The anaphylatoxins (AT) C3a and C5a are effector molecules of C3 and C5 exerting multiple biologic functions through binding and activation of their cognate G protein–coupled receptors. C3a interacts with the C3a receptor (C3aR), whereas C5a and its primary degradation product C5a-desArg engage C5aR1 and C5aR2. In the past, analysis of AT expression has been hampered by cross reaction of antibodies designed to recognize the different AT receptors. Furthermore, assessment of effects mediated by cell-specific activation has been difficult. Here, floxed AT receptor reporter mice are described as tools to monitor AT receptor expression in cells and tissues and to study the functions of C3a and C5a by cell-specific deletion of their cognate AT receptors. © 2020 The Authors.

**Basic Protocol 1:** Genotyping of floxed GFP-C5aR1 knockin mice

**Support Protocol 1:** Genotyping of LysMcre-C5ar1/− mice

**Basic Protocol 2:** Genotyping of floxed tdTomato-C3aR and -tdTomato-C5aR2 knockin mice

**Support Protocol 2:** Preparation of genomic DNA

**Basic Protocol 3:** Determination of C5aR1, C5aR2, and C3aR expression using floxed AT receptor reporter mice

**Support Protocol 3:** Determination of C3aR expression using a C3aR-specific antibody

**Support Protocol 4:** Determination of C5aR1, C5aR2, and C3aR mRNA expression in floxed GFP-C5aR1, floxed tdTomato-C5aR2 or -tdTomato C3aR positive cells

**Basic Protocol 4:** Analysis of C5aR1-driven ERK1/2 phosphorylation in GFP-C5aR1+ cells

**Basic Protocol 5:** Assessment of C3aR functions in cells obtained from floxed tdTomato-C3aR knockin mice- Determination of C3aR internalization

**Alternate Protocol:** C3a-induced increase in intracellular Ca2+

**Basic Protocol 6:** C5aR2-driven IFN-γ production from NK cells

**Support Protocol 5:** Isolation of splenic NK cells by FACS

Keywords: anaphylatoxin • anaphylatoxin receptor • C3a • C5a • complement
INTRODUCTION

In recent years, our view of the complement system as a guardian of the extracellular space has been markedly extended. Several reports have shown that complement is not activated systemically solely via the three canonical activation pathways, i.e., the classical, lectin, and alternative pathway, but by non-canonical pathways via specific proteases derived from pathogens, activated host cells, or the contact system (Hajishengallis, Reis, Mastellos, Ricklin, & Lambris, 2017). These newly discovered non-canonical activation pathways of complement, its unexpected contributions to cell homeostasis, metabolism, differentiation, and apoptosis, its crosstalk with several receptor classes and other cascade systems of innate immunity, as well as its impact on adaptive immune responses make the complement system more than ever an important field of research within immunology (Kolev, Le Friec, & Kemper, 2014; West, Kolev, & Kemper, 2018).

Many of these newly discovered functions can be attributed to the anaphylatoxins (ATs) C3a and C5a, which were originally considered mere proinflammatory molecules. Their pleiotropic functions are mainly mediated through activation of their G protein-coupled corresponding complement peptide receptors, i.e., C3aR for C3a and C5aR1 and C5aR2 for C5a. Until recently, AT receptor expression in professional and non-professional immune cells, as well as tissue-resident stroma cells, has been ill-defined, and is still controversial (Laumonnier, Karsten, & Köhl, 2017). To better understand the expression patterns of the AT receptors in health and disease, and to define the multiple roles of the ATs in the innate and adaptive immune networks, we have generated floxed AT receptor reporter mice that are now available to and widely used by the scientific community.

This article describes the use of C3aR and C5aR2 reporter mice with a tandem-dye (td)Tomato construct (Karsten et al., 2017; Quell et al., 2017), as well as the use of a C5aR1 knock-in mouse in which the Aequorea coeruleescens green fluorescent protein [(Ac)GFP] was added to monitor C5aR1 expression (Karsten et al., 2015). These mice are available from the Köhl laboratory upon request. Basic Protocol 1 outlines the procedure to genotype GFP-C5aR1 knockin mice. Support Protocol 1 describes as an example the genotyping of mice with conditional deletion of C5aR1 in LysM-expressing cells after Cre-mediated targeting. Basic Protocol 2 describes the genotyping of tdTomato-C3aR and C5aR2 knock-in mice, with Support Protocol 2 detailing the preparation of the genomic DNA. Basic Protocol 3, together with two support protocols that describe the analysis of AT receptor protein (Support Protocol 3) and mRNA (Support Protocol 4) expression, outlines a flow cytometric approach to assess AT receptor expression in GFP-C5aR1<sup>+</sup>, tdTomato-C5aR2<sup>+</sup>, or tdTomato-C3aR<sup>+</sup> cells. To link the expression of the AT receptor reporter constructs with AT receptor function, we provide three basic protocols to determine distinct functions of C5aR1 in GFP-C5aR1 knockin mice (Basic Protocol 4), C3aR in tdTomato-C3aR knockin mice (Basic Protocol 5), and C5aR2 in tdTomato-C5aR2 knockin mice (Basic Protocol 6). Finally, we have added Support Protocol 5 for the isolation of NK cells, which exclusively express C5aR2 but not C5aR1.

NOTE: All animals were used for organ removal according to protocols approved by local authorities of the Animal and Care and Use Committee (Ministerium für
All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

GENOTYPING OF FLOXED GFP-C5aR1 KNOCKIN MICE

The genes encoding for the three AT or complement peptide receptors are organized in a similar way, with a 5′-untranslated region (UTR), two exons, and a 3′-UTR after exon 2 (https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=5). This offers the opportunity to insert a fluorescent reporter sequence (tdTomato or GFP) before or at the beginning of exon 2. With the inserted construct, one locus of cross-over P1 (loxP) site is introduced at the 5′ end of the reporter sequence and another one after the coding region of the receptor before the 3′-UTR. According to the general design, the C5aR1 construct includes a GFP coding sequence ending with a stop codon, followed by an internal ribosomal entry site (IRES) placed upstream of exon 2 encoding for the C5ar1 gene, which is flanked by loxP sites at both ends. Between the loxP site at the 5′-UTR

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**Figure 1** Gene targeting strategy to generate floxed GFP-C5aR1 knockin mice and to excise the C5ar1 mutant locus by Cre-mediated deletion. (A) Genomic locus of the C5ar1 gene on chromosome 7 with two exons. (B) Targeting vector comprising an AcGFP-IRES cassette directly upstream of the 5′ end of exon 2 of the C5ar1 gene. Further, the neomycin marker gene was introduced at the 5′ end of the AcGFP cassette flanked by two FRT sites. Finally, two loxP sites were introduced; one at the 5′-end of the neomycin cassette next to the FRT site and another one downstream of exon 2 of the C5ar1 gene directly after the stop codon. (C) Floxed GFP-C5ar1 mutant locus after homologous recombination. Breeding of floxed GFP-C5ar1 mutant mice to flp deleter mice resulted in flp recombinase-mediated neomycin gene removal. (D) Crossing floxed GFP-C5aR1 knockin mice with mice expressing Cre recombinase results in the excision of the C5ar1 mutant locus at the loxP target sequence. FRT, recognition sequence for flp recombinase-mediated neomycin gene removal; loxP, recognition sequence for Cre recombinase-mediated exon deletion; neo: neomycin gene cassette for selection in ES cells.
| Use in PCR# | Primer/type/target | Sequence                     | bp  | Design          |
|------------|--------------------|------------------------------|-----|-----------------|
| 1          | AH7/F/C5ar1        | TAGAGTTGAGACTCAGAAAGACGG    | 24  | Ozgene          |
| 1          | AH9/R/GFP         | GGTTGGACAGTGAGTGGTTATC      | 22  | Ozgene          |
| 1          | GK91/F/Tcrd       | CAAATGTTGCTTTGCTCTGGT       | 20  | JAX Lab olMR8744|
| 1          | GK92/R/Tcrd       | GTCAGTCGAGTCAGAGTTTT        | 20  | JAX Lab olMR8745|
| 2          | AH7/F/C5ar1        | TAGAGTTGAGACTCAGAAAGACGG    | 24  | Ozgene          |
| 2          | AH8/R/C5ar1       | GTACACGAAGGATGGAATGGT       | 22  | Ozgene          |
| 2          | AH15/F/Il2        | CTAGGGCCACAGAATTGAAAGATCT   | 24  | Jax Lab olMR7338|
| 2          | AH16/R/II2        | GTAGGTGGAATTCTAGCAGATCC     | 25  | Jax Lab olMR7339|

Abbreviations: bp, base pair; F, forward; GFP, green fluorescent protein; Il2, IL-2 precursor short; R, reverse; Tcrd, T cell receptor delta chain.

and the GFP reporter gene, a neomycin cassette with flippase recognition target (FRT) sites at both ends was included. During the generation of mice, the neomycin cassette was removed by breeding to a flippase (FLP) deleter mouse strain (Fig. 1).

To determine the genotype of the AT receptor reporter mice, we have set up a PCR-based approach in which we detect the presence of the reporter sequence (GFP: PCR1) including the loxP sites and/or the AT receptor wild-type genes (C5ar1: PCR 2), in particular in the case of target cell—specific AT receptor deletion after breeding of the AT receptor reporter mice to defined Cre mice (H. Kim, Kim, Im, & Fang, 2018; Song & Palmiter, 2018). In the latter case, the Cre mouse strain used needs to be genotyped in a separate approach, as outlined in Support Protocol 1.

**Materials**

- 100 μM primers for PCR 1 and 2 (Table 1; Eurofins Genomics/Thermo Fisher Scientific)
- Nuclease-free water (Life Technologies GmbH, cat. no. AM9937)
- 2× KAPA2G Fast Hot Start Genotyping Mix (Sigma Aldrich, cat. no. KK5621; manufactured by Roche)
- Template DNA: genomic DNA (Support Protocol 2) from floxed GFP-C5aR1 knockin mouse (Köhl laboratory)
- 1.5% SB agarose gel (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001)
- Sodium borate (SB) buffer (see recipe)
- O’Gene Ruler DNA Ladder Mix (Thermo Fisher Scientific, cat. no. SM0333)
- GelRed working solution (see recipe)
- 1.5-ml screw-cap microcentrifuge tube with O-ring (Sarstedt, cat. no. 72.692.405)
- Microcentrifuge
- 0.2-ml 8-cap PCR strips without caps (BioRad (cat. no. TBS0201)
- 8-cap strips for PCR tubes (BioRad (cat. no. TBS0803)
- Filtered pipet tips (10, 250, and 1000 μl; Sarstedt, cat. no. 701.116.210, 701.189.215, 701.186.210)
- Microcentrifuge tubes (1.5-ml, Sarstedt, cat. no. 72.690)
- PCR machine (BioRad C1000 or C1000 Touch Thermal Cycler)
- Gel staining tray
- UV transilluminator and camera
Additional reagents and equipment for agarose gel electrophoresis (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001)

**CAUTION:** Remember the proper work safety requirements to protect eyes and skin against UV radiation. Boric acid belongs to the group of carcinogenic, mutagenic, reprotoxic (CMR) substances. Follow the national requirements for disposal and working safety.

**NOTE:** All chemicals need to be molecular grade (DNase-free). For all steps use filtered pipet tips only.

1. Prepare PCR1 primer mix by combining 20 μl each of primers AH7/AH9/GK91/GK92 (Table 1) from primer stock (100 μM) in a 1.5-ml screw-cap microcentrifuge tube with O-ring, and make up with 120 μl nuclease-free water to a final volume of 200 μl. Store at −20°C.

2. Prepare PCR2 primer mix by combining 20 μl each of primers AH7/AH8/AH15/AH16 (Table 1) from primer stock (100 μM) in a 1.5-ml screw-cap microcentrifuge tube with O-ring, and make up with 120 μl nuclease-free water to a final volume of 200 μl. Store at −20°C.

   The final concentration of the primer mix for PCR1 and PCR2 is 10 μM for each primer.

3. Prepare separate master mixes for PCR1 and PCR2 by pipetting the following reagents at the indicated volumes into a 1.5-ml microcentrifuge tube (per sample):
   
   - 6.25 μl nuclease-free water
   - 1.25 μl primer mix for PCR1 (step 1) or PCR2 (step 2)
   - 12.5 μl 2× KAPA2G Fast Hot Start Genotyping Mix

   Mix and microcentrifuge briefly to bring mixture to bottom of tube.

   The final concentration of each primer in PCR1 and 2 is 0.5 μM after adding the template.

4. Combine 5 μl template [genomic DNA (Support Protocol 2) from floxed GFP-C5aR1 knockin mouse diluted 1:10 with nuclease-free water] or nuclease-free water for control with 20 μl of the appropriate master mix in a 0.2-ml 8-tube PCR strip without caps, and run the following thermocycling conditions for both PCR1 and PCR2 after closing the tubes with the 8-cap strips for PCR tubes:

   | Step no. | Temp. | Time | No. of cycles |
   |----------|-------|------|--------------|
   | 1        | 95°C  | 180 s| 1 cycle      |
   | 2        | 95°C  | 15 s |              |
   | 3        | 62°C  | 15 s |              |
   | 4        | 72°C  | 60 s |              |
   | 5        | As in 2, 3, and 4 | As in 2, 3, and 4 | 35 cycles (total) |
   | 6        | 72°C  | 120 s | 1 cycle    |
   | 7        | 15°C  | Hold |              |

   The thermocycler protocol always follows the same layout: in an initial step, the protecting protein of the hot-start Taq polymerase is irreversibly denatured at 95°C (No.1). Then, a defined number of three-step cycles comprising DNA denaturation (No.2), primer annealing (No.3), and elongation (No.4) are carried out (here, for 35 cycles). After final elongation of unfinished sequences (step 6), samples are incubated indefinitely at 15°C (No.7).

   These conditions apply to the BioRad C1000 Thermal Cycler or C1000Touch. Temperatures need to be established for other thermocycler models. It is desirable to use a PCR
workstation (e.g., Analytic Jena) to avoid cross contamination and a PCR cooler (e.g., Eppendorf AG) to secure the template quality.

5. Prepare a 1.5% SB agarose gel as described in Brody & Kern (2004).

Also see Current Protocols article: Voytas (2001).

6. Run samples on 1.5% SB agarose gel using the following conditions: 30-40 min, 200 V, with a 7-cm running length. Include DNA markers. Load per lane: either 6 μl PCR reaction or 4 μl marker.

The selected markers (O’Gene Ruler DNA Ladder Mix) use the TriTrack DNA Loading Dye, which allows for three-color tracking of DNA migration. On a 1% agarose gel, bromophenol blue matches ~300-bp DNA, while xylene cyanol FF matches ~4000-bp DNA. Orange G matches small DNA fragments (~50 bp). Even though the electrophoresis running time at this high voltage is quite short, the band resolution of the gel is excellent when using sodium borate buffer instead of TAE buffer. Because of the low ionic strength of the buffer, no additional cooling of the gel is needed.

7. Stain the gel with GelRed working solution for 45 min in a tray.

![Figure 2](image)

**Figure 2** Amplions resulting from genotyping of wild-type and floxed GFP-C5aR1 knockin mice: PCR-based genotyping of wild-type, heterozygous GFP-C5aR1\(^{fl+/+}\), and homozygous GFP-C5aR1\(^{fl/fl}\) mice. (A) PCR1: The primer combination AH7/AH9 amplifies an 802-bp DNA fragment of the GFP cassette in GFP-C5aR1\(^{fl+/+}\) and GFP-C5aR1\(^{fl/fl}\) mice. The primer combination GK91/GK92 amplifies a 206-bp DNA fragment of the TCR delta chain gene as an internal template control in samples from all strains. (B) PCR2: The primer combination AH7/AH8 amplifies a 609-bp DNA fragment of the C5ar1 gene (from exon 2) in wild-type and GFP-C5aR1\(^{fl+/+}\) mice. In GFP-C5aR1\(^{fl/fl}\) mice, it amplifies an additional 2037-bp DNA fragment comprising the GFP gene, the IRES fragment, and a part of exon 2 of the C5ar1 gene. The primer combination AH15/AH16 amplifies a 324-bp DNA fragment of the IL-2 precursor gene as an internal template control in samples from all strains. Amplification products were separated on a 2.5% (PCR1) or 1.5% (PCR2) sodium borate (SB) agarose gel stained with GelRed. M = marker; W = water control; 1 = wild type; 2 = heterozygous GFP-C5aR1\(^{fl+/+}\) mice; 3 = homozygous GFP-C5aR1\(^{fl/fl}\) mice.

**Table 2** Amplion Size of Wild-Type and Floxed GFP-C5aR1 Knockin Mice

| Genotype  | Result PCR1 | Result PCR2 |
|-----------|-------------|-------------|
|           | Specific: GFP | Internal control: Tcrd | Specific: C5ar1 | Internal control: Il2 |
| +/-       | -           | 206 bp      | 609 bp         | 324 bp         |
| flx/+     | 802 bp      | 206 bp      | 609 and 2037 bp\(^a\) | 324 bp         |
| flx/flx   | 802 bp      | 206 bp      | 2037 bp\(^a\)  | 324 bp         |

\(^a\)The 2037 bp fragment is very faint and can even be missing.

Abbreviations: bp, base pair; GFP, green fluorescent protein; Il2, IL-2 precursor short; Tcrd, T cell receptor delta chain.
Tilting on a conventional rocking platform with ±7.5° at about 15 rpm will ensure uniform staining results. No destaining step is necessary. GelRed is non-mutagenic and safer for the environment than ethidium bromide.

8. Visualize the gel bands with UV light and document the result by photography.

Typical results showing the amplicons from genotyping of wild-type, GFP-C5aR1<sup>fl/+</sup>, and GFP-C5aR1<sup>fl/fl</sup> mice are shown in Figure 2. The sizes of the PCR fragments amplified from the different mouse strains are shown in Table 2.

**GENOTYPING OF LysMcre-C5aR1 MICE**

Breeding of the floxed GFP-C5aR1 knockin mice with strains that harbor a Cre recombinase will result in the targeted deletion of the GFP-C5aR1 cassette through excision at the two loxP sites (Fig. 1D). Similarly, the tdTomato-C3ar1 or C5ar2 genes will be excised in response to Cre (Figs. 4D and 5D). Here, we describe the genotyping of LysMcre-C5aR1 in which LysMcre mice (Clausen, Burkhardt, Reith, Renkawitz, & Forster, 1999) have been crossed with floxed GFP-C5aR1 knockin mice to specifically delete C5aR1 in granulocytes, monocytes, and macrophages (Karsten et al., 2015). Given that the C5ar1 gene is still expressed in many cell types, we have set up an analysis system that includes C5ar1 (Basic Protocol 1) and Lyz2, the gene encoding for lysozyme M(lysM). The C5ar1 and Lyz2 genes are expressed on two different chromosomes, i.e., chromosomes 7 and 10. To delete C5aR1 in lysM-expressing cells, GFP-C5aR1<sup>fl/fl</sup> mice need to harbor at least one functional Cre allele under the control of the lysM promoter.

**Materials**

- 100 μM primers for PCR3 (Eurofins Genomics or Life Technologies GmbH)
- Nuclease-free water (Life Technologies GmbH, cat. no. AM9937)
- 2× KAPA2G Fast Hot Start Genotyping Mix (Sigma Aldrich, cat. no. KK5621; manufactured by Roche)
- Template DNA: genomic DNA (Support Protocol 2) from LysMcre-C5aR1 mouse
- 1.5% SB agarose gel (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001)
- Sodium borate (SB) buffer (see recipe)
- O’Gene Ruler DNA Ladder Mix (Thermo Fisher Scientific, cat. no. SM0333)
- GelRed working solution (see recipe)
- 1.5-ml screw-cap microcentrifuge tube with O-ring (Sarstedt, cat. no. 72.692.405)
- Microcentrifuge
- 0.2-ml 8-tube PCR strips without caps (BioRad, cat. no. TBS0201)
- 8-cap strips for PCR tubes (BioRad, cat. no. TBS0803)
- Filtered pipet tips (10, 250, and 1000 μl; Sarstedt, cat. no. 701.116.210, 701.189.215, 701.186.210)
- Microcentrifuge tubes (1.5-ml, Sarstedt, cat. no. 72.690)
- PCR machine (BioRad C1000 or C1000 Touch Thermal Cycler)
- Gel staining tray
- UV transilluminator and camera

**CAUTION:** Remember the proper work safety requirements to protect eyes and skin against UV radiation. Boric acid belongs to the group of carcinogenic, mutagenic, reprotoxic (CMR) substances. Follow the national requirements for disposal and working safety.
### Table 3 Primers to Genotype LysMcre-C5aR1 Mice

| Use in PCR# | Primer/type/target | Sequence                       | bp | Design                  |
|-------------|---------------------|--------------------------------|----|-------------------------|
| 3           | GK199/R/cre recombinase | CCCAGAAATGCCAGATTACG             | 20 | Riken BRC 02302/3 = Jax Lab oIMR3066 |
| 3           | GK223/F/Lyz2         | GCAATTGCAGACTAGCTAAAGGCAG        | 24 | Riken BRC 02302/1       |
| 3           | GK224/R/Lyz2         | GTCGGCCAGGCTGACTCCATAG           | 22 | Riken BRC 02302/2       |

Abbreviations: bp, base pair; F, forward; Lyz2, Lysozyme M; R, reverse.

**NOTE:** All chemicals need to be molecular grade (DNase free). For all steps use filtered pipet tips only.

1. Prepare PCR3 primer mix to amplify Lyz2 by combining 20 μl of primers GK199/GK223/GK234 (Table 3) from primer stock (100 μM) in a 1.5-ml screw-cap microcentrifuge tube with O-ring and make up with 140 μl nuclease-free water to a final volume of 200 μl.

   *You can store the PCR primer mixes at −20°C for several months before use.*

2. Prepare a master mix for PCR3 by pipetting the following reagents and the indicated volumes into a 1.5-ml microcentrifuge tube (per sample):

   - 1.25 μl nuclease-free water
   - 1.25 μl primer mix for PCR3
   - 12.5 μl 2× KAPA2G Fast Hot Start Genotyping Mix.

   Mix and microcentrifuge briefly to bring mixture to bottom of tube.

3. Combine 10 μl template [genomic DNA (Support Protocol 2) from floxed LysMCre-C5aR1 mouse diluted 1:10 with nuclease-free water] or nuclease-free water for control with 15 μl master mix in a 0.2-ml 8-tube PCR strip without caps and run the following thermocycling conditions for PCR3 after closing the tubes with the 8-cap strips for PCR tubes:

   | Step no. | Temp. | Time | No. of cycles |
   |----------|-------|------|--------------|
   | 1        | 95°C  | 180 s| 1 cycle      |
   | 2        | 95°C  | 30 s |              |
   | 3        | 66.5°C| 30 s |              |
   | 4        | 72°C  | 60 s |              |
   | 5        | As in 2, 3, and 4 | 35 cycles (total) |
   | 6        | 72°C  | 120 s| 1 cycle      |
   | 7        | 15°C  | Hold |              |

4. Run samples on 1.5% SB agarose gel (Brody & Kern, 2004) using the following conditions: 30-40 min, 200 V, with a 7-cm running length. Include DNA markers (O’Gene Ruler DNA Ladder Mix). Load per lane: either 6 μl PCR reaction or 4 μl marker.

5. Stain the gel with GelRed working solution for 45 min in a tray.

6. Visualize the gel bands with UV light and document the result by photography.

*Typical results showing the amplicons from genotyping of wild-type, LysMcre-C5aR1cre/+,* and *LysMcre-C5aR1cre/cre* mice are shown in Figure 3. The sizes of the PCR fragments amplified from the different mouse strains are shown in Table 4.*
Figure 3  Amplicons resulting from genotyping of LysMcre-C5aR1 mice. PCR3: The primer combination GK223/GK224 amplifies a 429-bp DNA fragment of the Lyz2 gene in LysMcre-C5ar1<sup>+/+</sup> and LysMcre-C5ar1<sup>cre/+</sup> mice. In LysMcre-C5ar1<sup>cre/+</sup> and LysMcre-C5ar1<sup>cre/cre</sup> mice, an 800-bp fragment is amplified. Amplification products were separated in a 1.5% sodium borate (SB) agarose gel stained with GelRed. M = marker; W = water control; 1 = wild type; 2 = heterozygous LysMcre-C5ar1<sup>cre/+</sup> mice; 3 = homozygous LysMcre-C5ar1<sup>cre/cre</sup> mice.

Table 4  Amplicon Size of LysMcre-C5aR1 Mice

| Genotype   | Result PCR3          |
|------------|----------------------|
| Lyz2       | 429 bp               |
| cre/+      | 429 and 800 bp       |
| cre/cre    | 800 bp               |

Abbreviations: bp, base pair; Lyz2, lysozyme M.

GENOTYPING OF FLOXED tdTomato-C3aR AND tdTomato-C5aR2 KNOCKIN MICE

In addition to C5aR1, we have targeted the C3ar1 and C5ar2 genes to generate reporter mice that allow tracking of cellular C3aR and C5aR2 expression and cell-specific deletion. To provide a strong signal with a high stability, in particular upon standard fixation procedures, we used a tandem-dye (td)Tomato instead of GFP (de Felipe et al., 2006). Furthermore, although IRES is a widely used design to produce independent proteins from one mRNA, we and others have found that the translation of the coding sequence under the control of IRES is less efficient than the translation of the main ribosomal entry sequence (de Felipe et al., 2006; Karsten et al., 2015). As an alternative approach, we have used the short, self-cleaving peptide derived from porcine teschovirus-1 (P2A; Kim et al., 2011), which shows high cleavage efficiency and a stoichiometric expression of proteins flanking the 2A peptide (de Felipe et al., 2006; Kim et al., 2011). In the case of the C3ar1 gene, the resulting targeting construct encoded a 6.2-kb 5′ homology arm and a 3.0-kb 3′ homology arm flanking the floxed coding region of exon 2, in which tdTomato_P2A has been inserted in frame with the ATG of exon 2 (Fig. 4). Similarly, we have inserted the tdTomato sequence following the splice acceptor of exon 2 in-frame with the coding sequence of the C5ar2 gene (Fig. 5). In both cases, two loxP sites flank the constructs for potential excision of the C3ar1 or C5ar2 genes by Cre-mediated deletion.
Gene targeting strategy to generate floxed tdTomato-C3aR knockin mice and to excise the C3ar1 mutant locus by Cre-mediated deletion: (A) Genomic locus of the C3ar1 gene on chromosome 6 with two exons. (B) Targeting vector comprising a tdTomato-P2A cassette directly upstream of the 5′ end of exon 2 of the C3ar1 gene. Further, the neomycin marker gene was introduced at the 5′ end of the tdTomato-P2A cassette flanked by two FRT sites. Finally, two loxP sites were introduced: one at the 5′-end of the neomycin cassette next to the FRT site and another one downstream of exon 2 of the C3ar1 gene directly after the stop codon. (C) Floxed tdTomato-C3ar1 mutant locus after homologous recombination. Breeding of floxed tdTomato-C3arR knockin mice to flp deleter mice resulted in flp recombinase–mediated neomycin gene removal. (D) Crossing floxed tdTomato-C3arR knockin mice with mice expressing Cre recombinase results in the excision of the C3ar1 mutant locus at the loxP target sequence. 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.

Materials

100 μM primers for PCR4-6 (Eurofins Genomics or Life Technologies GmbH)
Nuclease-free water (Life Technologies GmbH, cat. no. AM9937)
2× KAPA2G Fast Hot Start Genotyping Mix (Sigma Aldrich, cat. no. KK5621; manufactured by Roche)
Template DNA: genomic DNA (Support Protocol 2) from floxed tdTomato-C3aR knockin mouse, or -tdTomato-C5aR2 knockin mouse
2.5% and 1% SB agarose gels (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001)
Sodium borate (SB) buffer (see recipe)
O’Gene Ruler DNA Ladder Mix (Thermo Fisher Scientific, cat. no. SM0333)
O’Gene Ruler Ladder, 50 bp (Thermo Fisher Scientific, cat. no. SM 0371)
GelRed working solution (see recipe)
1.5-ml screw-cap microcentrifuge tube with O-ring (Sarstedt, cat. no. 72.692.405)
Microcentrifuge
0.2-ml 8-tube PCR strips without caps (BioRad, cat. no. TBS0201)
8-capstrips for PCR tubes (BioRad, cat. no. TBS0803)
Filtered pipet tips (10, 250, and 1000 μl; Sarstedt, cat. no. 701.116.210, 701.189.215, 701.186.210)
Microcentrifuge tubes (1.5-ml, Sarstedt, cat. no. 72.690)
PCR machine (BioRad C1000 or C1000 Touch Thermal Cycler)
Figure 5  Gene targeting strategy to generate floxed tdTomato-C5aR2 knockin mice and to excise the C5ar2 mutant locus by Cre-mediated deletion:  (A) Genomic locus of the C5ar2 gene on chromosome 7 with two exons.  (B) Targeting vector comprising a tdTomato-P2A cassette directly upstream of the 5′-end of exon 2 of the C5ar2 gene. Further, the neomycin marker gene was introduced at the 5′-end of the tdTomato-P2A cassette flanked by two FRT sites. Finally, two loxP sites were introduced: one at the 5′-end of the neomycin cassette next to the FRT site and another one downstream of exon 2 of the C5ar2 gene directly after the stop codon. (C) Floxed tdTomato-C5ar2 mutant locus after homologous recombination. Breeding of floxed tdTomato-C5aR2 knockin mice to flp deleter mice resulted in flp recombinase-mediated neomycin gene removal. (D) Crossing floxed tdTomato-C5aR2 knockin mice with mice expressing Cre recombinase results in the excision of the C5ar2 mutant locus at the loxP target sequence. 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.

Gel staining tray
UV transilluminator and camera

Additional reagents and equipment for agarose gel electrophoresis (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001)

CAUTION: Remember the proper work safety requirements to protect eyes and skin against UV radiation. Boric acid belongs to the group of carcinogenic, mutagenic, reprotoxic (CMR) substances. Follow the national requirements for disposal and working safety.

NOTE: All chemicals need to be molecular grade (DNase free). For all steps use filtered pipet tips only.

1. Prepare PCR4 primer mix to amplify tdTomato by combining 20 μl of primers GK368/GK369/GK91/GK92 (Table 5) from primer stock (100 μM) in a 1.5-ml screw-cap microcentrifuge tube with O-ring and make up with 120 μl nuclease-free water to a final volume of 200 μl.

2. Prepare PCR5 primer mix to amplify C3ar1 as in step 1 using 20 μl of primers GK342/GK45/GK190/GK191 (Table 5) analogous to step 1.
### Table 5: Primers to Genotype Floxed tdTomato-C3aR and tdTomato-C5aR2 Knockin Mice

| Use in PCR# | Primer/type/target | Sequence | bp | Design |
|-------------|--------------------|----------|----|--------|
| 4           | GK368/R/tdTomato   | ATGACGGCCATGTTGTTGTC | 20 | Köhl lab |
| 4           | GK369/F/tdTomato   | CACCACCTGTTCTCTGGGG | 18 | Köhl lab |
| 4           | GK91/F/Tcrd        | CAAATGTGCTTTGTCTGGTG | 20 | JAX Lab oIMR8744 |
| 4           | GK92/R/Tcrd        | GTCACTGAGTGACACAGTT | 20 | JAX Lab oIMR8745 |
| 5           | GK342/F/C3ar1      | AACAACAGAAGTAGGGAGGTGTA | 24 | Köhl lab |
| 5           | GK45/R/C3ar1       | TCCAAATAGACAAGTGAGACCAA | 23 | Harvard primerbank #6753224a1 |
| 5           | GK190/R/Ii2        | CCGTGCTTTTCTCTCACCAC | 20 | Köhl lab |
| 5           | GK191/F/Ii2        | CGATTACCTCAGTCCCCCTTTAC | 23 | Köhl lab |
| 6           | GK360/F/C5ar2      | TGTCAGCCCGGGACCTTTA | 19 | Köhl lab |
| 6           | GK361/R/C5ar2      | CTTATCAGTGCTCGGGGTAA | 21 | Köhl lab |
| 6           | GK91/F/Tcrd        | CAAATGTGCTTTGTCTGGTG | 20 | JAX Lab oIMR8744 |
| 6           | GK92/R/Tcrd        | GTCACTGAGTGACACAGTT | 20 | JAX Lab oIMR8745 |

Abbreviations: bp, base pair; F, forward; Ii2, IL-2 precursor long; R, reverse; Tcrd, T cell receptor delta chain.

3. Prepare PCR6 primer mix to amplify C5ar2 as in step 1 using 20 μl of primers GK360/GK361/GK91/GK92 (Table 5) analogous to step 1.

You can store the PCR primer mixes at −20°C for several months before use.

4. Prepare master mixes for PCR 4, 5, and 6 separately by pipetting the following reagents at the indicated volumes into a 1.5-ml microcentrifuge tube (per sample):

- 6.25 μl nuclease-free water
- 1.25 μl primer mix (PCR4, 5 or 6)
- 12.5 μl 2× KAPA2G Fast Hot Start Genotyping Mix.

Mix and microcentrifuge briefly to bring mixture to bottom of tube.

5. Combine 5 μl template [genomic DNA (Support Protocol 2) from floxed tdTomato-C3aR knockin mouse or tdTomato-C5aR2 knockin mouse diluted 1:10 with nuclease-free water] or nuclease-free water for control with 20 μl of the respective master mix in a 0.2-ml 8-tube PCR strip without caps and run the following thermocycling conditions for PCR4 after closing the tubes with the 8-capstrips for PCR tubes:

| Step no. | Temp. | Time | No. of cycles |
|----------|-------|------|---------------|
| 1        | 95°C  | 180 s| 1 cycle      |
| 2        | 95°C  | 15 s |               |
| 3        | 67°C  | 15 s |               |
| 4        | 72°C  | 10 s |               |
| 5        | As in 2, 3, and 4 | 35 cycles (total) |
| 6        | 72°C  | 120 s| 1 cycle      |
| 7        | 15°C  | Hold |               |

For PCR5 (C3ar1), use the same conditions as in PCR4 except that the temperature in step 3 is 62°C and the cycle time in step 4 is 60 s. For PCR6 (C5ar2), use the same conditions as in PCR4, except that the temperature in step 3 is 63°C and the cycle time in step 4 is 90 s.
Amplicons resulting from genotyping of wild-type and floxed tdTomato-C3aR knockin mice: PCR-based genotyping of wild-type, heterozygous tdTomato-C3aR1+/+, and homozygous tdTomato-C3aR1/fl/fl mice. (A) PCR4: The primer combination GK368/GK369 amplifies an 89-bp DNA fragment of the tdTomato cassette in tdTomato-C3aR1/fl/fl and tdTomato-C3aR1+/+ mice. The primer combination GK91/GK92 amplifies a 206-bp DNA fragment of the TCR delta chain gene as an internal template control in samples from all strains. (B) PCR5: The primer combination GK342/GK45 amplifies a 400-bp DNA fragment of the C3ar1 gene (from exon 2) in wild-type and tdTomato-C3aR1+/+ mice. In tdTomato-C3aR1+/+ and tdTomato-C3aR1/fl/fl mice, it additionally amplifies a 2000-bp DNA fragment comprising the tdTomato gene, the p2A fragment, and a part of exon 2 of the C3ar1 gene. The primer combination GK190/GK191 amplifies a 547-bp DNA fragment of the IL-2 precursor gene as an internal template control from samples of all strains. Amplification products were separated in a 1% (PCR4) or 2.5% (PCR5) sodium borate (SB) agarose gel stained with GelRed. M = marker; W = water control; 1 = wild type; 2 = heterozygous tdTomato-C3aR1+/+ mice; 3 = homozygous tdTomato-C3aR1/fl/fl mice.

Table 6  Amplicon Size of Wild-Type and Floxed tdTomato-C3aR Knockin Mice

| Genotype | Result PCR4 | Result PCR5 |
|----------|-------------|-------------|
|          | Specific:tdTomato | Internal control:Tcrd | Specific:C3ar1 | Internal control:Il2 |
| +/-      | -           | 206 bp          | 400 bp          | 547 bp          |
| flx/+    | 89 bp       | 206 bp          | 400 and 2000 bp | 547 bp          |
| flx/flx  | 89 bp       | 206 bp          | 2000 bp         | 547 bp          |

*The 2000 bp fragment is very faint and can be even missing.
Abbreviations: bp, base pair; Il2, IL-2 precursor long; Tcrd, T cell receptor delta chain.

6. Run samples on a 2.5% (PCR5 and 6) or an 1% (PCR4) SB agarose gel (Brody & Kern, 2004; also see Current Protocols article Voytas, 2001). Run SB agarose gels for 30-40 min at 200 V with a 7 cm running length. Include DNA markers (O’Gene Ruler DNA Ladder Mix for PCR 5, and O’Gene Ruler DNA Ladder 50 bp for PCR 4 and 6). Load per lane: either 6 μl PCR reaction or 4 μl marker.

7. Stain the gel with GelRed working solution for 45 min in a tray. Visualize the gel bands with UV light and document the result by photography.

Typical results showing the amplicons of wild-type, tdTomato-C3aR1+/+ and tdTomato-C3aR1/fl/fl mice are depicted in Figure 6. The size of the PCR fragments amplified from the different mouse strains are shown in Table 6.

Typical results showing the amplicons of wild-type, tdTomato-C5aR2+/+ and tdTomato-C5aR2/fl/fl mice are depicted in Figure 7. The size of the PCR fragments amplified from the different mouse strains are shown in Table 7.
Figure 7  Amplicons resulting from genotyping of wild-type floxed tdTomato-C5aR2 knockin mice: PCR-based genotyping of wild-type, heterozygous tdTomato-C5aR2<sup>fl/+</sup>, and homozygous tdTomato-C5aR2<sup>fl/fl</sup> mice. (A) PCR4: The primer combination GK368/GK369 amplifies an 89-bp DNA fragment of the tdTomato cassette in tdTomato-C5aR2<sup>fl/fl</sup> and tdTomato-C5aR2<sup>fl/+</sup> mice. The primer combination GK91/GK92 amplifies a 206-bp DNA fragment of the TCR delta chain gene as an internal template control in samples from all strains. (B) PCR6: The primer combination GK360/GK361 amplifies a 428-bp DNA fragment of the C5ar2 gene (from exon 2 and the 3′-UTR) in wild-type and tdTomato-C5aR2<sup>fl/+</sup> mice. In tdTomato-C5aR2<sup>fl/+</sup> and tdTomato-C5aR2<sup>fl/fl</sup> mice, it amplifies a 478-bp DNA fragment of the C5ar2 gene (from exon 2 and the 3′-UTR) including the fragment encoding for the loxP site. Also, a heteroduplex of 528 bp occurs in tdTomato-C5aR2<sup>fl/+</sup> mice. The primer combination GK91/GK92 amplifies a 206-bp DNA fragment of the TCR delta chain gene as an internal template control from samples of all strains. Amplification products were separated in a 2.5% sodium borate (SB) agarose gel stained with GelRed. M = marker; W = water control; 1 = wild type; 2 = heterozygous tdTomato-C5aR2<sup>fl/+</sup> mice; 3 = homozygous tdTomato-C5aR2<sup>fl/fl</sup> mice.

Table 7  Amplicon Size of Wild-Type and Floxed tdTomato-C5aR2 Knockin Mice

| Genotype | Specific:tdTomato | Internal control:Tcrd | Specific:C5ar2 | Internal control:Tcrd |
|----------|-------------------|-----------------------|----------------|-----------------------|
| +/+      | -                 | 206 bp                | 428 bp         | 206 bp                |
| flx/+    | 89 bp             | 206 bp                | 428, 478, and 528 bp<sup>a</sup> | 206 bp |
| flx/flx  | 89 bp             | 206 bp                | 478 bp         | 206 bp                |

<sup>a</sup>Heteroduplex formation.
Abbreviations: bp, base pair; Tcrd, T cell receptor delta chain.

SUPPORT PROTOCOL 2

PREPARATION OF GENOMIC DNA

The prerequisite to amplify the different genomic regions of interest by the PCRs outlined in Basic Protocols 1 and 2 is the appropriate preparation of genomic DNA extracted from a piece of tissue. The most frequently used mouse tissues for genomic DNA preparation are from tail or ear. Our animal facility uses ear punch as the method of identification. Thus, we are using such ear punch biopsies for preparation of genomic DNA as outlined below. Several methods and kits are available to extract DNA from mouse tissue. We routinely use the KAPA Express Extract Kit, which results in high amounts of genomic DNA of good quality.

Materials

- Ear punch biopsy (max. 2 mm<sup>2</sup>) from floxed GFP-C5aR1, floxed tdTomato-C5aR2, floxed tdTomato-C3aR knockin or LysMcre-C5ar1 mouse (Köhl laboratory)
- Lysis master mix (see recipe)
- UltraPure™ 1 M Tris·HCl, pH 8.0 (Thermo Fisher Scientific Invitrogen, cat. no. 15567027), autoclaved
NOTE: We recommend using a PCR workstation (e.g., Analytic Jena AG) to avoid cross contamination and a PCR cooler (e.g., Eppendorf AG) to secure the template quality.

1. Transfer biopsy using a Blunt Fill cannula from the tube that was used to collect the specimen from the animal into a 0.2-ml PCR tube with lid under sterile conditions.

   Make sure that the biopsy is immediately stored at −20°C. In our hands, storing the biopsy for several months does not affect the yield of genomic DNA. Due to static charging, the tissue sticks to the metal during the transfer into the PCR tube.

2. Add 50 μl lysis master mix to the biopsy, vortex, and spin down in a microcentrifuge at room temperature.

   The tissue needs to be completely covered by the buffer. Any included air will lead to insufficient lysis, and should be removed during the centrifugation. If necessary, you may press the biopsy down with a pipet tip. In our hands, it is possible to handle up to 32 biopsies simultaneously.

3. Incubate 10 min at 75°C followed by 5 min at 95°C using a cycler or two independent heating blocks.

   Avoid opening of the microcentrifuge tubes, due to vapor pressure, which may result in a loss of material and or cross contamination of your sample.

4. Vortex at full speed for 2-3 s.

5. Centrifuge samples 1 min at 2000 × g, room temperature.

   This will pellet cellular debris.

6. Carefully transfer 40 μl supernatant to a 0.5-ml microcentrifuge tube containing 360 μl of 10 mM Tris·HCl (pH 8.0).

7. Use immediately or store at −20°C.

   Samples can be stored for years −20° without quality loss. However, avoid repeated thawing and freezing cycles.

**DETERMINATION OF C5aR1, C5aR2, AND C3aR EXPRESSION USING FLOXED AT RECEPTOR REPORTER MICE**

Floxed AT receptor reporter mice allow the assessment of AT receptor expression without the need for labeled ligands or antibodies. The GFP and tdTomato reporter genes that are under the control of the promoters of the *C5ar1*, *C5ar2*, or *C3ar1* genes encode for strongly fluorescent proteins that can easily be detected by imaging technologies, including flow cytometry and fluorescence microscopy. As an example, we describe here a protocol to monitor the expression of tdTomato as a surrogate for C3aR expression in peritoneal macrophages. The same procedure can be used to delineate the expression of GFP as a surrogate for C5aR1 and tdTomato as a surrogate for C5aR2 expression, since peritoneal macrophages are known to strongly express all AT receptors (Karsten et al., 2015; Karsten et al., 2017; Quell et al., 2017). Importantly, the genetic association of a reporter gene to AT receptor genes can impact the trafficking of the AT receptors...
to the cell surface (Dunkelberger, Zhou, Miwa, & Song, 2012). Thus, it is important to also assess the surface expression of ATRs in GFP-C5aR1+, tdTomato-C5aR2+, or tdTomato-C3aR+ cells using AT-receptor-specific antibodies, and control for the specificity using the respective AT receptor-deficient mice. For this purpose, we provide Support Protocol 3, which determines C3aR expression using a C3aR-specific antibody. Finally, Support Protocol 4 outlines the assessment of AT receptor mRNA expression in GFP-C5aR1+, tdTomato-C5aR2+, or tdTomato-C3aR+ cells to verify the AT receptor expression using GFP-C5aR1 mice as an example.

**Materials**

Floxed GFP-C5aR1, floxed tdTomato-C5aR2, or tdTomato-C3aR knock-in mouse (Köhl laboratory); C57BL/6 or BALB/c wild-type mice (The Jackson Laboratory, stock no. 000664 or 000651)

Thioglycolate-elicited peritoneal macrophages (see Current Protocols article: Zhang, Goncalves, & Mosser, 2008)

Dulbecco’s phosphate-buffered saline (D-PBS) without calcium or magnesium, pH 7.0-7.3 (Thermo Fisher Scientific, cat. no. 14190144)

Anti-CD16/32 (clone 93; Thermo Fisher Scientific, cat. no. 14-0161-82)

Anti-mouse F4/80 BrilliantViolet (BV)421 (Clone BM8; BioLegend, cat. no. 123131)

Anti-CD11b-BV510, (clone M1/70; BioLegend; cat. no. 101245)

D-PBS/BSA (see recipe)

75 × 12-mm polystyrene round-bottom FACS tubes (Sarstedt, cat. no. 50-809-212)

Refrigerated centrifuge

1.5-ml microcentrifuge tubes (Sarstedt, cat. no. 72.690.001)

Flow cytometer

Additional reagents and equipment for anesthesia (see Current Protocols article: Donovan & Brown, 2001) and euthanasia (see Current Protocols article: Donovan & Brown, 2006) of mice, and counting cells (see Current Protocols article: Strober, 2001)

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   *We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.*

2. Isolate peritoneal cells from floxed GFP-C5aR1, -tdTomato-C5aR2, -tdTomato-C3aR, C57BL/6, or BALB/c wild-type mice as described in Current Protocols article Zhang et al. (2008).

   *Depending on the background of the AT receptor reporter mice, C57BL/6 or BALB/c mice can serve as control mice to determine the baseline fluorescence for the GFP or the tdTomato signal (see Fig. 15). We regularly use 8- to 12-week-old mice of both sexes. While one mouse of each strain is sufficient to determine AT receptor expression, at least three to five animals should be used to confirm expression and to compare expression in different cell types.*

3. Count cells (see Current Protocols article: Strober, 2001). Adjust the cells to a density of 1 × 10⁷ cells/ml in D-PBS and transfer 100 μl of the cells to a 1.5-ml microcentrifuge tube.

4. Add 1 μl (0.5 μg diluted in D-PBS) of anti-CD16/32 antibody and incubate for 15 min at 4°C
The anti-CD16/32 antibody binds to FcyRII and FcyRIII, thus preventing non-specific binding to lineage-specific antibodies that define the peritoneal macrophages in peritoneal lavage cells.

5. Centrifuge the cell suspension 5 min at 500 × g, 4°C, in a refrigerated centrifuge.
6. Discard the supernatant by aspiration.
7. Break up the pellet by gently tapping the bottom of the tube.
8. Prepare an antibody master mix by diluting the anti-F4/80 antibody to a final concentration of 0.5 μg/ml and the anti-CD11b antibody to 0.25 μg/ml in 100 μl D-PBS/BSA.
9. Incubate the cells from each sample with 100 μl of the antibody master mix for 20 min at 4°C.
10. Add 1 ml D-PBS/BSA and centrifuge 5 min at 500 × g, 4°C, in a refrigerated centrifuge.
11. Discard the supernatant by careful aspiration.
12. Resuspend the cells in 300 μl D-PBS/BSA and transfer to a flow cytometer.
13. Plot FSC-H versus FSC-W and gate on singlet cells as shown in Figure 8A. Label the population “singlets.”

The FSC-A should be set at 25,000 to exclude any debris. This will ensure that the events counted will be cells and not any contaminants. Record 5 × 10^5 events for cell analysis.

Figure 8  tdTomato and C3aR expression in thioglycolate-elicited peritoneal macrophages. (A) Contour plot showing the gating strategy to identify thioglycolate-elicited peritoneal macrophages as CD11b+ and F4/80+ cells. Peritoneal macrophages stain positive for tdTomato-C3aR (middle panel) and C3aR (right panel) by flow cytometry. The solid black line depicts the signal from the tdTomato-C3aR knockin mice; the dashed line shows the signal obtained from C3ar1−/− mice; and the gray histogram shows the signal from wild-type Balb/c mice. (B) Contour plot of wild-type (left panel) and tdTomato-C3ar1fl/fl mice showing a tdTomato-C3aR signal only in tdTomato-C3ar1fl/fl mice. Part of this figure was originally published in Quell et al. (2017). Copyright © [2017] The American Association of Immunologists, Inc.
14. Considering the singlet cells, plot CD11b (on the y axis) versus F4/80 (on the x axis). Gate on the CD11b^+ F4/80^+ cells (Fig. 8A).

15. Record the tdTomato signal in the PE channel (also in case of tdTomato-C5aR2 knockin mice) or the GFP-signal in the FITC channel (in case of floxed GFP-C5aR1 knockin mice) (Fig. 8A).

The tdTomato^+ cells define either the tdTomato-C3aR^+ or tdTomato-C5aR2^+ macrophages, whereas the GFP^+ cells define the GFP-C5aR1^+ cells (depending on the mouse strain used). The autofluorescent emission of the CD11b^+ F4/80^+ macrophages in wild-type mice defines the baseline fluorescence for the GFP or the tdTomato signal (fluorescence minus one (FMO) control).

**DETERMINATION OF C3aR EXPRESSION USING A C3aR-SPECIFIC ANTIBODY**

The expression of the GFP or tdTomato AT receptor surrogate fluorochrome is not necessarily linked with surface or intracellular expression of the AT receptors. Although our previous results show a strong match of C5aR1 and C3aR surface expression with the GFP or tdTomato signal (Karsten et al., 2015; Quell et al., 2017), we strongly recommend also staining the GFP-C5aR1^+, tdTomato-C3aR^+, or tdTomato-C5aR2^+ cells with AT receptor—specific antibodies to verify AT receptor surface expression. In the case of tdTomato-C5aR2^+ cells, we have not yet been able to verify the surface expression with C5aR2-specific antibodies, as all antibodies that we have tested so far have also showed surface staining in C5aR2-deficient mice, suggesting cross-reactivity with other structures (Karsten et al., 2017). We strongly recommend using AT receptor—deficient mice to control for the specificity of a given AT receptor antibody and not to rely only on FMO or IgG isotype controls. Support Protocol 3 outlines an example of C3aR staining using an anti-C3aR antibody and an AF647-labeled secondary antibody, as no conjugated C3aR antibodies are commercially available yet. Alternatively, the anti-C3aR antibody might be conjugated with a fluorochrome by commercially available conjugation kits. For C5aR1 staining, several antibodies conjugated with different fluorochromes are available.

**Materials**

- BALB/c wild-type (The Jackson Laboratory, stock no. 000664), tdTomato-C3aR (Köhl laboratory), and C3ar1^−/− mice (The Jackson Laboratory, stock no. 005712)
- Thioglycolate-elicited peritoneal macrophages (see Current Protocols article: Zhang et al., 2008)
- D-PBS/20% FBS (see recipe)
- Anti-C3aR antibody (clone 14D4; Hycult Biotech, cat. no. HM1123)
- Anti—rat F(ab')2-AlexaFluor(AF)647 (clone ab150151; Abcam, cat. no. 150151)
- Fc blocking buffer (see recipe)
- Anti—mouse F4/80 BrilliantViolet (BV)421 (Clone BM8; BioLegend, cat. no. 123131)
- Anti-CD11b-BV510, (clone M1/70; BioLegend, cat. no. 101245)
- D-PBS/BSA (see recipe)
- 1.5-ml microcentrifuge tubes (Sarstedt, cat. no. 72.690.001)
- Refrigerated centrifuge
- 75 × 12—mm polystyrene round-bottom FACS tubes (Sarstedt, cat. no. 50-809-212)
- Flow cytometer

Additional reagents and equipment for anesthesia (see Current Protocols article: Donovan & Brown, 2001) and euthanasia (see Current Protocols article:
Donovan & Brown, 2006) of mice, and counting cells (see Current Protocols article: Strober, 2001)

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.

2. Isolate peritoneal exudate cells from BALB/c wild-type, tdTomato-C3aR1, and C3ar1−/− mice as described in the Current Protocols article Zhang et al. (2008).

   We regularly use 8- to 12-week-old mice of both sexes. While one mouse of each strain is sufficient to determine C3aR receptor expression, at least three to five animals should be used to confirm expression and to compare expression in different cell types.

3. Count cells (see Current Protocols article: Strober, 2001). Adjust the cells from each mouse strain to a density of \(1 \times 10^7\) cells/ml in D-PBS/20% FBS buffer, transfer 100 μl of the cells into a 1.5-ml microcentrifuge tube, and incubate for 30 min at 4°C.

   This step will block unspecific binding of the anti-C3aR.

4. Add 2 μl (0.4 μg/ml) of the C3aR antibody to each tube and incubate for 30 min at 4°C.

5. Add 500 μl of D-PBS/20% FBS to each tube and centrifuge the cells 5 min at 500 \(\times\) g, 4°C.

6. Discard the supernatants by aspiration.

7. Add 1 μl F(ab')2 antibody to 500 μl D-PBS/20% FBS per sample to prepare the F(ab')2 master mix.

8. Resuspend the cell pellet in each microcentrifuge tube with 50 μl of F(ab')2 master mix and incubate for 20 min at 4°C.

9. Add 500 μl D-PBS/20% FBS to each tube and centrifuge 5 min at 500 \(\times\) g, 4°C, in a refrigerated centrifuge.

10. Discard the supernatant by aspiration and repeat this procedure once.

11. Resuspend the cells in each tube in 100 μl of Fc blocking buffer and incubate for 15 min at 4°C.

12. Prepare an antibody master mix by diluting the anti-F4/80 antibody to a final concentration of 0.5 μg/ml and the anti-CD11b antibody to 0.25 μg/ml in 100 μl of sample volume of D-PBS/BSA.

13. Centrifuge 30 sec at 21,000 \(\times\) g, 4°C.

14. Discard the supernatant by aspiration.

15. Break up the pellet by tapping the bottom of the tube.

16. Add 100 μl of the CD11b/F4/80 antibody master mix to each tube and incubate the cells for 20 min at 4°C.

17. Add 500 μl D-PBS/BSA to each tube and centrifuge 30 sec at 21,000 \(\times\) g, 4°C.

18. Discard the supernatant by aspiration.

19. Resuspend the cells for flow cytometric analysis in 300 μl D-PBS/BSA and analyze the sample with a flow cytometer according to the following steps.
20. Gate on the CD11b⁺F4/80⁺ cells as described in the Basic Protocol 3, steps 13 to 14 (Fig. 8A).

21. Record the tdTomato-C3aR signal in the PE channel and C3aR-AF647 emission in the APC channel (Fig. 8B).

**DETERMINATION OF C5aR1, C5aR2, AND C3aR mRNA EXPRESSION IN FLOXED GFP-C5aR1, -tdTomato-C5aR2, OR -tdTomato C3aR–POSITIVE CELLS**

In addition to the determination of AT receptor expression using the GFP or tdTomato signal of floxed AT receptor knockin mice or the signal from fluorochrome-labeled AT receptor antibodies, Support Protocol 4 describes an example of the analysis of AT receptor mRNA expression in peritoneal macrophages from floxed GFP-C5aR1 knockin mice. This procedure is useful to verify AT receptor expression when AT receptor antibody staining has revealed cross-reactivity with structures in AT receptor-deficient mice, as is frequently observed with anti-C5aR2 antibodies.

**Materials**

- Floxed GFP-C5aR1, floxed tdTomato-C5aR2 or tdTomato-C3aR knockin mice (Köhl laboratory)
- Thioglycolate-elicited peritoneal macrophages (see Current Protocols article: Zhang et al., 2008)
- DMEM medium (Thermo Fisher Scientific Gibco, cat. no. 31885-023)
- Dulbecco’s phosphate-buffered saline (D-PBS) without calcium or magnesium, pH 7.0-7.3 (Thermo Fisher Scientific, cat. no. 14190144)
- RNAeasy Mini Kit (Qiagen, cat. no. 74104)
- Nuclease-free water (Thermo Fisher Scientific, cat. no. R0581)
- DNase I buffer (10×) with Mg²⁺: 100 mM Tris·HCl (pH 7.5 at 25°C)/25 mM MgCl₂/1 mM CaCl₂ (part of the DNase I kit; Thermo Fisher Scientific, cat. no. EN0525)
- DNase I (Thermo Fisher Scientific, cat. no. EN0525)
- 50 mM EDTA (part of DNase I kit; Thermo Fisher Scientific, cat. no. EN0525)
- Oligo(dT)₁₈ primer (Thermo Fisher Scientific, cat. no. S0132)
- 10× dNTP (Thermo Fisher Scientific, cat. no. R0191)
- RNaseOUT recombinant RNase inhibitor (Thermo Fisher Scientific, cat. no. 10777019)
- 5× RT buffer: 250 mM Tris·HCl (pH 8.3 at 25°C)/250 mM KCl/20 mM MgCl₂/50 mM DTT (part of the RevertAid kit; Thermo Fisher Scientific, cat. no. EP0441)
- RevertAid reverse transcriptase (Thermo Fisher Scientific, cat. no. EP0441)
- Primers for C5ar1, C5ar2, C3ar1 (100 μM; Eurofins Genomics or Thermo Fisher Scientific)
- iQ SYBR Green Supermix (BioRad, cat. no. 1708880)
- 1.5% TBE agarose gel (see Current Protocols article: Voytas, 2001)
- O’Gene Ruler Ladder, 50 bp (Thermo Fisher Scientific, cat. no. SM 0371)

- 24-well plates
- Cell scraper (Sarstedt, cat. no. 83.1830)
- 1.5-ml microcentrifuge tubes (Sarstedt, cat. no. 72.690.001), autoclaved
- Refrigerated centrifuge
- NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)
- Two heat blocks
- 0.2-ml 8-microtube strip without caps: high-profile (BioRad, cat. no. TBS0201) or low-profile (BioRad, cat. no. TLS0801) depending on your thermocycler
0.2-ml optical flat PCR-tube 8-cap strips, ultraclear (Bio-Rad, cat. no. TCS0803)
1.5-ml screw-cap microcentrifuge tubes with O-rings (Sarstedt, cat. no. 72.692.405)
Thermal cycler

Additional reagents and equipment for anesthesia (see Current Protocols article: Donovan & Brown, 2001) and euthanasia (see Current Protocols article: Donovan & Brown, 2006) of mice, counting cells (see Current Protocols article: Strober, 2001), and agarose gel electrophoresis (see Current Protocols article: Voytas, 2001)

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   *We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.*

2. Isolate peritoneal cells from floxed GFP-C5aR1, floxed tdTomato-C5aR2 or tdTomato-C3aR as well as C57BL/6 or BALB/c wild-type mice as described in the Current Protocols article Zhang et al. (2008).

   *We regularly use 8- to 12-week old mice of both sexes. While one mouse of each strain is sufficient to determine AT receptor expression, at least three to five animals should be used to confirm expression and to compare expression in different cell types.*

3. Count cells (see Current Protocols article: Strober, 2001). Adjust the cells from each mouse strain to a density of 1 × 10^7 cells/ml in DMEM medium without serum, add 500 μl/well to adherent 24-well-plates, and incubate 3 hr.

4. Discard the supernatant with the non-adherent cells.

5. Carefully wash the plate with 1 ml D-PBS, to remove remaining non-adherent cells.

6. Add 1 ml D-PBS to each well.

7. Harvest the adherent cells using a cell scraper into 1 ml of D-PBS and transfer detached cells into a 1.5-ml microcentrifuge tube.

8. Wash cells three times, each time with 1 ml D-PBS by centrifuging 5 min at 500 × g, 4 °C.

   *For the following steps, it is important to work quickly and on ice to prevent degradation of the mRNA.*

9. Discard the supernatant and isolate the RNA using the RNeasy Mini Kit according to the manufacturer’s instructions.

   *A minimum of 5 × 10^5 cells should be collected as a cell pellet, but not more than 1 × 10^7 cells*

10. Determine the concentration of isolated total RNA using a spectrophotometer (NanoDrop or equivalent).

11. Transfer up to 8 μl of total RNA into a fresh autoclaved 1.5-ml microcentrifuge tube

   *The total amount of RNA used for the reverse transcription reaction should not exceed 1 μg. In case your RNA yield is >1 μg, adjust the concentration to 1 μg with nuclease free water.*

12. Add 1 μl of 10 × DNase I Buffer with Mg^{2+}.
13. Add 1 μl of DNase I and mix by gently pipetting several times up and down. 

*DNase I is a fragile enzyme which requires careful handling. Do not vortex. The primers used to amplify the C5ar1, C3ar1, and C5ar2 genes are located in exon 2. Thus, treatment with DNase I is crucial to prevent contamination by genomic DNA.*

14. Incubate for 30 min at 37°C in a heat block.

15. Add 1 μl of 50 mM EDTA, transfer to a heat block (65°C), and incubate for 10 min. 

*This will stop the reaction, as EDTA chelates the Mg²⁺ ions, thus inhibiting the DNase I. Heating results in enzyme denaturation.*

16. Spin down for 5 s at 21,000 × g, then add 1.25 μl oligo(dT) primer, 1.25 μl 10× dNTPs, 0.5 μl RNaseOUT, and 5.5 μl nuclease-free water.

17. Heat the sample for 5 min in a heat block (65°C). 

*Heating ensures the linearization of the mRNA by breaking off mRNA hairpins prior incubation with the oligo(dT).*

18. Place the sample immediately on ice. 

*Placing the tubes on ice will result in immediate cooling of the solution, preventing the re-formation of secondary structures and favoring the maintenance of linear molecules. This will allow appropriate hybridization of oligo(dT) primers to poly(A) tails of mRNA.*

19. Spin down 5 s at 21,000 × g, add 5 μl of 5× RT buffer, and mix by tapping the bottom of the tube.

20. Remove 5 μl of the reaction and set aside.

*This sample will serve as the negative RT control.*

21. Add 0.5 μl of reverse transcriptase to the remaining 19.5 μl.

22. Pipet gently up and down and incubate for 30 min in a heat block (50°C)

23. Heat for 5 min on a heat block (85°C).

*This step will result in the denaturation of the reverse transcriptase and stop the reaction. Beware of excessive pressure in the microcentrifuge tube, which can result in the opening of the cap and spilling of the reaction buffer*.

24. Spin down 5 s at 21,000 × g.

*At this point, the cDNA can be stored for several months at −20°C*.

25. Place 2 μl of cDNA, 2 μl of RT negative control (from step 20), or 2 μl nuclease-free water as PCR negative control in each of three different tubes of a 0.2-ml, 8-microtube strip.

26. Prepare PCR 7 (C5ar1), PCR 8 (C5ar2), and PCR9 (C3ar1) primer mixes by adding 20 μl of each forward and reverse primer combination (Table 8) from their respective primer stocks (100 μM) to a 1.5-ml micro tube and add 160 μl nuclease-free water for a final volume of 200 μl. Store at −20°C.

*The final concentration of the primer mix for PCRs 7-9 is 10 μM for each primer.*

27. Prepare a master mix for these three samples by combining 36.5 μl of iQ SYBR Green Supermix with 3 μl each of C5ar1 (PCR7), C5ar2 (PCR8), or C3ar1 (PCR9) primer mix (step 26) and 28.5 μl nuclease-free water.

*Scale up the volume of master mix depending on the number of samples you want to test. For each RT, you need the corresponding RT negative control, and you always need one PCR negative control.*
Table 8 Primers to Amplify AT Receptor mRNA

| Use in PCR# | Primer/type/target | Sequence | bp | Design                      |
|-------------|--------------------|----------|----|-----------------------------|
| 7           | GK19/R/C5ar1       | CCGGAGAAGATCCTTATATGC | 22 | Köhl lab                   |
| 7           | GK18/F/C5ar1       | TTTCTGCTAAGGTTGTCAAAG | 18 | Köhl lab                   |
| 8           | GK82/R/C5ar2       | GCCCAGGAAGCCAAAGAGGA | 21 | (Atefi et al., 2011)       |
| 8           | GK81/F/C5ar2       | CTGGGCTCTCCTGTGACTTGCG | 22 | (Atefi et al., 2011)       |
| 9           | GK45/R/C3ar1       | TCCCCATAGACAAGTGAACCAA | 20 | Harvard primerbank # 6753224a1 |
| 9           | GK44/F/C3ar1       | TCGATGCTGACACCAATTCAAA | 20 | Harvard primerbank # 6753224a1 |

Abbreviations: bp, base pair; F, forward, R, reverse.

28. Add 23 μl of PCR master mix to each of the three tubes.

29. Place the microtube strip into a thermal cycler and run the following thermal cycling conditions:

   | Step no. | Temp. | Time  | No. of cycles |
   |----------|-------|-------|---------------|
   | 1        | 25°C  | 120 s | 1 cycle       |
   | 2        | 53°C  | 600 s |               |
   | 3        | 95°C  | 120 s |               |
   | 4        | 95°C  | 15 s  |               |
   | 5        | 55°C  | 15 s  |               |
   | 6        | 72°C  | 15 s  |               |
   | 7        | As in 4, 5, and 6 | As in 4, 5, and 6 | 35 cycles (total) |
   | 8        | 72°C  | 120 s | 1 cycle       |
   | 9        | 15°C  | Hold  |               |

   For PCR8 (C5ar2), use the same conditions as in PCR7, except that the temperature in step 5 is 54°C. For PCR9 (C3ar1), use the same conditions as in PCR7, except that temperature in step 5 is 60°C.

30. Run samples on a 1.5% TBE agarose gel for 30-40 min at 200 V with a 7-cm running length. Include DNA markers (O’Gene Ruler DNA Ladder, 50 bp). Load per lane: 6 μl PCR reaction and 4 μl marker.

31. Visualize the gel bands with UV light and document the result by photography.

Figure 9 C5ar1 mRNA expression in peritoneal macrophages from C57BL/6 wild-type and GFP-C5aR1 knockin mice. Lanes 1 and 3: β-actin; lanes 2 and 4: C5ar1; M: marker. The primers amplify an amplicon of 148 bp in exon 2 of the C5ar1 gene.
Typical results showing the amplicons of C5ar1 from peritoneal macrophages of C57BL/6 wild-type mice and GFP-C5aR1 knockin animals are shown in Figure 9. The amplicon sizes for C5ar2 and C3ar1 are 153 and 101 bp, respectively.

**ANALYSIS OF C5aR1-DRIVEN ERK1/2 PHOSPHORYLATION IN GFP-C5aR1+ CELLS**

C5aR1 is a G protein–coupled receptor (GPCR) that couples preferentially to pertussis toxin (PT)–sensitive G protein Ga2 or Gai3, but can also bind to the PT-insensitive G proteins Ga15 and Ga16 (Klos, Wende, Wareham, & Monk, 2013). Engagement of C5aR1 by its natural ligands C5a and C5a-desArg results in a series of signaling events that involve the activation of PI3K, Akt, and MAPK signaling, resulting in the phosphorylation of the MAPK ERK 1/2, in particular in bone marrow–derived neutrophils. Thus, phosphorylation of ERK1/2 can be used to assess the C5aR1 function in GFP-C5aR1+ cells. As an example, this protocol describes the analysis of C5a-driven ERK1/2 phosphorylation in GFP-C5aR1+ bone marrow–derived neutrophils.

**Materials**

- Floxed GFP-C5aR1 knockin mice (Köhl laboratory)
- Dulbecco’s phosphate-buffered saline, no calcium, no magnesium (D-PBS; Thermo Fisher Scientific, cat. no. 14190144)
- Recombinant human (rh) C5a, (Hycult Biotech, cat. no. HC2101)
- 37% formaldehyde (Sigma Aldrich, cat. no. 252549)
- Methanol, anhydrous, 99.8%, (Sigma Aldrich, cat. no. 322415)
- Anti-CD16/32 (clone 93; Thermo Fisher Scientific, cat. no. 14-0161-82)
- Anti-p-ERK MAPK-APC, Thr202, Tyr204, (clone MILAN8R; Thermo Fisher Scientific, cat. no. 17-9109-42)
- Anti-Ly6G-eF450 (clone 1A8; Thermo Fisher Scientific, cat. no. 48-9668-82)
- FACS flow buffer (BD Bioscience, cat. no. 342003)
- Refrigerated centrifuge
- Flow cytometer with lasers that can excite the APC and eF450 dyes [e.g., BD™ LSR II equipped with red (640 nm) and violet lasers (405 nm)]

Additional reagents and equipment for euthanasia of mice (see Current Protocols article: Donovan & Brown, 2006), isolation of bone marrow cells (see Current Protocols article Swamydas, Luo, Dorf, & Lionakis, 2015), counting cells (see Current Protocols article: Strober, 2001)

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.

   We regularly use 8- to 12-week-old mice of both sexes.

2. Isolate bone marrow cells as described in the Current Protocols article Swamydas et al. (2015).

   While one mouse of each strain is sufficient to harvest enough bone marrow cells to determine ERK phosphorylation, at least three to five animals should be used to validate the data.
3. Count cells (see Current Protocols article: Strober, 2001). Adjust the number of bone marrow cells to $1.0 \times 10^7$ cells/ml using D-PBS.

4. Add 100 μl of the bone marrow cells to a 0.5-ml low-retention tube and let the cells rest for 5 min at 37°C.

   Due to their basic pK, C3a and C5a have a very strong capacity to bind plastics. Thus, it is mandatory to use low-retention tubes and tips.

5. Add rhC5a at a final concentration of 10 nM using low-retention pipet tips.

6. Incubate the cells for 5 min at room temperature.

7. Quickly add 4 μl of 37% formaldehyde solution to the 100 μl cell suspension and place cells immediately on ice.

   This will stop the reaction. Make sure to use clear, colorless formaldehyde with no precipitates.

   CAUTION: Formaldehyde belongs to the group of carcinogenic, mutagenic, reprotoxic (CMR) substances. Follow the national requirements for disposal and working safety.

8. Keep the cells for 10 min on ice in the dark.

9. Centrifuge the cells 5 min at $500 \times g$, 4°C.

10. Aspirate the supernatant and break up the pellet by gently tapping the bottom.

11. Add 500 μl pre-cooled ($-20°C$) methanol and incubate for 10 min at $-20°C$ to permeabilize the cells.

   Once in methanol, cells can be stored at $-20°C$ for up to 4 weeks.

12. Centrifuge the cells 5 min at $500 \times g$, 4°C.

   The speed of $500 \times g$ must not be exceeded after permeabilization, as cell integrity can be easily destroyed. Also, a washing step is not recommended because of the difficulty in pelleting the cells.

13. Aspirate the supernatant.

   Because the cells do not always form a clear and visible pellet after centrifugation, take extra care while removing the supernatant.

14. Prepare Fc blocking buffer by diluting anti-CD16/32 antibody to a final concentration of 100 μg/ml in D-PBS.

15. Carefully resuspend the cells in 100 μl Fc blocking buffer and incubate the cells for 15 min at 4°C.

16. Centrifuge the cells 5 min at $500 \times g$, 4°C.

17. Discard the supernatant.

18. Prepare an antibody master mix by diluting anti-pERK antibody to a final concentration of 10 μg/ml and anti-Ly6G antibody to 5 μg/ml in D-PBS.

19. Incubate the cells with 100 μl of the antibody master mix for 60 min at 4°C.

   Ly6G is a specific marker to define the neutrophil population within the bone marrow cells. Antibodies recognizing Gr-1 (Ly6C and Ly6G) are not recommended, as they also stain monocytes (Daley, Thomay, Connolly, Reichner, & Albina, 2008).

20. Centrifuge the cells 10 min at $500 \times g$, 4°C

21. Discard the supernatant.
Figure 10  C5a-driven ERK1/2 phosphorylation in bone marrow–derived neutrophils from wild-type and GFP-C5aR1 knockin mice. The contour plot on the left shows the gating strategy to identify Ly6G+ bone marrow neutrophils. The histograms show phosphorylated (p-)ERK1/2 in bone marrow neutrophils from wild-type (middle panel) or GFP-C5ar1fl/fl mice (right panel), unstimulated (gray histogram) or stimulated with 10 nM hC5a for 5 min at room temperature (black line).

22. Resuspend the cells in 250 μl FACS flow buffer. Analyze ERK1/2 phosphorylation using an appropriate flow cytometer equipped with lasers that can excite the APC and eF450 dyes [e.g., BD™ LSR II equipped with red (640 nm) and violet lasers (405 nm)].

Figure 10 shows an example of the analysis of ERK phosphorylation in wild-type and GFP-C5aR1+ neutrophils.

ASSESSMENT OF C3aR FUNCTIONS IN CELLS OBTAINED FROM FLOXED tdTomato-C3aR KNOCKIN MICE

Engagement of C3aR by C3a on different human cell types including neutrophils, eosinophils, and endothelial cells results in activation of PT-sensitive and PT-insensitive G proteins, as well as a transient increase in intracellular Ca2+ which requires extracellular Ca2+ sources (Klos et al., 2013). Further, C3aR activation in mouse bone marrow–derived macrophages (Cui, Wu, Song, Chen, & Wan, 2019) has resulted in ERK1/2 phosphorylation. In contrast, we found only minor ERK1/2 phosphorylation in peritoneal macrophages that express high levels of C3aR at their surface (Quell et al., 2017), demonstrating that signaling pathways downstream of C3a do not always result in ERK1/2 phosphorylation. Here, we provide two assays that we have used to determine C3aR function in different cell types, i.e., the internalization of the receptor upon C3a ligation and the assessment of changes in intracellular Ca2+ concentration [Ca2+]i. These functions can be determined by flow cytometry and fluorescence microscopy.

**BASIC PROTOCOL 5**

**Determination of C3aR Internalization**

**Materials**

Floxed tdTomato-C3aR knockin mice (Köhl laboratory)
0.5 mg/ml human (h)C3a (Hycult Biotech, cat. no, HC2126)
Thioglycolate-elicited peritoneal macrophages (see Current Protocols article: Zhang et al., 2008)
Dulbecco’s phosphate-buffered saline, no calcium, no magnesium (D-PBS; Thermo Fisher Scientific, cat. no. 14190144)
37% formaldehyde, (Sigma Aldrich, cat. no.252549)
D-PBS/20% FBS (see recipe)
Anti-C3aR antibody (clone 14D4; Hycult Biotech, cat. no. HM1123)
Anti–rat F(ab’)2–AlexaFluor(AF)647 (clone ab150151; Abcam, cat. no. 150151)
Fc blocking buffer (see recipe)  
Anti-F4/80-BV510 (clone BM8; BioLegend, cat. no.123135)  
D-PBS/BSA (see recipe)  

Low-retention tubes (0.5 and 1.5 ml; Eppendorf, cat. no. 0030108094, 0030108116)  
Low-retention pipet tips (10, 200, and 1000 μl; Eppendorf, cat. no. 0030072006, 0030072022, 0030072030)  
Heat block  
1.5-ml microcentrifuge tubes (Sarstedt, cat. no. 72.690.001)  
Flow cytometer  

Additional reagents and equipment for anesthesia (see Current Protocols article: Donovan & Brown, 2001) and euthanasia (see Current Protocols article: Donovan & Brown, 2006) of mice, and counting cells (see Current Protocols article: Strober, 2001)  

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).  

   We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.  

   We regularly use 8- to 12-week-old mice of both sexes.  

2. Isolate peritoneal exudate cells from the peritoneum of BALB/c wild-type and tdTomato-C3aR knockin mice as described in the Current Protocols article Zhang et al. (2008).  

   While one mouse of each strain is sufficient to harvest enough peritoneal cells to determine C3aR internalization, at least three to five animals should be used to validate the data.  

3. Count cells (see Current Protocols article: Strober, 2001) Resuspend peritoneal cells from both mouse strains at a density of $1 \times 10^6$ cells/ml in D-PBS.  

4. Distribute 100 μl of the cell suspension into four low-retention 1.5-ml microcentrifuge tubes.  

5. Pre-incubate the cells for 5 min at 37°C in a heat block.  

6. Prepare hC3a working solution by adding 1 μl of hC3a (0.45 mg/ml in D-PBS, pH 7.2) to a 1.5-ml low-retention tube containing 1 ml of D-PBS and vortex. From this solution, add 1 μl to 99 μl of D-PBS, resulting in a 0.5 μM hC3a working solution.  

7. Add 2 μl of the hC3a working solution to three of the four low-retention 1.5-ml microcentrifuge tubes prepared in step 4 (10 nM final concentration). Add 2 μl D-PBS to one of the four low-retention 1.5-ml microcentrifuge tubes as a control.  

8. Gently mix the cells by pipetting up and down and incubate one of the three hC3a-stimulated tubes for 1 min, the second one for 3 min, and the third one for 9 min at 37°C in a heat block. Also, incubate the unstimulated control tube for 9 min at 37°C on the heat block.  

9. Add 2 μl of 37% formaldehyde and immediately transfer the tubes on to a pre-cooled rack placed on ice and incubate on ice for 30 min.  

   The 1.5% formaldehyde solution will stop the reaction and fix the cells.
CAUTION: Formaldehyde belongs to the group of carcinogenic, mutagenic, reprotoxic (CMR) substances. Follow the national requirements for disposal and working safety.

10. Centrifuge the cells 30 s at 21,000 × g, 4°C.
11. Discard the supernatant by careful aspiration.
12. Resuspend the fixed cells in 100 μl D-PBS/20% FBS and incubate for 30 min at 4°C.

   This step will block unspecific binding of the anti-C3aR.
13. Perform steps 4 to 11 of Support Protocol 3.
14. Add F4/80-specific antibody to each microcentrifuge to to reach a final concentration of 0.5 μg/ml, and incubate the cells for 20 min at 4°C.
15. Add 500 μl D-PBS/BSA to each microcentrifuge tube and centrifuge 30 s at 21,000 × g, 4°C.
16. Discard the supernatant by aspiration.

Figure 11  C3a drives rapid C3aR internalization and mobilization of intracellular calcium in peritoneal macrophages from wild-type and tdTomato-C3ar1<sup>fl/fl</sup> mice. (A) Comparison of C3aR surface expression in thioglycolate-elicited F4/80<sup>+</sup> peritoneal macrophages (gating shown in contour blot on the left) before as well as 1, 3, and 9 min after stimulation with 10 nM C3a at 37°C (graph on the right). Shown is the ΔMFI of C3aR staining normalized to the C3ar1<sup>−/−</sup> peritoneal macrophage equivalents. Values shown are the mean ± SEM; n = 5-6 per group. (B) Microscopic evaluation of the C3a-mediated change of [Ca<sup>2+</sup>]<sub>i</sub> in thioglycolate-elicited peritoneal macrophages from wild-type and tdTomato-C3ar1<sup>fl/fl</sup> mice. Adherent thioglycolate-elicited peritoneal macrophages were loaded with Fluo4-AM and challenged with C3a (37 nM). The images show the fluorescence emission in the FITC (Fluo4 emission) and PE channels (tdTomato), as well as from polarized light before and 6 s after C3a stimulation (×40 objective). Scale bar, 10 μm. Data are representative of three independent experiments. Originally published in Quell et al. (2017). Copyright © [2017] The American Association of Immunologists, Inc.
17. Resuspend the cells in each tube in 300 μl of D-PBS/BSA and measure the samples on a flow cytometer.

*Figure 11A shows the gating strategy to define the F4/80<sup>+</sup> peritoneal macrophages and the rapid internalization of the tdTomato-C3aR upon C3a stimulation.*

### C3a-Induced Increase in Intracellular Ca<sup>2+</sup>

**Additional Materials** (also see *Basic Protocol 5*)

- Complete RPMI medium (see recipe)
- 5 mM Fluo-4AM stock solution (see recipe)
- hC3a working solution (see *Basic Protocol 5*, step 6)
- Dulbecco’s phosphate-buffered saline, calcium, magnesium (D-DBS, with calcium and magnesium; Thermo Fisher Scientific, cat. no. 14040133)
- Biocoat fibronectin coverslips, 3 cm<sup>2</sup> (BD Bioscience, cat. no. 354088)
- 6-well plates (Sarstedt, cat. no. 83.3920.300)
- Cover glass forceps (Fine Science Tools, cat. no. 11073-10)
- 50-ml centrifuge tube (Sarstedt, cat. no. 62.547.254)
- Confocal microscope with 40× objective
- Camera

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   *We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.*

   *We regularly use 8- to 12-week-old mice of both sexes.*

2. Isolate peritoneal exudate cells from the peritoneum of BALB/c wild-type and tdTomato-C3aR knockin mice as described in the Current Protocols article Zhang et al. (2008).

   *While one mouse of each strain is sufficient to harvest enough peritoneal cells to determine C3aR internalization, at least three to five animals should be used to validate the data.*

3. Centrifuge the thioglycolate-elicited peritoneal cells 5 min at 350 × g, 4°C.

4. Remove supernatant and resuspend the cells at a density of 1 × 10<sup>6</sup> cells/ml with pre-warmed complete RPMI 1640 medium at 37°C.

   *Cell counting is described in Current Protocols article Strober (2001).*

5. Place 3-cm<sup>2</sup> circular glass coverslips into wells of a 6-well plate.

6. Add 3 ml of the peritoneal cell suspension to each well and incubate for 2 hr at 37°C in an incubator.

   *This step allows the macrophages to adhere to the glass cover slips.*

7. Gently swirl the plates and then aspirate medium.

   *The swirling will detach loosely adhering cells, but not firmly adhering macrophages, to get rid of cells other than macrophages. Work quickly and make sure that the coverslips are still covered by RPMI medium, to avoid cell damage.*

8. Add 3 ml D-PBS, mix, aspirate, and discard the supernatant.

9. Add 500 μl D-PBS to the glass coverslips.
10. Pipet 350 μl of D-PBS into a 0.5-ml microcentrifuge tube. Add 0.7 μl of 5 mM Fluo-4AM stock solution, resulting in a 10 μM working solution.  

Avoid light exposure of the Fluo-4AM solution by wrapping the microcentrifuge tube with aluminum foil.

11. Remove the glass coverslip with forceps and transfer it into an unused well of the 6-well plate.

12. Place 300 μl of the Fluo-4AM working solution on top of the coverslip.  

One coverslip can be loaded with as much as 500 μl of the Fluo-4AM working solution, but covering the slide with 300 μl is sufficient.

13. Keep the coverslip in the dark at room temperature for 30 min.

14. Take the coverslip out of the 6-well plate using forceps and discard the excess fluid by blotting it on a clean paper towel.

15. Transfer the coverslip into a 50-ml centrifuge tube filled with 40 ml of D-PBS.  

During the washing step, continue to hold the coverslip with the forceps.

16. Transfer the coverslip into a fresh well of the 6-well plate containing 5 ml D-PBS and incubate for 30 min at 37°C in the dark.  

The lipophilic transport of Fluo-4AM is made possible due to the acetylmethylester (AM) tail. However, the presence of that group prevents the binding of calcium, and it should be removed. This is done by cellular acetylmethylesterases during that extra 30 min incubation time.

17. Take the coverslip out using the forceps and blot the excess fluid again with a paper towel.

18. Insert the coverslip into the glass coverslip holder of an immunofluorescence or confocal microscope with a 40× objective, and add 463 μl D-PBS.

19. Set the focus using the polarized filter and analyze your sample in the FITC (Fluo-4AM) and PE channel (tdTomato).

20. Take a picture.  

Make sure that the focus is properly set by visualizing the tdTomato signal. This picture serves as the pretreatment control.

21. Carefully add 37 μl of the hC3a working solution (see step 6 of Basic Protocol 5) to the cells.  

This results in a final hC3a concentration of 37 nM.

22. Record a second picture 6 s after the addition of hC3a.

23. Process and analyze images using the data analysis software of your microscope.  

Figure 11B shows the microscopic evaluation of the C3a-mediated change of [Ca²⁺]ᵢ in thioglycolate-elicited PMs from wild-type and tdTomato-C5ar1flu/fl mice.

**BASIC PROTOCOL 6**  

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**C5aR2-DRIVEN IFN-γ PRODUCTION FROM NK CELLS**  

C5aR2 expression frequently overlaps with C5aR1 expression, making it difficult to differentiate between C5aR1 and C5aR2 effects in response to C5a stimulation. One solution to this problem is to use the recently described specific C5aR2 agonist P32 (Croker et al., 2016). Another option is to use cell types that exclusively express C5aR2. Using floxed tdTomato-C5aR2 knockin mice, we uncovered that some naïve NK cells from
spleen and blood show this property and express C5aR2 but not C5aR1 (Karsten et al., 2017). Also, naïve B cells from blood and spleen express C5aR2 but not C5aR1. Thus, NK cells are a perfect tool to study the function of C5aR2 independently of C5aR1. NK cells are innate immune cells that exert different effector functions. In addition to their cytotoxic effects critical for killing of tumor cells, they can produce a wide array of cytokines that play important roles in viral infections and the regulation of adaptive immune responses including IFN-γ, TNF-α, and IL-22 (see Current Protocols article: Zamora, Grossenbacher, Aguilar, & Murphy, 2015). In Basic Protocol 6, we therefore describe C5aR2-driven suppression of IL-12/IL-18-induced IFN-γ production using P32 as an example of C5aR2-mediated function in NK cells.

**Materials**

- Floxed tdTomato-C5aR2 knockin mice (Köhl laboratory)
- Splenic NK cells (see Support Protocol 5)
- Complete RPMI medium (see recipe)
- IL-12p70 (Peprotech, cat. no. 210-12)
- IL-18 (BioLegend, cat. no. 767002)
- Dulbecco’s phosphate-buffered saline, no calcium, no magnesium (D-PBS; Thermo Fisher Scientific, cat. no. 14190144)
- C5aR2-specific agonist P32 (T. M. Woodruff, University of Queensland, Brisbane, Australia) or recombinant human (rh)C5a (Hycult Biotech, cat. no. HC2101)
- Duo-Set IFN-γ ELISA kit (R&D Systems, cat. no. DY485-05)
- 48-well plate (Sarstedt, cat. no. 83.3923.500)
- CO₂ incubator

Additional reagents and equipment for euthanasia of mice (see Current Protocols article: Donovan & Brown, 2006), counting cells (see Current Protocols article: Strober, 2001), and isolation of splenic NK cells (Support Protocol 5)

1. Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

   *We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.*

   *We regularly use 8- to 12-week-old mice of both sexes.*

2. Isolate splenic NK cells as described in Support Protocol 5.

   *While one mouse of each strain is sufficient to harvest enough splenic cells to determine the IFN-γ production, at least three to five animals should be used to validate the data.*

3. Count cells (see Current Protocols article: Strober (2001). Adjust the cell number to $5 \times 10^5$ cells/ml in complete RPMI medium

4. Transfer 200 μl to a 48-well plate.

5. Prepare IL-12 and IL-18 working solutions by adding 1 μl of the IL-12p70 (10 μg/ml) to 9 μl D-PBS. Prepare IL-18 working solution by adding 1 μl of IL-18 (100 μg/ml) to 99 μl D-PBS.

6. Add 2 μl each of the IL-12 and IL-18 working solution (10 ng/ml each final concentration) or D-PBS (as control) to the cells.

   *This step will stimulate the NK cells to produce IFN-γ. Make duplicates or triplicates for each condition to minimize variation.*
Figure 12  C5aR2 controls IL12-/IL18-induced IFN-γ production from NK cells: (A) IFN-γ production from sorted splenic NK cells of wild-type and C5ar2−/− mice 24 hr after stimulation with IL-12/IL-18. (B) IFN-γ production from sorted wild-type splenic NK cells 24 hr after stimulation with IL-12/IL-18 in the presence or the absence of the C5aR2 agonist P32. Shown is the relative decrease in IL-12/IL-18–mediated IFN-γ production in response to C5aR2 stimulation by the C5aR2 agonist P32. Originally published in Karsten et al. (2017). Copyright © [2017] The American Association of Immunologists, Inc.

7. Add 4 µl of the specific C5aR2 agonist P32 at 5 mM for a 100 µM final concentration, or an equal amount of D-PBS (as control) to the cells. 

This step will test the impact of C5aR2 activation on the IL-12/IL-18-induced IFN-γ production. Alternatively, you can add 2.88 µl of rhC5a (0.87 µM; 12.5 nM final concentration) instead of the specific C5aR2 agonist, as NK cells express only C5aR2 but not C5aR1. Make duplicates or triplicates for each condition to minimize variation.

8. Incubate cells for 24 hr in a CO₂ incubator at 37°C (5% CO₂).

9. Collect the supernatant and measure IFN-γ concentration using a Duo-Set IFN-γ ELISA kit according to the manufacturer’s instructions.

Figure 12 shows the impact of C5aR2 activation on IL-12/IL-18-induced IFN-γ production from splenic NK cells.

SUPPORT PROTOCOL 5

ISOLATION OF SPLENIC NK CELLS BY FACS

Several methods have been published to isolate NK cells from spleen, some of which use selected mAbs to deplete contaminating cells such as T, B, dendritic cells, granulocytes, and monocytes by magnetic separation (see Current Protocols article: Pak-Wittel, Piersma, Plougastel, Poursine-Laurent, & Yokoyama, 2014). The advantage of magnetic isolation is that it is fast, easy to carry out, and cost-effective, and still results in a purity of up to 95%. However, to reach higher purities (up to 99%), we describe a protocol below for positive selection by FACS.

Materials

Floxed tdTomato-C5aR2 knockin mice (Köhl laboratory)
Dulbecco’s phosphate-buffered saline (D-PBS) without calcium or magnesium, pH 7.0-7.3 (Thermo Fisher Scientific, cat. no. 14190144)
Red blood cell lysis (RBCL) buffer (see recipe)
Anti–mouse CD16/32 (Clone 93; Thermo Fisher Scientific, cat. no. 16-0161-82)
Live/Dead Staining Viability kit eFluor™ 780 for flow cytometry (Thermo Fisher Scientific, cat. no. 65-0865-18)
Anti-CD3e-PerCP-Cy5.5 (clone 145-2C11; BD Bioscience, cat. no. 551163)
Sacrifice mice by cervical dislocation (see Current Protocols article: Donovan & Brown, 2006) under anesthesia (see Current Protocols article: Donovan & Brown, 2001).

We routinely anesthetize mice using 300 μl ketamine (100 mg/kg body weight) and xylazine (7.5 mg/kg body weight) via intraperitoneal injection.

We regularly use 8- to 12-week-old mice of both sexes.

Remove the spleen (see Current Protocols article: Reeves et al., 2001), transfer it into a 1.5-ml tube filled with ice-cold D-PBS, and store it on ice.

Pass the cells through a nylon cell strainer (40-μm pore size) into a 50-ml tube using a syringe plunger to obtain a single-cell suspension.

Add 15 ml of D-PBS to wash the cells and centrifuge 5 min at 400 × g, 4°C.

Add 3 ml of RBCL and incubate for 3 min at room temperature.

Add 37 ml of D-PBS and centrifuge cells 5 min at 400 × g, 4°C.

This will stop the lysis of red blood cells.

Resuspend pellet in 30 ml D-PBS.

Prepare a 10-μl aliquot to count the cell number.

We routinely use a Neubauer chamber (hemocytometer; see Current Protocols article: Strober, 2001).

Centrifuge the cells 5 min at 400 × g, 4°C.

Adjust the cell number to 1 × 10⁸ cells/ml with D-PBS.

Example: 6.5 × 10⁷ cells were counted from the pellet resuspended in a total volume of 30 ml D-PBS using the Neubauer chamber. To obtain 1 × 10⁸ cells/ml, cells are centrifuged for 5 min at 400 × g, 4°C, and resuspended in 650 μl D-PBS.

Add 3.33 μl of anti-CD16/CD32 antibody per ml of cells and incubate for 15 min at room temperature.

This treatment blocks unspecific binding of the antibodies to IgG Fc receptors. No Fc block buffer is used in this protocol because the Fc block is prepared in D-PBS/BSA; however, Live/Dead staining has to be done in D-PBS without BSA.

Prepare Live/Dead staining working solution (50 μM) by adding 10 μl Live/Dead stain from the kit to 10 μl D-PBS.

Use Live/Dead reagent at room temperature because this will allow the dye to permeate the damaged membranes of dead cells more efficiently due to increased Brownian motion,
compared to incubation at 4°C. Once inside the cell, the dye will bind to intracellular amines and its fluorescent properties will be activated.

13. Add 2 μl of the Live/Dead working solution per ml cell suspension.

14. Add 2.5 μl of each of the following antibodies per ml of cells:
   
   CD3e (1.25 μg/ml)
   NKp46 (0.5 μg/ml)
   NK1.1 (1.25 μg/ml).

15. Incubate cells for 15 min at room temperature in the dark.

16. Add 0.5 ml of D-PBS and centrifuge cells for 5 min at 400 × g, 4°C.

17. Resuspend cells in 350 μl D-PBS.

18. Run the cells on a cell sorter using the gating strategy outlined in Figure 13

   The number of splenic NK cells is quite low. About 2% of spleen cells are NK cells. Thus, to obtain a highly pure NK cell population, the gates need to be set very conservatively to avoid contamination with other cells. The typical yield is 5 × 10^7 total cells/spleen, i.e., 1 × 10^6 NK cells/spleen.

19. Continue with Basic Protocol 6.

REAGENTS AND SOLUTIONS

NOTE: For all buffers use at least ultrapure water; for PCR related-buffers always use nuclease-free ultrapure water.

**Complete RPMI 1640 medium**

Supplement RPMI 1640 medium (Gibco, cat. no. 42401042) medium with:

- 10% fetal bovine serum (FBS; PAA, cat. no. A15-043), heat inactivated 30 min at 56°C (stored in 50-ml aliquots)
- 100 U/ml penicillin, 100 μg/ml streptomycin (add from penicillin/streptomycin; Gibco, cat. no. 15-140-122)
2 mM L-glutamine (Thermo Fisher Scientific Gibco, cat. no. 25-030-081)
Store up to 1 month at 4°C

**D-PBS/BSA**

Supplement 47.5 ml of D-PBS (Thermo Fisher Scientific, cat. no. 14190144) with 2.5 ml MACS BSA stock solution (20× concentrated, 10% solution; Milteny Biotec GmbH, cat. no. 130-091-376) in a 50-ml tube. Store up to 1 day at 4°C.

**D-PBS/20% FBS**

Supplement 40 ml of D-PBS (Thermo Fisher Scientific, cat. no. 14190144) with 10 ml fetal bovine serum (FBS; PAA, cat. no. A15-043; heat-inactivated 30 min at 56°C) in a 50-ml tube. Store up to 1 day at 4°C.

**Fc block buffer**

Dilute anti-CD16/32 antibody to a final concentration of 100 μg/ml in D-PBS/BSA (see recipe).

**Fluo-4AM stock solution (5 mM)**

Add 13.6 μl DMSO to a 50 μg vial of Fluo4AM (Thermo Fisher Scientific cat. no. F14201).

**GelRed working solution**

Add 150 μl 10,000× GelRed™ Nucleic Acid Stain (Biotrend, cat. no. 41003) to 500 ml SB working buffer (see recipe). Store up to 1 week at room temperature.

**Lysis master mix**

Combine 44 μl nuclease-free water (Life Technologies GmbH, cat. no. AM9937), 5 μl 10× KAPA Express Extract Buffer, and 1 μl 1 U KAPA Express Extract Enzyme from the KAPA Express Extract Kit (Sigma Aldrich, cat.no. KK7100; manufactured by Roche). Prepare immediately before use.

**Red blood cell lysis (RBCL) buffer**

Prepare in distilled H₂O:

- 155 mM NH₄Cl
- 10 mM KHCO₃
- 0.1 mM EDTA
- pH 7.2
Store up to 6 months at room temperature

**Sodium borate (SB) buffer**

*Stock (200 mM, pH 8.5):* Dissolve 8 g NaOH per L distilled H₂O and add solid H₃BO₃ to adjust pH to 8.5. Store up to 6 months at room temperature.

*SB working buffer (10 mM; pH 8.5):* Dilute SB stock 1:20 with distilled H₂O. Store up to 1 week at room temperature.

**COMMENTARY**

**Background Information**

The ATs were first identified more than 50 years ago (Cochrane & Muller-Eberhard, 1968). For many years, before the age of molecular biology, AT receptor expression was determined by biochemical methods through binding studies with radiolabeled ligands (Huey & Hugli, 1985). With the rise of molecular biology, human (Gerard & Gerard, 1991) and mouse C5aR1 (Gerard et al., 1992), human (Crass et al., 1996) and mouse C3aR (Hsu et al., 1997) and eventually, C5aR2, initially named C5L2 or GPR77 (Okinaga et al., 2003), were cloned.
which allowed the genetic targeting of the AT receptors. Since then, considerable work has been done to develop new tools to track, delineate, and/or block the functions of AT receptors in mice, humans, and several other species including rats, guinea pigs, and even fish (trout) or amphibia (axolotl). Among the existing tools, the generation of AT receptor knock-out mouse strains has been one of the most important achievements for studying the role of AT receptors in steady state and under pathophysiological conditions. In the past decades, several laboratories have generated such mice, which are now broadly available and used by the scientific community. Another important advancement was the generation of AT receptor–specific antibodies that helped to identify AT receptor–expressing cells by immunohistochemical methods or by flow cytometry and associated techniques (e.g., live stream imaging). However, controversial results have been obtained using antibodies to determine AT receptor expression, in particular by immunohistochemical methods (Drouin et al., 2001; Quell et al., 2017; Tscherneig, Kifafard, Dibbert, Neumann, & Zwirner, 2007). Monitoring mRNA expression of AT receptors in the cells of interest in addition to antibody staining, either by northern blot or by PCR, can help to test the results obtained with antibody staining. However, mRNA expression of AT receptors does not necessarily match protein expression. Furthermore, the structure of the AT receptor genes, consisting of 2 exons with most of the coding sequence located in exon 2, strengthen the need for appropriate controls to prevent unspecific amplification of genomic DNA by PCR or recognition by radioactive probes via blot hybridization.

Recently, our laboratory has developed reporter mouse strains for C5aR1, C5aR2, and C3aR to overcome these inherent problems and ease expression studies of AT receptors. During the past few years, we generated floxed GFP-C5aR1– (Karsten et al., 2015), tdTomato-C3aR (Quell et al., 2017), and tdTomato-C5aR2–knockin mice (Karsten et al., 2017), which allowed ourselves and more than 30 academic laboratories in the U.S., Europe, and Australia to re-evaluate AT receptor expression in many tissues under homeostatic and multiple disease conditions. Further, many conditional AT receptor knockout strains have already been generated or are currently being developed targeting several professional and non-professional immune cells. We expect that these mice will further broaden our understanding of the multiple roles of the AT receptors in allergy, autoimmunity, infection, cancer, metabolic disease, transplantation, ischemia reperfusion injury, hypertonia and several other diseases or disorders.

**Critical Parameters**

**Working with anaphylatoxins**

One critical issue in handling ATs is their tendency to bind to plastic surfaces such as polypropylene walls of microcentrifuge tubes due to their basic nature. To avoid absorption to plastic surfaces, use low-retention pipet tips whenever you use pure AT solutions. To achieve this, several companies provide special tubes with a highly polished surface. Further, it is important to avoid repeated freezing/thawing processes.

**Analysis of C5aR1-driven ERK1/2 phosphorylation in GFP-C5aR1+ cells**

For staining of intracellular proteins using methanol-mediated fixation and permeabilization, the centrifugation steps and the handling of cells after treatment are critical. Methanol treatment makes the cells very fragile and sensitive to centrifugal forces. Thus, handle cells with care during washing and in particular the centrifugation steps. Because methanol-treated cells change buoyancy, the pellet is very fragile, unstable and difficult to spin down. Do not exceed 500 × g in your centrifugation steps.

**C3a-induced increase in intracellular Ca2+**

Depending on the cell type, you have to consider use of D-PBS supplemented with Ca2+ when measuring C3a-induced increase in [Ca2+]. In contrast to C5aR1 activation, which mobilizes intracellular Ca2+ stores of the endoplasmic reticulum (Norgauer et al., 1993), C3aR pathway activation requires uptake of extracellular calcium in human neutrophils (Norgauer et al., 1993). In some cells, C3aR activation drives the mobilization of intracellular calcium from intracellular stores as in transfected cell lines (Chao et al., 1999) or astrocytes (Sayah et al., 2003). When the C3aR signaling pathway has not been evaluated yet in the cell type under investigation, we recommend using D-PBS supplemented with Ca2+.

**Troubleshooting**

**Determination of AT receptor expression using antibodies**

Several polyclonal and monoclonal antibodies have been reported that have been used
to determine C5aR1, C5aR2, or C3aR expression in immune and tissue cells (Laumonnier et al., 2017). Unfortunately, many of the available AT receptor antibodies not only recognize the AT receptors, but can also bind additional structures and/or epitopes (Karsten et al., 2017). Thus, we recommend using AT receptor−deficient mice or additional PCR measurements to control for the specificity of a given AT receptor antibody staining, and not rely solely on fluorescence minus one (FMO) controls. In our hands, C5aR1 mAb 20/70 shows high specificity for C5aR1, and frequently matches results obtained with GFP-C5aR1 reporter mice. For C5aR2, mAb 468705 is widely used, but we found that this mAb not only stained bone marrow-derived neutrophils and macrophages as well as peritoneal macrophages from the tdTomato-C5ar2fl/fl mice, but also those from C5ar2−/−, suggesting that it cross reacts with other structures on these cells (Karsten et al., 2017). However, this antibody was found to specifically stain C5aR2 in endothelial cells (Miyabe, Miyabe, Mani, Mempel, & Luster, 2019), indicating that the specificity of this antibody is dependent on the cell type used. Regarding C3aR, we found that the anti-C3aR clone 14D4 matched the results obtained with floxed tdTomato-C3aR knock-in mice. In contrast, we found positive staining in different immune cells from C3ar1−/− mice using antibodies widely used in the past to stain C3aR, i.e., the D12, D20, and H300 clones (Quell et al., 2017) (Fig. 14).

**Impact of fixatives on fluorochrome stability**

Several fluorochromes (such as PE, PerCP), their respective tandem dyes (such as PE-Cy7 or PerCP-Cy5.5), and to a certain extent APC, are sensitive to methanol treatment and lose their structural integrity and hence their capacity to emit light. Because of their instability, we do not recommend use of such dyes prior to methanol treatment but advise the reader to consider alternative fluorochromes for Basic Protocol 4. APC might be used when the expression of the target molecule/receptor is high.

**Understanding Results**

**Genotyping of floxed AT receptor reporter mice**

For good lab practice, include water controls, internal controls, i.e., primers targeting the wild-type gene in the PCR reaction and run reference lanes for wild-type, heterozygous, and homozygous genotypes with each experiment, as shown in the examples in Figures 2, 3, 6 and 7.

**Strength of the reporter molecules**

Due to the fact that the GFP protein emits quite strongly, the signal can be easily observed in cells expressing high levels of C5aR1 such as neutrophils using...
either homozygous or heterozygous C5aR1 reporter mice. The use of heterozygous mice should be preferred whenever the full functionality of the AT receptor is required, since we have shown previously that, although still functional, homozygous cells react somewhat more weakly to C5a than wild-type or heterozygous cells (Karsten et al., 2017). Interestingly, heterozygous animals can also be used to investigate the expression of C5aR1 in cells with high autofluorescence, such as alveolar macrophages (Fig. 15). Although very useful, a couple of things have to be considered regarding GFP as a reporter fluorochrome. It is well appreciated that it exerts high immunogenicity and cytotoxicity, and there is phototoxicity associated with the blue light used to excite GFP (Ansari et al., 2016). Also, GFP is sensitive to fixation by formaldehyde or ethanol dehydration (Jockusch, Voigt, & Eberhard, 2003). In contrast, the tdTomato protein is more stable in this regard (Shaner, Steinbach, & Tsien, 2005). It is well suited for immunofluorescence microscopy (Fig. 11B, [Morris, Klanke, Lang, Lim, & Crombleholme, 2010], [Quell et al., 2017]) without extensive optimization (Zhanmu et al., 2019). Furthermore, tdTomato emits more strongly than most other chromophores (Shaner et al., 2004; Shaner et al., 2005) making it a very interesting surrogate for cells with minor expression of the target molecule.

**Flow cytometer settings used with AT receptor reporter mice**

GFP is excited by the blue laser (488 nm) with an emission maximum at 509 nm. Therefore, GFP is typically detected in the FITC channel (530/25). Using a narrower-bandpass filter (510/12) that is closer to the emission maximum can improve the detection of dimly expressed GFP. Further, tdTomato is best excited by the yellow-green laser (561 nm), and is typically detected in the PE channel using a 585/15 bandpass filter. Consequently, antibodies labeled with fluorochromes that are detected in the FITC channel cannot be used with the GFP-C5aR1 reporter mouse, nor can antibodies labeled with fluorochromes detected in the PE channel with tdTomato-C3aR and tdTomato-C5aR2. Depending on the signal intensity of the fluorescent proteins, it is recommended to minimize the spillover into their detection channels. Autofluorescence from cells like macrophages, due to their high metabolic activity involving NADPH, might be a source of false positive signals in GFP and tdTomato detection channels. Therefore, when working with highly autofluorescent cells (like macrophages),

**Figure 15** Comparison of the GFP signal in wild-type, GFP-C5ar1<sup>fl/fl</sup> and GFP-C5ar1<sup>fl/+</sup> mice in alveolar macrophages with strong autofluorescence. Histogram showing the GFP expression in SiglecF<sup>-</sup>CD11c<sup>+</sup> alveolar macrophages from wild-type (gray histogram), GFP-C5ar1<sup>fl/+</sup> mice (light green), and GFP-C5ar1<sup>fl/fl</sup> mice (dark green). Data are representative of three independent experiments.
fluorochromes emitting in the FITC or PE channel, but also emitting in the violet channels (BV421, 450, and 510), should be used with care.

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