3′ (downstream, italicized for the XbaI site). The 5.3-kb fragment corresponded to the coding region and a portion of the 3′-UTR of exon 2. It was amplified by PCR using the following two primers: 5′-TCT-AGA-ATA-ATC-TCT-TAT-ATA-GTA-TGC-TAT-AAG-TTA-TCA-GAG-GTGG-CAC-TTC-3′ (upstream, italicized for the XbaI site and underlined for the loxP sequence), 5′-CTG-AGG-GGG-TGG-ACA-AGG-TAG-CGA-AGA-A-3′ (downstream, italicized for the XhoI site). The 5.3- and 1.5-kb fragments were then digested with XhoI and XbaI/XhoI restriction digestion, respectively. They were then ligated into a pND1 vector, containing the short arm prepared by NotI/XhoI digestion, in a three-piece ligation reaction. Finally, a GFP cassette containing an IRES-GFP-SV40 cassette then was excised with NotI and ligated into pND1 and underlined for the XhoI site). The SV40 sequence was cloned into pCR2 and then excised by SacI restriction digestion and subcloned into the Migr-1 vector at the SalI site. The IRES-GFP-SV40 cassette then was excised from Migr-1 by XhoI digestion and cloned into pND1 as outlined previously. The targeting vector was linearized with NotI and used for gene targeting to produce a GFP-KI mouse by gene targeting and used GFP as a sensitive surrogate marker to examine C5aR expression. Our studies shed new light on the expression and regulation of C5aR and represent a major step forward in understanding the sites and mechanism of action of C5aR signaling in T cell biology.

**Northern blot analysis**

Total RNAs from mouse tissues were isolated using the TRIzol reagent (Life Technologies). RNA samples (10 μg each lane) were separated on a 1% formaldehyde-agarose gel and transferred onto a nylon membrane (Hybond-N; Amersham Biosciences) via capillary action overnight in 20× SSC. The membrane was cross-linked under UV illumination and hybridized first with a 32P-labeled C5ar1 cDNA probe. After being probed, the membrane was stripped by boiling in 0.1× SSC-0.1% SDS and rehybridized with a 32P-labeled GFP cDNA probe. Both DNA probes were labeled with [32P]° using random primers. Northern hybridizations were conducted in QuikHyb solution (Stratagene) at 68°C for 1 h. The membrane was washed, first in 2× SSC-0.1% SDS at 55°C for 15 min and then in 0.1× SSC-0.1% SDS at 55°C, and exposed to X-ray film.

**Western blot analysis**

Western blot analyses were performed according to standard protocols (35). Peritoneal macrophages were lysed in 0.5× SDS-containing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer, and protein concentrations were determined by Pierce bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Western blots were exposed to ECL (Amersham Biosciences) and developed by the Fuji image reader.

**Neutrophil migration assay**

Neutrophils were isolated from mice via peritoneal lavage with PBS 6 h after the injection of 1 ml 4% (w/v) Brewer thioglycollate (Becton Dickinson Microbiology Systems). Transwell chemotaxis assays were performed as described previously (36). Briefly, peritoneal neutrophils were suspended in DMEM with 10% FBS in the top well of a Costar Transwell 24-well plate (Corning). Media or media containing 10 nM recombinant murine C5a (Hycult) was added to the bottom chamber and incubated for 6 h, after which time an accurate count of the number of neutrophils that had migrated to the bottom chamber was determined by running the contents of the bottom chamber through a flow cytometer.

**Cell isolation**

PBMCs were isolated from peripheral blood collected via either retroorbital bleeding or cardiac puncture using 0.2 M EDTA as an anticoagulant (1:10 volume). PBMCs were washed twice in staining buffer and were stained with a variable panel of Abs covering the cell surface Ags used in analysis. Panels were made up of some combination of fluorochromes including PE, PE-Texas Red, PerCP-Cy5.5, PE-Cy7, allophycocyanin, Alexa Fluor 700,
allophyocyanin-Alexa Fluor 750, and Pacific Blue. All of the Abs were obtained from eBioscience except anti-C5aR (clone 20/70; Cedarlane). Isotype controls for Abs (rat IgG1/2a/2b and Armenian hamster IgG) were obtained in the appropriate colors from eBioscience. Aqua Live/Dead (Invitrogen) was included with every sample to gate out dead cells, with only Aqua low cells being used in further analyses. Samples were analyzed on a FACS Canto flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star). Data are representative of three to five independent experiments.

**Immunostaining and fluorescence microscopy**

Peritoneal neutrophils were isolated according to the above protocol and were fixed, and splenocytes were isolated as described above and further subjected to cytometric analyses.

**FLT3L-secreting tumor implantation**

GFP-KI mice and WT controls were implanted with 5 × 10⁵ FLT3L-secreting B16 melanoma cells (kindly provided by Dr. U. H. Von Andrian, Harvard Medical School, Boston, MA) by s.c. route according to published protocols (37, 38). After 15 d, tumor-bearing mice were sacrificed, and splenocytes were isolated as described above and further subjected to cytometric analyses.

**T cell activation assays**

Nonspecific T cell activation was performed using plate-bound anti-mouse CD3 mAb (5 μg/ml for plate-coating; BD Pharmingen) in the presence of 2 μg/ml soluble anti-mouse CD28 mAb (BD Pharmingen) or using Con A (1 μg/ml; Sigma-Aldrich) in 200 μl complete medium (DMEM containing 10% FBS, 2 mM l-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 50 μM 2-ME, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin) for 6 h. Cells were collected hourly in complete medium and analyzed using flow cytometry. All of the reagents and columns used for cell isolation were purchased from Miltenyi Biotec (Auburn, CA). Splenocytes from OT-I-GFP-KI⁺/⁻ and OT-II-GFP-KI⁺/⁻ mice were prepared by the lysis of RBCs with lysis buffer and passage through a cell strainer as described previously. In each well of a 96-well U-bottom plate, 1 × 10⁶ splenocytes were plated in 200 μl complete medium. OVA peptides (OVA264-278:α for OT-I and OVA213-229 for OT-II; ABBIOTEC) were added at concentrations of 1 μg/ml at the time of cell mixing. Triplicate wells were set up for each condition. Forty-eight hours after being mixed, cells were collected for flow cytometric analysis, and proliferation was measured with the CellTiter cell proliferation kit (Promega).

**In vivo TLR stimulation**

LPS (E. coli 026:B6; Sigma-Aldrich) and CpG 1826 (5'-CCATACGTTACGTCTGACGTT-3'; synthesized by Oligos Etc.) were injected i.p. into GFP-KI mouse or nontransgenic littermate controls at doses of 20 mg/kg. Animals were sacrificed after 12 h, and relevant tissues were harvested for analysis. Polyniosin-polyctidylic acid [poly(I:C)] (high molecular weight; Invivogen) was injected i.p. at doses of 15 mg/kg, and animals again were sacrificed after 12 h for analysis.

**BM-derived macrophage and DC cultures**

BM-derived macrophages were generated as described previously (40). Briefly, BM cells were isolated as described above and plated in RPMI 1640 containing 10 mM HEPES, 10% FBS, and 15% (v/v) of L-cell conditioned medium, which was produced according to previous protocols (39). Cells were collected at day 7 and stained for flow cytometry. BMDCs were generated as described previously (40). BM cells were cultured in RPMI 1640 (10% FCS, 2 mM l-glutamine, 50 μM 2-ME, 100 U penicillin/streptomycin, 20 ng/ml GM-CSF, and 10 ng/ml IL-4). Medium was changed every other day. On day 6, DCs were stimulated with LPS at a concentration of 1 μg/ml, and cells were collected 24 h later for flow cytometry analysis.

**Results**

**Generation of C5ar1 GFP-KI mice**

We used gene targeting to insert a GFP cDNA gene cassette into the 3' flanked region of the C5ar1 gene (Fig. 1A). We obtained several targeted ES cell clones that had the GFP cassette correctly inserted but without the upstream loxP site incorporated as intended (Fig. 1A and data not shown). The latter outcome was the result of preferential homologous recombination between the C5ar1 gene and the sequences downstream of the loxP in the targeting vector. Insertion of the GFP cDNA into the 3'-UTR of the C5ar1 gene would allow both genes to be transcribed bicistronically under the control of the C5ar1 gene promoter, producing a single chimeric mRNA from which C5ar1 and GFP can be synthesized as separate proteins.

We successfully produced heterozygous (GFP-KI⁺/⁻) and homozygous (GFP-KI⁺/⁺) GFP knockin mice from one of the positive ES clones. Northern blot analysis revealed two C5ar1 mRNA species in the spleen and lung of GFP-KI⁺/⁻ mice, one of normal size and the other of a larger size (Fig. 1B). As expected, only the larger C5ar1 mRNA species could be detected by a GFP cDNA probe (Fig. 1B), confirming the presence of a bicistronic C5ar1-GFP mRNA in GFP-KI⁺/⁻ mice. Western blot analysis showed that C5ar1 protein was present in peritoneal macrophages of both WT and GFP-KI⁺/⁻ mice, whereas GFP protein could be detected only in GFP-KI⁺/⁺ mice (Fig. 1C). Similarly, green fluorescent PBMCs were detected easily by immunofluorescence microscopy on a blood smear of GFP-KI⁺/⁻ mice but not of WT mice (Fig. 1D). Collectively, these data established that GFP mRNA was transcribed under the control of the C5ar1 gene promoter in GFP-KI⁺/⁻ and GFP-KI⁺/⁺ mice and that GFP protein was synthesized successfully and easily detectable.

By FACS analysis, we also detected high levels of GFP in elicited peritoneal neutrophils of GFP-KI⁺/⁻ and GFP-KI⁺/⁺ mice but not in those of WT controls (Fig. 1E). Unexpectedly, although C5ar1 expression was detected by FACS on WT and GFP-KI⁺/⁻ cells, it was reduced markedly on GFP-KI⁺/⁺ mouse cells (Fig. 1E). Furthermore, in a chemotactic assay, neutrophils from WT and GFP-KI⁺/⁻ mice, but not GFP-KI⁺/⁺ mice, responded to C5a stimulation (Fig. 1F). These results suggested that the insertion of GFP into the 3' flanked region of the C5ar1 gene interfered with C5ar1 expression on the cell surface. The inhibitory effect of C5ar1 expression appeared to be posttranslational, because Western blot analysis detected a higher level of C5ar1 protein in the cell lysate of GFP-KI⁺/⁺ mouse cells (Fig. 1G). Immunostaining of peritoneal neutrophils from GFP-KI⁺/⁺ mice confirmed the presence of intracellular C5ar1 protein that was localized to the perinuclear region (Supplemental Fig. 1). In light of this result, we used GFP-KI⁺/⁻ mice in subsequent GFP expression studies to avoid potential underestimation of C5ar1 expression in case the receptor is autoregulated.

**GFP expression on peripheral blood cells and in secondary lymphoid organs**

Using GFP-KI⁺/⁻ mice, we first set out to identify GFP-expressing cells in the periphery and secondary lymphoid organs, including the spleen and lymph nodes. We found that ∼10–20% of PBMCs in GFP-KI⁺/⁻ mice were positive for GFP (Fig. 2A). Staining with cell type-specific markers showed that a majority of Gr-1⁺ cells and approximately half of CD11b⁺ cells expressed GFP (Fig. 2A). In contrast, very few CD8⁺, CD4⁺, and B220⁺ PBMCs expressed GFP (Fig. 2A). These results demonstrated that GFP-expressing PBMCs were primarily neutrophils and monocytes and that peripheral T and B lymphocytes were negative for GFP expression. In a separate analysis, we further examined GFP expression on three distinct populations of PBMCs defined by Gr-1 and CD11b double staining (Fig. 2B). Gr-1⁺CD11b⁺ cells were negative for F4/80 and CD11c and had high GFP expression. These cells represented neutrophils and granulocytes. Gr-1⁺CD11b⁻ cells
FIGURE 1. Generation of a C5ar1 GFP knockin mouse. (A) Schematic of the gene targeting strategy. An IRES-enhanced GFP-SV40 gene cassette was inserted into the 3′-UTR of the C5ar1 gene by homologous recombination. (B) Northern blot demonstrating the presence of a larger chimeric C5ar1-GFP mRNA species in the spleen and lung of GFP-KI+/- mice. Note that WT and mutant mRNA bands in the GFP-KI+/- mouse were of similar intensities, suggesting that the transcription of the mutant C5ar1 gene allele was not affected. (C) Western blot analysis showing that C5aR protein was detected in the cell lysates of both WT and GFP-KI+/- mouse peritoneal macrophages, whereas GFP was detected only in GFP-KI+/- mouse cell lysate. (D) Peripheral blood smear showing fluorescent PBMCs in GFP-KI+/- but not WT mice. (E) FACS analysis of peritoneal neutrophils showing GFP+ cells in GFP-KI+/- and GFP-KI+ mice but not in WT controls. It also shows staining of C5aR with mAb 20/70 in WT and GFP-KI+/- mice. C5aR staining was absent on C5ar1-/- cells and markedly reduced on GFP-KI+/- cells. (F) Neutrophil assays showing GFP-KI+/- mouse neutrophils did not respond to C5a stimulation, confirming the essential absence of C5aR on their surfaces. (G) Western blot demonstrating higher intracellular C5ar protein levels in the cell lysate of elicited peritoneal macrophages of GFP-KI+/- mice.
expressed low levels of F4/80, CD11c, and GFP, and these cells most likely represented monocytes. Gr-1<sup>-</sup>CD11b<sup>-</sup> cells had no expression of F4/80, CD11c, or GFP and presumably consisted of lymphocytes (Fig. 2B).

Similar results were obtained from the analysis of splenocytes of naive mice. In general, ~3–5% splenocytes were positive for GFP (Fig. 2C). As in PBMCs, GFP expression was mostly coincidental with Gr-1 staining and, to a lesser extent, with CD11b staining.
(Fig. 2C), and very few splenic T (CD8+ or CD4+) and B (B220+) lymphocytes expressed GFP (Fig. 2C). Notably, we also failed to detect GFP expression on splenic CD11c+ or PDCA-1+ cells, suggesting that C5aR is expressed neither on myeloid DCs (mDCs) nor on plasmacytoid DCs (pDCs) of the mouse spleen (Fig. 2C). To further examine GFP expression on splenic DCs, we implanted GFP-KI+/- mice with FLT3L-expressing tumor cells to expand the DC populations (37, 38). As in naive mice, we found that splenic mDCs and pDCs from FLT3L-stimulated GFP-KI+/- mice were also essentially negative for GFP expression (Fig. 2C). Finally, no GFP was observed on any of the inguinal lymph node cells (lymphocytes) of naive mice (Fig. 2D). Importantly, staining of native C5aR with the 20/70 mAb detected good correlations between GFP and C5aR signals in both GFP+ and GFP- spleen cell populations (Supplemental Fig. 2).

**GFP expression on BM cells and peritoneal and BM-derived macrophages**

In contrast to peripheral blood or secondary lymphoid organs, C5aR expression was more prevalent on BM cells. We found that ~50% of primary BM cells expressed GFP (Fig. 3A). As is the case in peripheral blood and the spleen, much of GFP expression tracked with Gr-1+ and CD11b+ cells, which likely represented the pool of mature and maturing neutrophils that can be mobilized after immune challenges (41). Interestingly, we detected small populations of CD11clowGFP high cells and B220+GFP+ cells (Fig. 3A).}

**FIGURE 3.** GFP expression on primary BM cells and macrophages from different sources. (A) Close to half of primary BM cells were positive for GFP, and most GFP+ cells also are Gr-1+ and CD11b+. Small populations of CD11clowGFP high and B220+GFP+ cells were present in the BM. Both populations also were Gr-1+, but they appeared to be separate populations. (B) The majority of resident peritoneal macrophages were negative for GFP, but a small population had high levels of GFP expression. Thioglycollate-elicited peritoneal macrophages were relatively homogeneous in expressing moderate levels of GFP. BM-derived macrophages appeared to have two separate populations with regard to GFP expression, with GFP-expressing cells constituting the larger population. Data are representative of three independent experiments.
We wondered if these might be the same cells, representing a type of C5aR-expressing pDCs or their precursors with a phenotype of CD11c<sup>B220<sup>+</sup></sup> as described previously (42). Further analysis showed however that both cell populations were Gr-1<sup>+</sup> and that there was little overlap between CD11c and B220 markers (Fig. 3A). Thus, they appeared to represent minor subpopulations of the Gr-1<sup>+</sup>C5aR<sup>+</sup> cell lineage with unknown significance.

We found that resident peritoneal macrophages consisted of two distinct populations differing in GFP expression. The majority of the cells were negative for GFP, although a small population (∼10% of F4/80<sup>+</sup> cells) expressed high levels of GFP (Fig. 3B). The pattern of thioglycollate-elicited peritoneal macrophages was quite different in that most cells expressed intermediate levels of GFP (Fig. 3B). We also examined GFP expression in BM-derived macrophages and detected low to intermediate levels of GFP in a majority of the cells (Fig. 3B).

**GFP expression on activated T cells**

The finding that GFP is essentially absent from T lymphocytes and splenic DCs contradicted recent reports describing C5aR signaling on these cell types (15, 22, 24, 25). It has been suggested that local complement activation in the immunological synapse produces C5a that triggers C5aR signaling on both T cells and DCs, providing a costimulatory signal to enhance T cell activation (15, 43, 44). To determine if C5aR expression might be induced upon T cell activation, we examined GFP expression on polyclonal T cells activated by anti-CD3/anti-CD28 Abs or Con A. As shown in Fig. 4A, either treatment strongly and time-dependently activated GFP-KI<sup>+</sup> mouse T cells, as indicated by CD69 and CD25 upregulation. However, no GFP expression was observed on the activated T cells (Fig. 4B).

In separate experiments, we assessed GFP expression on T cells activated by specific Ags. We crossed GFP-KI<sup>+</sup> mice with OT-I or OT-II TCR transgenic mice and generated OT-I-GFP-KI<sup>+</sup> and OT-II-GFP-KI<sup>+</sup> mice. The OT-I and OT-II mice bear transgenic TCRs that recognize specific OVA peptides, in the context of MHC classes I and II, respectively, to activate CD8<sup>+</sup> or CD4<sup>+</sup> T cells. Splenocytes from OT-I-GFP-KI<sup>+</sup> mice were stimulated with 10<sup>4</sup> M OVA<sub>257–264</sub> for 72 h before being collected and analyzed via flow cytometry for activation markers and proliferation. Nonspecific stimulation with anti-CD3/anti-CD28 Abs was included as a control. Using CD69 and CD25 expression and cell proliferation assays, we showed that CD8<sup>+</sup> T cells from both WT and OT-I-GFP-KI<sup>+</sup> mice were activated by anti-CD3/anti-CD28 Abs stimulation, but only cells from OT-I-GFP-KI<sup>+</sup> mice were activated by OVA<sub>257–264</sub> (Fig. 4C, 4D). Importantly, we found no GFP expression on OVA peptide-activated TCR transgenic CD8<sup>+</sup> T cells (Fig. 4C–E). Likewise, we detected no GFP expression on TCR transgenic CD4<sup>+</sup> T cells from OT-II-GFP-KI<sup>+</sup> mice activated by OVA<sub>323–339</sub> peptide (Fig. 4C–E). Taken together, our

![Figure 4](http://www.jimmunol.org/Downloaded_from%20http%3A//www.jimmunol.org/)

**FIGURE 4.** GFP is not expressed on activated T cells. (A) Splenocytes from GFP-KI<sup>+</sup> mice were left nonstimulated (NS) or stimulated with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab or with Con A. Samples were collected at hourly intervals, and CD3-gated T cells were analyzed by FACS for the expression of CD25 and CD69, two activation markers. (B) Analysis of GFP levels on CD3-gated T cells from GFP-KI<sup>+</sup> mouse splenocytes at various time points after anti-CD3/anti-CD28 Ab or Con A stimulation. WT CD3<sup>+</sup> cells (top trace) are displayed as a negative control, and Gr-1<sup>+</sup> (second from top trace) cells are plotted as a positive control for GFP expression. **Inset numbers** represent mean fluorescence intensities. Data are representative of three independent experiments. (C) Splenocytes from OT-I-GFP-KI<sup>+</sup> and OT-II-GFP-KI<sup>+</sup> mice were stimulated with OVA<sub>257–264</sub> and OVA<sub>323–339</sub>, respectively, as a specific Ag or with anti-CD3/anti-CD28 Abs as a nonspecific activator (positive control). CD8<sup>+</sup>-gated (for OT-I) or CD4<sup>+</sup>-gated (for OT-II) T cells then were analyzed for the expression of the activation markers CD25 and CD69 at 48 h. (D) Proliferation assays of splenocytes stimulated as described in (C). (E) Despite robust activation by specific peptide Ags, GFP was not induced on OT-I CD8<sup>+</sup> or OT-II CD4<sup>+</sup> T cells. **Inset numbers** represent mean fluorescence intensities. Data are representative of two independent experiments.
results suggested that C5aR expression is not induced on activated T cells.

**GFP expression in peripheral blood and the spleen after systemic inflammatory challenge**

We next studied GFP expression on peripheral leukocytes and in the spleen in response to inflammatory challenge. We treated GFP-KI+/− mice with LPS, poly(I:C), or CpG, ligands for TLR4, TLR3, and TLR9, respectively, and examined GFP expression 12 h later by FACS. We found that systemic inflammation triggered by the activation of any of the three TLRs caused a profound increase in GFP+ cells in peripheral blood mononuclear leukocytes and in the spleen (Fig. 5). For example, the percentage of GFP+ cells in peripheral blood increased from a baseline of 10–20% to 50–80%, depending on the length of time of stimulation and specific ligand used (Fig. 5A and data not shown). Likewise, ~3% of splenocytes in naive mice were GFP+, but such cells constituted 10–20% of the splenocytes in TLR ligand-stimulated mice (Fig. 5B). As observed

**FIGURE 5.** GFP expression on PBMCs and in the spleen after systemic TLR stimulation. (A) GFP+ cells were markedly increased (5- to 8-fold) in PBMCs 12 h after GFP-KI+/− mice were treated with LPS, poly(I:C), or CpG. As in naive mice, most GFP+ cells in TLR ligand-stimulated mice were Gr-1+ and CD11b+. (B) GFP+ cells also were expanded significantly (3- to 5-fold) in the spleens of TLR ligand-stimulated GFP-KI+− mice when analyzed 12 h after stimulation. Again, the expanded GFP+ cell population was found to consist mainly of neutrophils (Gr-1+ and CD11b+). Note that TLR activation did not increase the number of GFP−CD11c+ cells in the spleens of GFP-KI+− mice. (C) A population of GFP−CD11c− cells expanded moderately in the spleens of TLR ligand-treated GFP-KI+− mice. These cells were all Gr-1+. Data are representative of more than three independent experiments. NS, nonstimulated.
in naive mice, the vast majority of GFP⁺ cells in the blood and spleen of stimulated mice were found to express Gr-1 and CD11b (Fig. 5), suggesting that systemic inflammation caused rapid and large-scale mobilization of neutrophils and monocytes, presumably by driving their migration from the BM to peripheral sites. None of the treatment conditions caused GFP expression on circulating or splenic T and B lymphocytes (data not shown). Notably, systemic inflammation also did not induce C5aR expression on splenic CD11c⁺ DCs, because very few of the CD11c⁺ and CD11c⁺ GFP cells expressed GFP regardless of the nature and length of time of TLR ligand stimulation (Fig. 5B and data not shown). However, we observed a moderate expansion in the spleen of GFP⁺ cells with a CD11c⁺ Gr-1⁺ phenotype (Fig. 5C). It is not known if these cells have DC function or are simply part of the Gr-1⁺ neutrophil/granulocyte lineage.

**GFP expression on BMDCs**

One type of DCs commonly used in T cell immune response studies in vitro are BMDCs. Given our findings that a substantial fraction of BM cells are GFP⁺, we investigated if DCs differentiated in vitro from BM in the presence of GM-CSF and IL-4 might express C5aR. As shown in Fig. 6A, we produced CD11c⁺ BMDCs in culture that expressed characteristic DC markers, including MHC class II, CD80, CD11b, and CD86. Interestingly, in multiple independent experiments, we found that 5–25% of CD11c⁺ BMDCs expressed GFP (Fig. 6B). GFP expression was constitutive and independent of the maturation status of the BMDC, because stimulation with LPS, a prototypical inducer of DC maturation, did not significantly augment GFP expression (Fig. 6B).

**Discussion**

C5aR mediates many of the proinflammatory activities of activated complement. It also plays a key role in the regulation of T cell immunity by complement and in the cross talk between complement and TLRs, topics that have attracted much attention recently. Most evidence suggesting a role for C5aR signaling in T cell immune responses came from disease modeling studies in mice, including T cell-mediated allogeneic organ transplantation, experimental autoimmune encephalomyelitis, allergic asthma, and viral infection (14–16, 18, 20, 21, 43–46). How C5aR signaling impacted T cell reaction in these settings remains incompletely understood. Some studies have indicated an intrinsic role for C5aR signaling on T cells and APCs such as macrophages and DCs in providing costimulation during T cell activation (15, 17, 24, 25), whereas other models have considered the possibility of indirect regulation (e.g., through the generation of proinflammatory cytokines by cells other than T cells or DCs via C5aR-dependent mechanisms) (13, 14, 19, 20, 30). Of relevance to these hypotheses is knowledge of C5aR expression and regulation on peripheral blood mononuclear cells (PBMCs) and various APCs. Unfortunately, there is considerable uncertainty on this issue, particularly with regard to C5aR expression on T cells and DCs. Part of the reason may relate to limitations in prior studies in Ab specificity, choice of appropriate controls, and activation status of the cells. Additionally, there may be species differences between humans and experimental animals.

In the current study, we generated a GFP knockin mouse by gene targeting and used GFP as a surrogate marker to track C5aR expression on cells of the immune system under various conditions. A similar approach has been used previously for the examination of other immunologically relevant promoters, including CD83, IL-23, and IL-13 (47–49). The advantage of this approach is the noninvasive nature of the labeling, which allows for direct visualization of the cells without Ab staining of the target protein, thus eliminating potential nonspecific binding of primary and secondary Abs. Using this approach, we demonstrated that C5aR was most abundantly expressed on Gr-1⁺CD11b⁺ neutrophils in peripheral blood, spleen, and BM. It also was detected on blood monocytes in naive mice but at a much lower level than that on neutrophils. In the resting mouse spleen, a similar pattern was observed (i.e., the vast majority of Gr-1⁺ neutrophils expressed high levels of C5aR, whereas Gr-1⁺CD11b⁺ splenic macrophages were either negative or had low levels of C5aR expression). Of interest, we found a significant difference in C5aR expression between resident and thioglycollate-elicited peritoneal macrophages. Only a small fraction of resident peritoneal macrophages were positive for GFP, whereas most elicited F4/80⁺ cells expressed GFP. We also found that TLR activation profoundly increased the abundance of C5aR-expressing cells in peripheral blood and the spleen. However, the increased cells were overwhelmingly Gr-1⁺CD11b⁺ neutrophils that expressed levels of C5aR similar to those in naive mice. Thus, this outcome represented the well-known phenomenon of neutrophil mobilization and migration from BM to peripheral sites in response to pathogen infection (41).

A clear finding of the current study is the lack of C5aR expression on T lymphocytes in both peripheral blood and secondary lymphoid organs. We did not detect GFP expression on naive T cells or on T cells activated by nonspecific stimulators such as Con A and anti-CD3/CD28 mAbs or by specific Ags. Through the
use of similar bicistronic gene constructs, GFP has been detected as a surrogate marker for genes expressed in T cells (48, 49), which rules out potential technical explanations for the failure to observe GFP expression in T cells. Furthermore, we observed good correlations between GFP and native C5aR expression in both GFP+ and GFP− cell populations (Supplemental Fig. 2). Thus, our data do not support an intrinsic role for C5aR signaling on T cells during their activation. Likewise and somewhat unexpectedly, we did not detect significant C5aR expression on CD11clow and CD11cint splenic mDCs or pDCs from naive or TLR ligand-stimulated mice or on splenic DCs expanded by FLT3L stimulation. We did however note an increase in CD11clowGFP+ cells in TLR ligand-stimulated mouse spleens. Further analysis showed these cells to express high levels of Gr-1. Interestingly, cells with similar characteristics (CD11clowGr-1+GFP+) also were observed in BM, suggesting that they may have migrated from BM to peripheral sites as did other Gr-1+ cells under inflammatory conditions. It is not known if these CD11clowGFP-Gr-1+ cells represented a subset of neutrophils or an uncharacterized type of DCs with APC function. In this context, it is relevant to mention a group of Gr-1+ cells that have been characterized previously as inflammatory monocytes or their downstream progeny, the so-called ‘inflammatory’ DCs that serve as important informers of the adaptive immune response to infection through Ag trapping, presentation, and cytokine production (50).

If T lymphocytes and conventional DCs (as defined by CD11chigh and CD11cint) do not express C5aR, how do we explain the effects of C5aR on T cell immunity in vivo in models? First, C5aR signaling could play a role in macrophages, which almost certainly can function as APCs in vivo. Indeed, many of the published in vitro studies demonstrating a role for C5aR signaling in T cell biology used elicited peritoneal macrophages as APCs (19, 20, 22, 26, 44). Second, although we did not find C5aR expression on CD11chigh and CD11cint DCs from naive and TLR ligand-stimulated mouse spleens, we cannot yet exclude the possibility that DCs differentiated and matured in other endpoints under certain disease settings (e.g., asthma, experimental auto-immune encephalomyelitis, organ transplantation, etc.) may up-regulate C5aR expression in response to specific cytokine stimulation. In support of this possibility, we found that a sub-group of DCs differentiated in vitro from BM cells in the presence of GM-CSF and IL-4 expressed C5aR. Hopefully, the availability of the C5aR GFP-KI mouse now will allow this hypothesis to be tested experimentally. Third, as mentioned above, it is possible that some CD11clow (or even CD11c) C5aR+ cells, such as the CD11clowGr-1+GFP+ cells that we have observed or other C5aR+ cells in the monocytic cell lineage, may function as unconventional APCs in vivo. Finally, C5aR signaling on cells other than T cells or APCs may extrinsically regulate T cell activation and differentiation by affecting the production of proinflammatory cytokines and chemokines (13). It is likely that one or several of these mechanisms in concert work to influence T cell immunity and link the complement and adaptive immune systems.

The finding that the 3'-UTR of the mouse C5ar1 gene regulates C5ar protein targeting to the cell surface was rather unexpected. We found that C5ar was largely absent from the cell surfaces of GFP-KI+/+ mice. This outcome was not due to decreased C5ar mRNA stability, which the 3'-UTR is generally known to regulate. We detected normal levels of the bicistronic C5ar-GFP mRNA, and both GFP and cytoplasmic C5ar protein were made at normal levels, and, in the case of C5ar, higher levels. To our knowledge, this is the only second example of a 3'-UTR sequence critically regulating cell surface expression of a G protein-coupled receptor protein. Previously, it has been reported that disruption of the 3'-UTR of the β2-adrenergic receptor (β2-AR) mRNA interfered with its recognition by the RNA-binding protein HuR, and this in turn led to impaired cell surface expression of β2-AR (51, 52). Nuclear binding of HuR to the 3'-UTR of β2-AR mRNA is thought to prevent the translation of β2-AR mRNA while allowing its transit to the cell periphery where unknown mechanisms re-release the translational block and the locally translated β2-AR protein can efficiently traffic to the cell surface. Disruption of the 3'-UTR of β2-AR mRNA or knockdown of HuR led to premature initiation of translation, resulting in the overproduction of β2-AR protein in perinuclear polyribosomes but defective trafficking to the cell surface (51). This is reminiscent of the increase in C5aR protein in the GFP-KI+/+ cytosol seen by Western blot analysis and its perinuclear localization on immunofluorescence microscopy in our study. It is likely that a similar mechanism accounted for impaired cell surface expression of C5aR in GFP-KI+/+ mice.

In summary, we have generated and used a novel GFP knockin mouse to comprehensively characterize C5aR expression. Our results shed new light on the regulation of C5aR in vivo and have implications for understanding the mechanism of action of C5aR signaling in T cell immune responses. The GFP-KI mouse described here may serve as a valuable tool to further elucidate the biology of C5aR in health and disease.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1

WT

GFP-KI+/+

C5aR−/−

WT

(secondary ab only)

[Blue]
Hoechst 33258
(DNA stain)

[Red]
Primary: Biotinylated C5aR mAb 20/70
Secondary: Alexa-Flour 594-conjugated streptavidin
Supplemental Fig 2

A. GFP

WT  GFP KI +/-  C5aR-/-

GFP

Gr-1

B. GFP

CD4

C. GFP

CD11c
Supplemental Fig 1. Alterations in the localization of C5aR protein between WT and homozygous GFP-KI mice. Peritoneal neutrophils were stained with a combination of biotinylated anti-C5aR 20/70 and Alexa 594-conjugated streptavidin secondary antibody to detect C5aR protein (red staining). Nuclei were counterstained with Hoechst 33258 (blue staining). Two representative images of neutrophils from each strain (WT, GFP-KI+/+, and C5aR−/−; left three columns) are displayed along with two representative images of WT neutrophils stained with secondary antibody alone (far right column). Data are representative of two independent experiments.

Supplemental Fig 2. Correlation of GFP and C5aR protein expression in both GFP+ and GFP− spleen cell populations. Splenocytes from WT, GFP-KI +/- and C5aR−/− mice were stained for Gr-1 (A), CD4 (B), or CD11c (C) as well as C5aR (using mAb 20/70). There is correlation between GFP and C5aR expression on Gr-1+ cells (A). There is also correlation between the lack of GFP and C5aR expression on CD4+ (B) and CD11c+ cells (C). Data are representative of at least 3 independent experiments.