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Monitoring and Cell-Specific Deletion of C5aR1 Using a Novel Floxed GFP-C5aR1 Reporter Knock-in Mouse

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Many of the biological properties of C5a are mediated through activation of its receptor (C5aR1), the expression of which has been demonstrated convincingly on myeloid cells, such as neutrophils, monocytes, and macrophages. In contrast, conflicting results exist regarding C5aR1 expression in dendritic cells (DCs) and lymphoid lineage cells. In this article, we report the generation of a floxed GFP-C5aR1 reporter knock-in mouse. Using this mouse strain, we confirmed strong C5aR1 expression in neutrophils from bone marrow, blood, lung, and spleen, as well as in peritoneal macrophages. Further, we show C5aR1 expression in lung eosinophils, lung- and lamina propria–resident and alveolar macrophages, bone marrow–derived DCs, and lung-resident CD11b+ and monocyte-derived DCs, whereas intestinal and pulmonary CD103+ DCs stained negative. Also, some splenic NKT cells expressed GFP, activated CD4+ Th cells in vitro or in vivo. Mating the floxed GFP-C5aR1 mouse strain with LysMCre mice, we were able to specifically delete C5aR1 in neutrophils and macrophages, whereas C5aR1 expression was retained in DCs. In summary, our findings suggest that C5aR1 expression in mice is largely restricted to cells of the myeloid lineage. The novel floxed C5aR1 reporter knock-in mouse will prove useful to track C5aR1 expression in experimental models of acute and chronic inflammation and to conditionally delete C5aR1 in immune cells. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: AcGFP, Aequorea coerulescens GFP; AT, anaphylatoxins; BALF, bronchoalveolar lavage fluid; BM, bone marrow; BMDC, BM-derived DC; [Ca2+]i, intracellular Ca2+; cDC, conventional DC; DC, dendritic cell; ES, embryonic stem; IRES, internal ribosome entry site; LN, lymph node; LP, lamina propria; MFI, mean fluorescence intensity; moDC, monocyte-derived DC; pEC50, negative logarithm of EC50; RBCL, RBC lysis; UTR, untranslated region; wt, wild-type.

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that C5a regulates signal 1 (MHC class II), signal 2 (costimulatory molecules), and signal 3 (cytokines) as a critical means to modulate the regulation of T cell proliferation and differentiation by APCs (reviewed in Ref. 37).

Recently, a new concept has emerged suggesting that the ATs control T cell activation by a paracrine mechanism (i.e., their impact on DC activation and maturation), as well as through a direct autocrine effect on T cells. Further, this concept includes production of complement factors of the alternative pathway by APCs and T cells resulting in local complement activation, generation of ATs, and activation of AT receptors, which then partake in the subsequent T cell–activation events (28, 29, 38–40). On T cells, AT receptor–mediated signals were suggested to induce upregulation of IL-12R, activation of PI3Kγ, and inhibition of cAMP–dependent protein kinase activation by interfering with 3′–5′-cAMP generation, which is associated with phosphorylation of Akt. Together, such events enable mammalian target of rapamycin (mTOR) activation of ATs, and activation of AT receptors, which then partake in the subsequent T cell–activation events (28, 29, 38–40).

Reagents

Materials and Methods

Animals

Generation of a C5aR1 GFP–knock-in mouse strain allowing for conditional C5aR1 deletion

C5aR1–GFP–knock-in mice were generated by gene targeting. The targeting strategy is shown in Fig. 1A. The targeting vector, targeted embryonic stem (ES) cells, and targeted mice were generated by Ozone. Our strategy was to insert Aequorea coerulescens (Ac)GFP and an internal ribosomal entry site (IRES) adjacent to the coding exon of C5aR1 and to simultaneously flank the AcGFP IRES C5aR1 cassette with two IRES sites. This strategy was designed to produce a C5aR1 GFP–knock-in mouse, as well as a C5aR1 floxed mouse for conditional knockout studies.

The targeting construct was inserted by the sequential cloning of five amplified fragments, using a recipient plasmid containing a phosphoglycerate kinase neo polA selectable marker cassette. The first fragment was a 2.8-kb 5′ homology arm containing a segment of the first intron of C5aR1 gene, as well as the first IRES site. The second fragment was amplified by PCR from C57BL/6 mouse genomic DNA using the following primers: SH_fwd: 5′-AAATACATTGGCCCTCACTTCA-3′ and SH_rev: 5′-TTCCGAATAACTCGTATAAGTGATCTAGTGAGTGTTCTAGATTGGCGCTTCTTCTGAGTCTCAACTA-3′ (includes Xba_I, IoxP, BstB_I). The second fragment of 0.8 kb contains the ox 2 splice acceptor and AcGFP coding sequence and was amplified using the following primers: AcGFP_fwd: 5′-TATATTAGCTCCATCCGTCGATGTTAC-3′ and AcGFP_rev: 5′-GACTCTCACCTGATCCTCAACTCCGTCGATGTTAC-3′ (contains Aat_II).

The third fragment of 0.6 kb contains the IRES sequence and was amplified using the following primers: IRES_fwd: 5′-GACGTCGACCCTCTCGCTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTAC-
was inserted upstream of the fifth fragment (described above) using AsclI thereby completing the targeting vector. The Phusion PCR system (Thermo Scientific) was used in all of the PCR reactions. Gene targeting in ES cells was performed according to standard protocols.

The targeting vector was linearized with PvuII digestion and transfected into ES cells (Bruce4, a cell line derived from the C57BL/6 mouse strain) as described (47). The neomycin gene in the vector allows for selection of ES cells that have undergone homologous recombination and incorporated GFP and flox C5aR1 gene loci. Positive clones were screened by Southern blot analysis of EcoRI-digested ES cell DNA using a 580-bp probe 3′ to the homology arm. The probe was amplified by PCR using the following primers: P3_fwd: 5′-GGGTTCATTCTGGGCTATCA-3′ and P3_rev: 5′-GAGGAAGGGAGGCATAAAGG-3′. Positive clones were expanded, confirmed in second-round screening, and microinjected into C57BL/6 blastocysts before being transferred to pseudo-pregnant females. Subsequently, male chimeras were mated with female C57BL6J mice (The Jackson Laboratory) to achieve germline transmission.

Genotyping of floxed GFP-C5aR1 knock-in and GFP C5aR1 LysMCre mice

For genotyping, we used tail or ear biopsies. Extraction of DNA from tissue was performed using the KAPA Extraction kit (PEQLAB), following the manufacturer’s instructions. For amplification of the different fragments, we used the following primers: Primer_01F: 5′-TAGAGTT- GGAAGGTAGGTGG-3′, Primer_02F: 5′-GTACACGAAGGATGGTG-3′, Primer_03R: 5′-GGGTTCATTCTGGGCTATCA-3′, and Primer_04R: 5′-CTCTGTGTCTCTGTTACACCCT-3′ (all from R&D Systems). The PCRs were run under the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, 62°C for 15 s, and 72°C for 30 s, followed by 72°C for 120 s. Then, the samples were loaded onto a 1% sodium borate agarose gel. Amplification products were detected by GelRed staining.

Cell preparation from different organs

Generally, mice were killed by cervical dislocation under anesthesia before organ removal. For bone marrow (BM) preparation, femurs, tibias, and humeri were removed, placed in PBS on ice, and flushed with PBS. RBCs were removed by incubating the cells in RBCC buffer for 3 min and washing in PBS to stop the lysis. For lung cell preparation, lungs were digested with Liberase, and single-cell lung suspensions were prepared as described (8). Bronchoalveolar lavage fluid (BALF) samples were obtained as described (36). To collect cells from the peritoneal cavity, mice were lavaged with 5 ml ice-cold PBS. Collected cells were washed once with PBS. RBCs were removed by incubating the cells in RBCC buffer for 3 min and washing with PBS. Blood was collected by cardiac puncture and immediately transfused into tubes containing 10 mM EDTA to prevent coagulation. Diluted blood samples were washed several times in RBCC buffer to remove RBCs and then washed with PBS. Isolation of cells from the spleen and lymph nodes (LN) was performed by mechanical disruption using a cell strainer (40 μm Nylon) and the plunger of a 5-ml syringe (both from BD). The cell strainer was flushed three times with 5 ml PBS. Cells were collected from the RBCC buffer for 3 min and washed with PBS. The proper (LP) cell suspension was obtained using an LP Disassociation Kit, following the manufacturer’s recommendation. Briefly, the small intestine was quickly cleared of feces, residual fat, and Peyer’s patches. Then, it was cut longitudinally and incubated twice in HBSS supplemented with EDTA (5 mM) and once in HBSS only. The tissue was digested using the manufacturer’s enzyme mixture in HBSS supplemented with Ca2+ and Mg2+ for 30 min at 37°C and homogenized with a gentleMacs dissociator. After the last washing step, the cell number was determined using a Neubauer chamber, and then the cells were resuspended in PBS containing 1% BSA. Viability of the collected cells was determined by trypan blue exclusion or a LIVE/DEAD kit using an LSR II flow cytometer (BD).

Chemotaxis assay

Chemotaxis of BM-derived cells was performed as described (48). Briefly, cells were resuspended in chemotaxis medium (HBSS containing 2% BSA) at a density of 5 × 106 cells/ml. The chemotaxaerator C5a (12.5 nM) was diluted in chemotaxis medium, placed in the bottom wells of a micro Boyden chemotaxis chamber (Neuroprobe), and overlaid with a 3-μm polycarbonate membrane. Then, 50 μl of the cell suspension were placed in the top wells and incubated for 30 min at 37°C. Subsequently, the membranes were removed and the cells on the bottom side of the membrane were stained with propidium iodide (Merk). The membranes in five high-power fields were counted, and the number of cells/mm² was calculated by computer-assisted light microscopy, as described (49). Results are expressed as the mean value of triplicate samples.

C5a-mediated increase in intracellular calcium

The C5a-induced increase in intracellular Ca2+ ([Ca2+]i) was determined, as described (48), using neurophils (Ly6Ghi) or macrophages (F4/80hi) from BM or the peritoneal cavity, respectively. Briefly, cells were collected as described above and loaded with 5 μM the Ca2+-sensitive fluorophore Fluo-4, AM for 30 min. Nonincorporated dye was washed away using PBS. Cells were then gated on Ly-6Ghi or F4/80hi cells and analyzed on the LSR II flow cytometer. The background signal was recorded for 30 s. Then, C5a was added at the indicated concentrations, and recording continued for another 90 s. The increase in [Ca2+]i was calculated by assessment of the maximal Ca2+ peak using the kinetic plug-in tool of FlowJo software (Vert. 9, TreeStar, Ashland, OR). The background signal was subtracted from the signal obtained in response to the specific stimulus and recorded as the Δ maximal peak.

Determination of C5aR1 expression on BM neutrophils by immunofluorescence staining

Cells were collected from the BM and stained with anti-CD88 mAb (clone 20/70), anti-Ly6G mAb, and DAPI. The cells were incubated at 4°C for 15 min, fixed with BD Cytofix/Cytperm, transferred to a slide, and mounted with Fluoromount-G. Images were obtained using an Olympus FV 1000 confocal microscope (Olympus) with a 60× oil 1.35 NA objective. Image analysis and capturing were performed using FluoView 2.1c software (Olympus).

Flow cytometry and cell sorting

Phenotypic characterization of cells was performed using a BD LSR II flow cytometer. The FITC channel was used to determine the GFP-derived fluorescence signal. BM-derived neutrophils and peritoneal macrophages also were analyzed for C5aR1 expression using a C5aR1-specific mAb. This procedure allowed us to correlate the GFP signal with C5aR1 surface expression. In some experiments, we permeabilized BM-derived neutrophils and peritoneal macrophages using BD Cytofix/Cytperm to determine intracellular C5aR1 expression, following the manufacturer’s instructions. Flow cytometric data were analyzed using FlowJo 9 software (TreeStar). C5ar1 mRNA expression was determined in cells purified using a BD Aria III cell sorter. The different cell populations were identified using a panel of lineage- and cell-specific Abs (Supplemental Fig. 1). Lung or BALF macrophages were identified and sorted using a recently published gating strategy (50). Briefly, macrophages were SiglecF+CD11c+ cells, and lung eosinophils were SiglecF+CD11c− cells. In the SiglecF− and lineage (CD49b, Ly6G, CD19, CD3)− negative population, different pulmonary CD11c+MHCII+ DC subsets were identified and sorted using the gating strategy displayed in Fig. 4A. Briefly, conventional DCs (cDCs) were identified as CD103+CD11b+CD64+ or CD103+CD11b+CD64− cells, and moDCs were identified as CD103−CD11b+CD64+ cells. In the lineage− fraction of the LP of the small intestine, macrophages and DCs were identified and sorted as shown in Fig. 4C using Abs against CD11c, CD103, CD11b, and F4/80, as described (51). DCs were identified as CD11c+CD103+CD11b+LCs and CD11c+CD103+CD11b−F4/80+ DCs. LP macrophages were identified as CD11c+CD103+CD11b+F4/80+ cells. Splenic DCs were identified by the expression of CD11c+ and differentiated into CD11c+CD80+ and CD11b+CD80+ cells. Splenic red pulp macrophages were identified as CD11c+ F4/80− cells. NK and NKT cells were identified by NK1.1 expression and were differentiated by anti-CD3 (CD3+ NKT; CD3− NK cells). B2 cells were identified by double staining with anti-CD19 and anti-CD45R. Neutrophils were sorted from BM using a Ly-6G−specific mAb.

CD4+ T cell preparation from spleen

The spleen was extracted and processed as described above. CD4+ T cells were isolated from the single-cell suspension by MACS using a CD4+ T Cell Isolation Kit II mouse, LS columns, and a QuadroMACS Separator, according to the manufacturer’s instructions.

In vitro activation of CD4+ T cells

To stimulate CD4+ T cells, a 48-well cell culture plate (Greiner Bio-One International) was coated with anti-CD3 or anti-CD28 in 150 μl PBS at a final concentration of 2 or 10 μg/ml, respectively. The plate was kept in an incubator at 37°C, 5% CO2 for ≥2 h before discarding the coating solution and adding the cell suspension. Recombinant mouse IL-2 (final concentration 5 ng/ml) was added to every well. Isolated CD4+ T cells were cultured in complete RPMI 1640 culture medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured for the indicated times and analyzed by flow cytometry for C5aR1 or GFP expression.
**Immunization of mice with OVA peptide**

Mice were immunized in the footpad with 100 μg OVA23-33 peptide emulsified in CFA and boosted once in the same way 8 d later. After an additional 7 d, mice were sacrificed. Popliteal and inguinal LNs, as well as the spleen, were collected and processed as described above. Subsequently, LN and spleen cells were used and assessed for GFP and C5aR1 expression by flow cytometry. Further, C5ar1 mRNA expression was determined by RT-PCR. Also, CD4+ T cells from the spleens were isolated using the CD4+ T Cell Isolation Kit II, mouse and used for ex vivo activation experiments by CD3 and CD28 stimulation, as described above.

**Generation of DCs from BM cell cultures**

BM-derived DCs (BMDCs) were generated as described (8). Briefly, BM cells were isolated from C57BL/6 wt mice or the different floxed GFP-C5aR1 knock-in mice, as described above. BM cells were washed and cultured at 1 x 10^6 cells/ml in complete RPMI 1640 culture medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml recombinant murine GM-CSF. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 9 d.

**RNA isolation and real-time PCR**

RNA was isolated using TRIzol reagent, according to the manufacturer’s instructions. After DNase I treatment of the RNA, a reverse transcription reaction was performed in a ReverTaid First Strand cDNA Synthesis Kit. Real-time PCR was performed using iQ SYBR Green Supermix on a CFX96 Real Time System (Bio-Rad) with the following primers: β-actin 5’-GACCCACACCCTTCTACATGAG-3’ and 5’-AAATAGCACCAGGCC-TGTGATGACAC-3’ and C5ar1 5’-TTCCTGCTGGTTCTCAAG-3’ and 5’-CTGAGTAGAAGTCCTTATATGC-3’. The temperature profile of quantitative PCR was 95°C for 3 min, followed by 40 cycles at 95°C for 5 s, 58°C (β-actin) or 52°C (C5ar1) for 5 s, and 72°C for 1 s. All reactions were performed in triplicates and expected PCR products were confirmed by melting curve analysis. Real-time RT-PCR data were analyzed using CFX Manager Software 3.1 (Bio-Rad).

**Western blot**

Cell lysates from 1 x 10^6 BM cells were separated by SDS-PAGE, according to standard procedures, using Mini-Protean TGX Precast gels 12% (Bio-Rad). Protein was transferred onto a Trans-Blot Nitrocellulose membrane using a Trans-Blot SD system (both from Bio-Rad). Western blot analysis was performed according to standard procedures. Briefly, the membrane was incubated with C5ar1-specific Ab (clone 1092; 0.2 μg/ml) in TBS + 5% Rockland Blotto low-fat dry milk for 2 h at room temperature. To control for protein loading, membranes were stripped for 5 min at room temperature in 0.2 M glycine (pH 2.5), washed, and reprobed with a polyclonal anti-β-actin Ab (Santa Cruz Biotechnology; 1:1000 in TBS + 5% Rockland Blotto low-fat dry milk) for 2 h at room temperature. To control for surface expression, we stained BM-derived Ly6G+ neutrophils with a C5aR1-specific Ab with (Supplemental Fig. 3) or without previous permeabilization of the cells. As shown in Fig. 2B and 2C, GFP-expressing neutrophils also stained positive for C5aR1 on the cell surface. Confocal microscopy confirmed the GFP and surface expression of C5aR1 in BM-derived neutrophils from GFP-C5aR1flox/fox mice (Fig. 2D). Interestingly, GFP-C5aR1flox/fox mice showed a slightly reduced surface expression of C5aR1, as evidenced by flow cytometry, using mAb 20/70 (mean fluorescence intensity [MFI]; C57BL/6 wt = 2039; GFP-C5aR1flox/fox = 1406; GFP-C5aR1flox/fox = 869; Fig. 2C). This reduced C5aR1 staining in C5aR1 mutant mice did not increase when BM neutrophils were permeabilized (Supplemental Fig. 3), suggesting that the reduced surface expression was not due to a shift from the surface toward intracellular C5aR1 expression or due to dysfunctional transport of C5aR1 from the intracellular compartment to the cell surface. The reduced surface expression most likely results from the IRES cassette, which was previously shown to decrease protein expression (53).

To determine the impact of reduced C5aR1 surface expression in C5aR1 mutant mice on C5a-mediated effector functions in neutrophils, we assessed the C5a-mediated increase in [Ca^2+], by FACS (Fig. 2E) and chemotaxis (Fig. 2F) using BM-derived neutrophils from C57BL/6 wt, GFP-C5aR1flox/fox, and GFP-C5aR1flox/fox mice. We observed a strong and dose-dependent increase in [Ca^2+], with neutrophils from all variants. However, the negative logarithm of EC_{50} (pEC_{50}) values obtained with GFP-C5aR1flox/fox or GFP-C5aR1flox/fox neutrophils were slightly lower than those obtained with wt mice (pEC_{50} wt: 8.7 ± 0.2; GFP-C5aR1flox/fox: 8.5 ± 0.1; GFP-C5aR1flox/fox: 8.1 ± 0.4). Similarly, we found that neutrophils from C57BL/6 wt, GFP-C5aR1flox/fox, and GFP-C5aR1flox/fox mice migrated toward C5a, but with different potencies. Cells from GFP-C5aR1flox/fox and GFP-C5aR1flox/fox mice showed a reduced response compared with cells from wt mice (Fig. 2F).

**GFP and C5aR1 expression in tissue macrophages**

To determine C5aR1 expression in the floxed GFP-C5aR1 knock-in mouse, we first focused on neutrophils, which were described to express C5aR1 in high numbers (52). Using flow cytometry, we found that most of the Ly6G+ neutrophils from BM, lung, spleen, and peripheral blood of GFP-C5aR1flox/fox mice were positive for GFP, whereas those from C57BL/6 wt mice stained negative (Fig. 2A). C5aR1 expression was confirmed at the mRNA level (Supplemental Fig. 2). To correlate the GFP signal with C5aR1 surface expression, we stained BM-derived Ly6G+ neutrophils with a C5aR1-specific Ab with (Supplemental Fig. 3) or without previous permeabilization of the cells. As shown in Fig. 2B and 2C, GFP-expressing neutrophils also stained positive for C5aR1 on the cell surface. Confocal microscopy confirmed the GFP and surface expression of C5aR1 in BM-derived neutrophils from GFP-C5aR1flox/fox mice (Fig. 2D). Interestingly, GFP-C5aR1flox/fox mice showed a slightly reduced surface expression of C5aR1, as evidenced by flow cytometry, using mAb 20/70 (mean fluorescence intensity [MFI]; C57BL/6 wt = 2039; GFP-C5aR1flox/fox = 1406; GFP-C5aR1flox/fox = 869; Fig. 2C). This reduced C5aR1 staining in C5aR1 mutant mice did not increase when BM neutrophils were permeabilized (Supplemental Fig. 3), suggesting that the reduced surface expression was not due to a shift from the surface toward intracellular C5aR1 expression or due to dysfunctional transport of C5aR1 from the intracellular compartment to the cell surface. The reduced surface expression most likely results from the IRES cassette, which was previously shown to decrease protein expression (53).

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**Generation of floxed GFP-C5aR1 knock-in and GFP-C5aR1 LysMCre mice**

We aimed at generating a GFP-C5aR1 reporter mouse that allows for conditional deletion of the C5ar1 gene. A recent report described blocked C5ar1 surface expression in response to disruption of the 3' UTR by insertion of a GFP cDNA cassette (43). Based on this finding, we cloned an AcGFP IRES C5ar1 cassette directly upstream of the 5’-end of exon 2 of the C5ar1 gene. To allow for conditional deletion of the C5ar1 gene, we placed two loxP sites upstream of the AcGFP gene and downstream of exon 2 of the C5ar1 gene (Fig. 1A). We obtained several ES cells that incorporated AcGFP and the two loxP sites into the C5ar1 gene locus, one of which was successfully used to produce heterozygous (GFP-C5aR1flox/+ and homozygous (GFP-C5aR1flox/flox) floxed GFP-C5aR1 knock-in mice (Fig. 1B). Mating GFP-C5aR1flox/flox mice with homozygous LysMCre mice (LysMCre^{tg/tg} mice) eventually resulted in GFP-C5aR1flox/flox/LysMCre^{tg/tg} mice, in which we found complete deletion of the AcGFP IRES C5ar1 cassette in cells expressing the mutated M Lysosome gene (i.e., neutrophils and macrophages) (Fig. 1A, 1C).
FIGURE 1. Generation of floxed GFP-C5aR1–knock-in and GFP-C5aR1 LysMCre–transgenic mice. (A) Gene-targeting strategy. An AcGFP1-IRES gene cassette flanked by two loxP sites was inserted directly upstream of exon 2 of the C5ar1 gene by homologous recombination. This strategy was designed to produce a floxed GFP-C5aR1 knock-in mouse that allows for conditional knockout studies. (B) PCR-based genotyping of wt, heterozygous GFP-C5aR1flox/+, and homozygous GFP-C5aR1flox/flox mice. The primer combination 01_F:02_R (left panel) amplifies a 609-bp DNA fragment in C57BL/6 wt mice and GFP-C5aR1flox/+ mice and a 2037-bp DNA fragment in GFP-C5aR1flox/+ and GFP-C5aR1flox/flox mice. The primer combination 01_F:03_R amplifies an 802-bp DNA fragment that makes up the AcGFP1 cassette (right panel) in mutant, but not in wt, mice. The arrows point to the amplified PCR fragments. (C) PCR-based control of a functional cre recombinase within GFP-C5aR1flox/floxLysMCre/kg mice compared with C57BL/6 wt mice. The primer combination 01_F:04_R amplifies a 1480-bp DNA fragment in C57BL/6 wt mice and a 403-bp DNA fragment in GFP-C5aR1flox/floxLysMCre/kg mice after successful deletion of the sequence flanked by the two loxP sites. FRT, recognition sequence for flp recombinase-mediated neo removal; loxP, recognition sequence for cre recombinase–mediated exon deletion; neo: neomycin cassette for selection in ES cells.
FIGURE 2. GFP and C5aR1 expression and function in Ly6G+ neutrophils of floxed GFP-C5aR1 knock-in mice. (A) Flow cytometric analysis of GFP signal expression in Ly6G+ neutrophils from BM, lung, blood, and spleen. The graphs depict the comparison of GFP-C5aR1flox/flox mice (filled graph) with C57BL/6 wt littermate mice (open graph). Plots are representative of at least four experiments. Numbers indicate the percentage of GFP+ cells within the Ly6G+ gate. (B) Counterstaining of GFP signal (y-axis) with C5aR1-specific mAb clone 20/70 in BM-derived Ly6G+ neutrophils using either IgG isotype control (left panel, x-axis) or C5aR1-specific mAb (right panel, x-axis). Numbers in quadrants indicate the percentages of positive cells within the Ly6G+ gate. Plots are representative of at least four experiments. Numbers indicate the percentage of C5aR1+ cells in GFP-C5aR1flox/+ mice (left panel) or GFP-C5aR1flox/flox mice (right panel). Typical MFI of C5aR1 signals in C57BL/6 wt and mutant mice are given. Plots are representative of at least three experiments. (C) Confocal microscopy of BM cells from C5aR1flox/flox mouse (left panel), GFP-C5aR1flox/flox mouse (middle panel), or C57BL/6 wt mouse (right panel), which were analyzed for GFP expression (green) and stained with C5aR1-specific mAb (clone 20/70; red) without permeabilization (original magnification ×600). Pictures are representative of at least three experiments. (E) C5a-mediated increase in [Ca2+]i in Ly6G+ BM-
we counterstained macrophages from the peritoneal cavity with the C5aR1-specific Ab and analyzed them by flow cytometry for C5aR1 surface expression (Fig. 3F). Cells from GFP-C5aR1flox/+ mice showed almost the same C5aR1 expression levels as did peritoneal macrophages from wt mice. In line with the results obtained from BM-derived neutrophils, we found a reduced C5aR1 surface expression in peritoneal macrophages from GFP-C5aR1flox/flox mice. The expression pattern did not change when the cells were permeabilized, suggesting no additional intracellular C5aR1 expression in cells from C5aR1flox/flox mice (data not shown).

To determine the impact of the reduced C5aR1 surface expression on C5a-mediated effector functions in macrophages, we assessed the C5a-mediated increase in [Ca2+]i (Fig. 3G). As with neutrophils, we observed a strong and dose-dependent increase in [Ca2+]i, in macrophages from wt and C5aR1 mutant mice. Although GFP-C5aR1flox/flox macrophages showed a lower pEC50 value compared with wt mice (pEC50 wt: 8.2 ± 0.7; GFP-C5aR1flox/flox: 7.6 ± 0.1), the differences did not reach statistical significance. To confirm C5aR1 expression in the different macrophage populations, we determined C5ar1 mRNA levels by RT-PCR in GFP-sorted macrophages. All GFP+ macrophages expressed C5aR1 at the mRNA level (Supplemental Fig. 2).

**GFP expression in DC subpopulations from lung, spleen, intestine, and BM**

Conflicting results have been obtained with regard to C5aR1 expression in DCs. In this study, we tested several DC subsets from GFP-C5aR1flox/+ mice for GFP expression. We focused first on the different DC populations of the lung because C5aR1 has been implicated in the regulation of allergic asthma as a result of its impact on allergen-presenting DC subpopulations (36). We differentiated CD103+ cDCs (CD11c+CD103+CD11b+CD64+) from CD11b+ cDCs (CD11c+CD103+CD11b+CD64+) and moDCs (CD11c+CD103+CD11b+CD64+). Importantly, we found GFP expression in CD11b+ cDCs and moDCs but not in CD103+ cDCs (Fig. 4A). This C5aR1 expression pattern was confirmed by RT-PCR (Supplemental Fig. 4). In the spleen, we found only a small population of GFP+ DCs, which belonged to the CD11c+CD11b+CD8+ subset (Fig. 4B). Interestingly, the DC analysis in LP did not mirror the results from lung tissue, because CD103+CD11b+ and CD103+CD11b+ cDCs in LP did not express GFP (Fig. 4C) or C5ar1 mRNA (Supplemental Fig. 4), suggesting a tissue-specific expression of C5aR1 on pulmonary CD11b+ cDCs and moDCs. In addition to primary DC populations from lung, spleen, and LP, we determined C5aR1 expression in BMDCs differentiated in response to GM-CSF treatment. Such cells are used frequently in in vitro functional assays and in vivo adoptive DC transfer models (54–56). After 9 d of GM-CSF culture, ~90% of the CD11c+CD11b+ DCs stained positive for GFP (Fig. 4D); this was confirmed by RT-PCR (Supplemental Fig. 4).

**GFP expression in eosinophils and NK, NKT, and B cells**

GFP expression was very limited in other cell types compared with neutrophils, macrophages, and DCs. We did not find GFP expression under steady-state conditions in B2 cells from spleen (Fig. 5A) or in NK cells from any of the investigated organs (BM, lung, spleen, peritoneal cavity, peripheral blood) (Fig. 5B, data not shown). A small fraction of NKT cells within the spleen was slightly GFP+ (Fig. 5B). mRNA expression was confirmed by RT-PCR (Supplemental Fig. 2). The only significant GFP expression in other cells was found in eosinophils from lung tissue (Fig. 5C), which was confirmed by mRNA analysis using sorted cells (Supplemental Fig. 2). Of note, we did not find any significant C5aR1 expression in pulmonary or intestinal cells not belonging to the myeloid compartment, suggesting that pulmonary and intestinal epithelial cells do not express C5aR1 under steady-state conditions.

**GFP and C5aR1 expression in CD4+ T cells**

Naive CD4+ T cells from the spleen, which were reported to express C5aR1 (28, 31, 57), showed no GFP signal under steady-state conditions (Fig. 6A), confirming previous reports using another GFP-C5aR1 knock-in mouse (43). To explore C5aR1 expression in CD4+ T cells in more detail, we took advantage of several additional in vivo and in vitro approaches. First, we used anti-C5aR1-specific mAb for cell surface staining of spleen-derived naive CD4+ T cells and assessed C5ar1 mRNA expression by RT-PCR in C57BL/6 wt mice. Confirming our data with GFP-C5aR1flox/+ CD4+ T cells, we found no C5aR1 surface or mRNA expression (data not shown). Next, we stimulated such CD4+ T cells with anti-CD3 and anti-CD28 mAbs for 3, 6, or 12 h or 1, 2, 3, 4, or 5 d and assessed GFP and C5aR1 surface and mRNA expression. We did not observe any C5aR1 expression (data not shown). When we cultured CD4+ T cells for 4 d in the absence of CD3 or CD28 stimulation, we found a CD4+ T cell population that stained positive for C5aR1 compared with the iso-type control (Fig. 6B). However, when we gated such C5aR1+ T cells on dead and living cells, we found that the positive C5aR1 signal derived exclusively from dead cells. Importantly, we observed that dead, but not living, CD4+ T cells from C5ar1−/−/C5ar1+/− mice also stained positive when mAb 20/70 was used for C5aR1 surface staining. In contrast, isotype staining was similar in dead and living cells from wt and C5ar1−/−/C5ar1+/− mice, suggesting that mAb 20/70 may recognize a C5aR1-independent neoepitope on apoptotic T cells. To assess the impact of Ag-specific T cell activation on C5aR1 expression in vivo, we immunized GFP-C5aR1flox/+ and wt mice with OVA323–339 peptide in CFA in the footpad and restimulated these mice 8 d later using the same immunization protocol. Seven days after boosting, mice were sacrificed, and draining LNs and spleens were collected. Cells from both organs were analyzed immediately by flow cytometry for GFP expression. Additionally, CD4+ Th cells from spleen were sorted and used for ex vivo culture in the presence of anti-CD3 and anti-CD28 mAbs for 5 d, as well as for RNA extraction. We found a strong and similar increase in CD4+ T cell numbers in the spleens and popliteal LNs of wt and GFP-C5aR1flox/+ mice, suggesting that the GFP knock-in does not affect Ag-driven T cell activation in vivo (Fig. 6C, upper and lower right panels). Next, we determined GFP expression in spleen- and popliteal LN-derived CD4+ and CD8+ T cells from GFP-C5aR1flox/+ mice. We were not able to detect any GFP+CD4+ or GFP+CD8+ T cells in the LN or spleen of immunized mice (Fig. 6C, middle panels). Finally, we determined C5ar1 expression in ex vivo cultures of CD4+ T cells from GFP-C5aR1flox/+ mice directly after preparation from spleen (day 0) and 1, 3, and 5 d after stimulation with anti-CD3 and anti-CD28 mAbs. In line with our previous observations, no GFP expression was detectable in living ex vivo stimulated T cells at any time point (Fig. 4D). We also assessed C5ar1 mRNA expression in such CD4+ T cells by RT-PCR. We were not able to amplify any C5ar1-specific fragment (Supplemental Fig. 2).
FIGURE 3. GFP and C5aR1 expression and function in macrophages of floxed GFP-C5aR1 knock-in mice. Flow cytometric analysis of the GFP signal from Siglec-F+/CD11c+ macrophages in lung (A) and BALF (B). Gating strategy for F4/80+ macrophages from LP of small intestine (C), peritoneal cavity (D), and spleen (E) in GFP-C5aR1flox/+/+ mice (shaded graphs) or C57BL/6 wt mice (open graphs). The numbers in the panels are the percentages of GFP+ cells within the gated populations. Plots are representative of at least four experiments. (F) C5aR1 surface staining of F4/80+ macrophages from C57BL/6 wt, GFP-C5aR1flox/+, and GFP-C5aR1flox/flox mice using C5aR1-specific mAb (clone 20/70). GFP-C5aR1flox/+/+ mice (dashed line, left panel), GFP-C5aR1flox/flox mice (dashed line, right panel), C57BL/6 wt mice (filled graphs), and C5aR1−/− mice (open graphs). The numbers indicate the percentages of C5aR1+ cells in GFP-C5aR1flox/+/+ (left panel) or GFP-C5aR1flox/flox (right panel) mice. Typical MFIs of C5aR1 signals in C57BL/6 wt and mutant mice are shown on the right. Plots are representative of at least four experiments. (G) C5a-mediated increase in [Ca2+]i in F4/80+ macrophages from C57BL/6 wt and mutant mice. pEC50 values ± SEM (n = 4) are shown below the graph. Data were analyzed using ANOVA. n.s., not significant.
FIGURE 4. GFP expression in different DC subsets from floxed GFP-C5aR1 knock-in mice. (A) Flow cytometric analysis of the GFP signal in different subpopulations of DCs in the lung. Cells were gated first on SiglecF^+CD11c^+ cells (same sample as in Fig. 3A). Cells were then gated negatively for lineage markers (Lin) and subsequently on CD11c^+MHCII^+ cells. These cells were subdivided into CD103^-CD11b^- or CD103^+CD11b^+ cDCs. The latter population was subdivided into cDCs (CD11b^-CD64^+) and moDCs (CD11b^-CD64^+). GFP expression was determined in CD103^-CD11b^- cDCs (upper left panel), CD103^-CD11b^+ cDCs (upper middle panel), and CD11b^-CD64^+ moDCs (upper right panel). GFP signals from GFP-C5aR1^flox/flox^ DCs (shaded graphs) are shown together with GFP signals from C57BL/6 wt controls (open graphs). (B) Flow cytometric analysis of the GFP signal in different DC subpopulations from the spleen. DCs were gated on CD11c expression (shaded graph; solid line = isotype control, left panel) and subdivided into CD11b^-CD8^- DCs (middle panel) or CD11b^-CD8^+ DCs (right panel). Signals from GFP-C5aR1^flox/flox^ DCs are depicted as shaded graphs; solid lines represent signals from C57BL/6 wt controls. (C) Flow cytometric analysis of the GFP signal in different subpopulations of DCs from the LP of the small intestine (same sample as in Fig. 3C). DCs were subdivided into CD11c^-CD11b^- (gate I) or CD11c^-CD11b^- positive (gate II) (left panel). Gate I contains CD103^-CD11b^- cDCs (middle panel), whereas gate II is composed of CD103^-CD11b^- cDCs (right panel). Shaded graphs represent the GFP signal from DCs of GFP-C5aR1^flox/flox^ mice, and solid lines represent the GFP signal from DCs of C57BL/6 wt mice. (D) Flow cytometric analysis of the GFP signal from BMDCs. Cells were gated on CD11c^-CD11b^- cells (left panel). Subsequently, the GFP signal was analyzed in cells from GFP-C5aR1^flox/flox^ mice (shaded graph) or C57BL/6 wt mice (open graph) (right panel). The numbers in the panels represent the percentages of GFP^+^ cells within the gated populations. Data are representative of at least three experiments.
The numbers in the panels represent the percentages of GFP+ cells within the gated populations. All data are representative of at least three experiments.

Neutrophil- and macrophage-specific deletion of C5aR1 expression in floxed GFP-C5aR1 LysMCre–transgenic mice

To specifically delete C5aR1 expression in neutrophils and macrophages, we generated GFP-C5aR1flox/floxF4/80+LysMCreCre mice. First, we determined GFP and C5aR1 surface expression in BM-derived neutrophils and F4/80+ peritoneal macrophages in such mice. Although we found, at best, residual GFP and C5aR1 expression in neutrophils, GFP and C5aR1 expression in macrophages was abrogated (Fig. 7A, 7C). In line with these findings, C5a failed to induce any increase in [Ca2+]i in neutrophils or macrophages (Fig. 7A, 7C). In line with these findings, C5a failed to induce any increase in [Ca2+]i in neutrophils or macrophages (Fig. 7A, 7C). In line with these findings, C5a failed to induce any increase in [Ca2+]i in neutrophils or macrophages (Fig. 7A, 7C).

Next, we determined GFP expression in CD103+ CD11b+ and BMDCs from GFP-C5aR1flox/floxF4/80+LysMCreCre mice using flow cytometry. We found a strong GFP signal in both DC populations that was comparable to the one obtained in DCs from GFP-C5aR1flox/flox mice (Fig. 7F, 7G). The slight shift in the GFP signal observed in the BMDC culture (Fig. 7G) is most likely due to the presence of macrophages (46). Taken together, our data provide evidence for C5aR1 deletion in GFP-C5aR1flox/floxF4/80+LysMCreCre mice that specifically targets C5aR1 in neutrophils and macrophages but not in DCs.

Discussion

The complement system is an ancient danger-sensing system, recognizing and tagging pathogens for elimination. It is also important when coping with endogenous danger, such as apoptotic cells or cell debris. Additionally, it is well appreciated that complement is involved in the regulation of adaptive and innate immune responses (36, 58). Of specific importance, when looking at the regulation of the adaptive and innate immune responses, the ATs C5a and C3a and their corresponding receptors (C5aR1/2 and C3aR) exert critical roles. C5aR1 plays an important role in regulating T cell immunity, and it is involved in the cross-talk between complement and TLRs (35, 59), as well as with FcγRs (60). Giving its importance in the immunological network, it is surprising that there is ongoing controversy about the distribution of C5aR1 on various immunological cell types. Many studies tried to address this question using classical Ab-based approaches, with all of their limitations and pitfalls. Recently, a GFP-C5aR1 reporter mouse was generated to avoid this problem. Dunkelberger et al. (43) inserted the GFP construct into the 3′ UTR of the C5ar1-coding region, which resulted in cytoplasmic arrest of the C5aR1 protein. Consequently, mice carrying the reporter construct on both chromosomes were functional C5aR1 knockouts, because cells from these mice failed to respond to C5a stimulation. We aimed at designing a floxed GFP-C5aR1 mouse that allows the tracing of C5aR1+ cells in vivo and in vitro, as well as cell and tissue–specific C5aR1 deletion.

First, we assessed whether insertion of the GFP construct into the 5′ end of exon 2 has any impact on C5aR1 surface expression and function. We observed strong surface expression of C5aR1 on neutrophils and macrophages from heterozygous GFP-C5aR1flox/+ mice using C5aR1 mAb staining. Moreover, such cells were fully functional and responsive to C5a stimulation, as shown by signaling and migration assays. However, we observed slightly reduced surface expression using cells from GFP-C5aR1flox/flox mice, which is most likely due to the introduction of the IRES motif. It has been repeatedly reported that this insert can affect the translation efficiency, specifically it can interfere with the translation of small-sized mRNAs (53). In contrast to the accumulation of C5aR1 protein within the cells, which was observed when the GFP construct was inserted into the 3′ UTR (43), we found no increased intracellular accumulation of C5aR1. However, we noticed slightly reduced C5a-mediated biological functions, at least when we used BM-derived neutrophils. Independent of the heterozygous or homozygous expression of the GFP construct, we demonstrated strong GFP expression in virtually all neutrophil populations investigated. Further, we found GFP expression in lung-resident eosinophils, confirming previous data that demonstrated C5aR1 expression in human, guinea pig, and mouse eosinophils by flow cytometry and by functional assays (61–63). With regard to macrophages, we found GFP+ populations in lung, BALF, and the peritoneal cavity. We found that most of the F4/80+ macrophages in the peritoneal cavity also stained positive for GFP, which is in accordance with previous publications showing that the majority of resident peritoneal macrophages express C5aR1 (32, 64). In contrast, Dunkelberger et al. (43) detected only a small fraction of GFP+ resident F4/80+ peritoneal macrophages in their GFP-C5aR1flox/flox mice. The lower GFP expression may result from differences in cell viability. It is well appreciated that resident peritoneal macrophages are prone to undergo apoptosis after isolation. Under these circumstances, the GFP signal can be missing as a result of the loss of cytosolic GFP under these circumstances, the GFP signal can be missing as a result of the loss of cytosolic GFP. Additionally, it is well appreciated that complement is involved in the regulation of adaptive and innate immune responses (36, 58). Of specific importance, when looking at the regulation of the adaptive and innate immune responses, the ATs C5a and C3a and their corresponding receptors (C5aR1/2 and C3aR) exert critical roles. C5aR1 plays an important role in regulating T cell immunity, and it is involved in the cross-talk between complement and TLRs (35, 59), as well as with FcγRs (60). Giving its importance in the immunological network, it is surprising that there is ongoing controversy about the distribution of C5aR1 on various immunological cell types. Many studies tried to address this question using classical Ab-based approaches, with all of their limitations and pitfalls. Recently, a GFP-C5aR1 reporter mouse was generated to avoid this problem. Dunkelberger et al. (43) inserted the GFP construct into the 3′ UTR of the C5ar1-coding region, which resulted in cytoplasmic arrest of the C5aR1 protein. Consequently, mice carrying the reporter construct on both chromosomes were functional C5aR1 knockouts, because cells from these mice failed to respond to C5a stimulation. We aimed at designing a floxed GFP-C5aR1 mouse that allows the tracing of C5aR1+ cells in vivo and in vitro, as well as cell and tissue–specific C5aR1 deletion.
FIGURE 6. GFP and C5αR1 expression in CD4⁺ T cells from floxed GFP-C5αR1–knock-in mice. (A) Flow cytometric analysis of the GFP signal in untouched, naive CD4⁺ T cells from spleen. (B) Flow cytometric analysis of C5αR1 expression in CD3⁺ T cells from spleen 4 d after in vitro culture without any stimulation. T cells were divided into living cells (left peak, middle panel) and dead cells (right peak, middle panel) using LIVE/DEAD staining. C5αR1 expression in both populations was determined using cells from C57BL/6 wt mice (upper right panels) or C3ar⁻/⁻/C5ar1⁻/⁻ double-deficient mice (lower right panels) using C5αR1-specific mAb (clone 20/70). Shaded graphs represent C5αR1-specific staining, solid lines represent isotype-control staining. (C) Flow cytometric analysis of GFP signals in CD4⁺ T cells from popliteal LNs (upper left panel) or spleen (lower left panel) of GFP-C5αR1floxed and C57BL/6 wt control mice. Mice were immunized with OVA323–339 peptide in CFA and boosted after 8 d using the same immunization regimen. Seven days after the second immunization, T cell populations were examined for GFP expression. GFP expression in CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells is depicted (middle panels). Numbers of CD3⁺ CD4⁺ T cells in LNs (upper right panel) or spleen (lower right panel) from C57BL/6 wt and GFP-C5αR1floxed mice. (D) Flow cytometric analysis of GFP signals obtained from living CD4⁺ T cells isolated ex vivo from spleen of mice immunized with OVA323–339 peptide. Shown is the GFP signal obtained after ex vivo stimulation with CD3- and CD28-specific mAbs on days 0, 1, 3, and 5. The GFP signals were analyzed in cells from GFP-C5αR1floxed mice (shaded graphs) or C57BL/6 wt mice (open graphs). The numbers in the panels represent the percentages of GFP⁺ cells within the gated population.

A group of F4/80⁺ red pulp macrophages. This population was described to contribute to the clearance of old and damaged RBCs (67).

Similar to what we observed with BM-derived neutrophils, C5αR1 surface expression was lower on peritoneal macrophages from GFP-C5αR1floxed mice than those from C57BL/6 wt mice. However, in contrast to neutrophils, we found only a minor impact of the decreased C5αR1 expression on the C5a-induced signaling pathways that drive increased [Ca²⁺]. This difference may result from different G-protein coupling in neutrophils and macrophages (7) and/or ligand concentration-dependent activation of different signaling pathways (68).

NK cells are another population of the innate immune system on which C5αR1 was observed, at least under inflammatory conditions in severe sepsis in mice (27). Further, C5αR1 expression was reported in human CD56⁺ and CD56⁻ NK cells (69). We did not find any C5αR1 expression in spleen- or lung-derived NK cells from naive mice, suggesting that C5αR1 is not expressed in mouse NK cells under steady-state conditions but, rather, needs inflammatory stimuli for its upregulation. In contrast, in the human system, the receptor is already expressed in a noninflamed environment.

Overwhelming evidence has accumulated that C5αR1 activation regulates CD4⁺ T cell survival, proliferation, and differentiation into Th1 (35), Th2 (36), or Th17 (70–72) effector cells, as well as induced (31, 70, 73) and natural (57) regulatory CD4⁺ T cells. However, conflicting results exist about whether such regulation is driven primarily by the impact of C5a on APC activation or whether C5a can directly modulate T cell activation. To address this issue, we focused first on C5αR1 expression on different DC populations in spleen, BM, lung, and the small intestine. In the spleen, two main populations of cDCs can be differentiated: CD8⁺ and CD8⁻ cDCs (67). CD8⁺ cDCs regulate tolerance against self-Ags, mediate cross-presentation, and are strong producers of the Th1-inducing cytokine IL-12p70. We found GFP expression in ~40% of the CD8⁺ cDCs but not the CD8⁻ cDCs. In line with this finding, it was reported that DCs from C5-deficient mice (74) or from mice lacking C5αR1 expression (39, 70) have an impaired ability to produce IL-12p70, resulting in defective Th1 responses. Also, DCs lacking C5αR1 showed reduced allostimulation in a mouse transplant model (30). In contrast to our findings, no
C5aR1 expression has been described in spleen-derived CD11c+ cDCs using the 3' UTR GFP-C5aR1 construct (43). At this point, it remains unclear whether the defective C5aR1 surface trafficking and the strong cytoplasmic receptor accumulation in this mouse strain may affect the development, differentiation, and/or migration of C5aR1-expressing CD8+ cDCs into the spleen. In addition to CD8+ cDCs in the spleen, we found that CD103+CD11b+ cDCs from the lung and CD11b+CD64+ pulmonary moDCs expressed C5aR1, which is in line with our previous finding (36). In contrast, CD103+CD11b- cDCs from the lung stained negative for GFP. Several reports showed that C5aR1 is critical for the development of Th2 (36) and Th17 (72) maladaptive immune responses in experimental allergic asthma. More recently, CD103+CD11b+ and, to a lesser extent, moDCs were described to drive such Th2 and Th17 development (50), suggesting that C5aR1 activation on pulmonary CD103+CD11b+ and moDCs could be critical for their maturation and subsequent activation of naïve CD4+ T cells in draining LNs during allergen sensitization. In addition to allergy, the importance of the activation of C3aR and C5aR1 on pulmonary cDCs for the induction of effector T cell responses was demonstrated in a model of experimental influenza infection (75). In this model, AT receptors on lung cDCs were critical for cell trafficking into draining LNs.

In accordance with previous studies (8, 28, 30, 70), we also demonstrate that CD11c+CD11b+ BMDCs express C5aR1. BMDCs express the CD11c, CD11b, CD47, and SIRP-α markers, but not CD103, suggesting that they functionally resemble CD11b+CD103+ SIRP-α+ inflammatory moDCs (50). To our surprise, we did not observe any C5aR1-expressing tissue DCs in the LP of the small intestine. In summary, our data suggest a complex and heterogeneous C5aR1 expression in tissue-resident and inflammatory DCs.

**FIGURE 7.** GFP and C5aR1 expression and function in neutrophils, macrophages, and DCs from GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice. (A) Flow cytometric analysis of GFP (left panel) or C5aR1 surface expression (using C5aR1-specific mAb clone 20/70; right panel) on BM-derived Ly6G+ neutrophils from GFP-C5aR1<sup>lox/lox</sup> mice (shaded graphs), GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice (dashed line), and C57BL/6 wt mice (for GFP signal, solid line) or C5aR<sup>-/-</sup> mice (for C5aR1 surface staining, solid line). Data are representative of at least four experiments. (B) C5a-mediated increase in [Ca<sup>2+</sup>], in Ly6G<sup>+</sup> BM-derived neutrophils from GFP-C5aR1<sup>lox/lox</sup> mice and GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice. Data are mean ± SEM (n = 3). (C) As in (A), except that F4/80<sup>+</sup> macrophages from the peritoneal cavity were used instead of neutrophils. Data are representative of at least four experiments. (D) As in (B), except that thioglycollate-elicited F4/80<sup>+</sup> peritoneal cavity macrophages were used instead of neutrophils. Data are mean ± SEM (n = 3). (E) Western blot analysis of C5aR1 and β-actin (control) expression using lysates from Ly6G<sup>+</sup> BM-derived neutrophils (1 × 10<sup>6</sup>) from GFP-C5aR1<sup>lox/lox</sup> mice and GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice. Results are representative of at least three independent experiments. (F) GFP signals in lung CD103<sup>-</sup>CD11b<sup+</sup> DCs from C57BL/6 wt mice (open graph), GFP-C5aR1<sup>lox/lox</sup> mice (shaded graph), and GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice (dashed line). Data are representative of two independent experiments. (G) GFP signals in CD11c<sup+</sup>CD11b<sup+</sup> BMDCs from C57BL/6 wt mice (open graph), GFP-C5aR1<sup>lox/lox</sup> mice (shaded graph), and GFP-C5aR1<sup>lox/lox</sup> LysMC<sup>retg/tg</sup> mice (dashed line). Data are representative of two independent experiments.
Based on several recent reports suggesting C5aR1 expression in mouse CD4+ T cells (28, 57), we expected a clear GFP signal in naïve CD4+ T cells. Surprisingly, naïve CD4+ T cells from the spleen did not show GFP expression. Similarly, there was no GFP expression from GFP-C5aR1 reporter mice. Taken together, our model by which C5a regulates CD4+ T cell responses in the human system, in which C5aR1 expression was observed in the human system, in which C5aR1 expression was demonstrated in T cells (76). Based on our findings, we suggest a model by which C5a regulates CD4+ T cell responses in the mouse exclusively at the APC level. However, at this point, we cannot exclude that mouse CD4+ T cells can express, like human T cells, C5aR1 under certain conditions (e.g., when they differentiate into Th1, Th2, Th9, or Th17 effector or regulatory T cells in acute or chronic inflammatory conditions).

We also evaluated C5aR1 expression on other cells of the adaptive immune system, such as B2 cells and NK cells. In contrast to a previous report showing C5aR1 expression on 10% of human naive and 40% of human memory B cells (77), we did not find GFP signals in spleen-derived B2 cells of naïve mice. Similar to NK cells, C5aR1 expression on NK cells was reported in the context of severe inflammation (27). In accordance with the previous observation, we found only a minor GFP signal in naive spleen-derived NK T cells. In future studies, the novel GF-C5aR1 reporter mouse will prove useful to assess the expression of C5aR1 in other tissues, including liver, spleen, brain, fat tissue, placenta, testis, and heart. Our findings that nonmyeloid cells from lung and the small intestine did not express GFP argues against a strong C5aR1 expression in pulmonary or intestinal epithelial cells.

Finally, we generated cell-specific C5aR1-knockout mice, in which C5aR1 expression and function are specifically deleted in neutrophils and macrophages. In contrast, C5aR1 expression and function are preserved in DCs of such mice. It is well appreciated that C5aR1 activation plays critical roles as a proinflammatory mediator in experimental models of allergy (36), autoimmunity (60), infection (78), and cancer (79). However, the individual contribution and the functional relevance of C5aR1 activation in neutrophils and macrophages in such models remain elusive. C5aR1flox/floxLysMcRcre22 mice will be instrumental in defining the importance of C5aR1 activation in neutrophils and macrophages in these models.

In summary, we generated a fully functional novel floxed GFP-C5aR1 reporter knock-in mouse, which enabled us to determine the C5aR1 expression profile of cells from the innate and the adaptive immune system under in vitro, ex vivo, and in vivo conditions. Further, we generated the first mouse strain, to our knowledge, that allows for conditional deletion of the receptor in neutrophils and macrophages. The loxP sites may prove useful for specifically targeting other cells of the myeloid lineage, such as mast cells, eosinophils, basophils, and DCs, to address the individual role of C5aR1 in such cells in models of allergy, infection, autoimmunity, cancer, and transplantation.

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Disclosures
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