Supplementary figure legends

**Supplementary s1.** Generation of c5l2−/− mice. **a**, Targeting strategy. The entire C5L2 coding sequence was replaced with a LacZ/Neo cassette. Primers a, b and c used for PCR analysis are indicated, as is the probe used to distinguish the WT and mutated C5L2 alleles. **b**, Genomic Southern blot analysis of two c5l2+/− ES clones plus the parental Lex1 clone. **c**, Expression of C5L2 and C5aR on thioglycollate-induced peritoneal macrophages (F4/80 gated) purified from B6 and BALB/c background wild type and c5l2−/− mice. **d**, Intracellular flow cytometry analysis of total C3aR and C5aR expression in wild type, heterozygous and c5l2−/− bone marrow derived neutrophils. Bold line, LPS stimulated cells. Dashed line, IgG control. **e**, Western blotting analysis. HSP70 as an endogenous protein loading control.

**Supplementary s2.** Impaired activation in c5l2−/− neutrophils and macrophages. **a**, Flow cytometric analysis of Mac-1 induction on neutrophils after 1hr stimulation with C5a (100ng/ml), C5adesArg (10μg/ml), C3a (10μg/ml) and C3adesArg (10μg/ml). **b**, Impaired induction of co-stimulatory molecules. Surface CD40 and CD86 expression on WT and c5l2−/− macrophages after stimulation with C5a (1μg/ml) and/or LPS (1μg/ml). The percentage of positive cells is indicated in each quadrant.

**Supplementary s3.** Decreased C3a-induced ERK activation and F-actin formation in c5l2−/− cells. **a**, (upper) ERK activation in bone marrow derived macrophages stimulated with C3a (1μg/ml) and C5a (100ng/ml). (lower) Comparable intracellular staining of C3aR and C5aR in the same wild type (WT) and c5l2−/− (KO) bone marrow derived macrophages utilized for the ERK study above. **b**, Decreased F-actin formation. WT and
c5l2−/− neutrophils were treated with C3a (12.5μg/ml) to induce F-actin formation. Data shown are representative of 20 fields examined per mouse.

**Supplementary s4** Reduced inflammatory cells in air pouches. Total cell numbers, numbers of neutrophils and macrophages that had infiltrated into dorsal air pouches in response to TG and/or C5a were recovered and counted.

**Supplementary s5.** Histological analyses (40x) of OVA-challenged lung tissues. HE stained. NS: no stimulation.

**Supplementary s6.** Reduced surface C5aR expression on C5L2−/− Neutrophils. 

a. Surface staining of C5aR by clone 20/70 on c5ar+/+, c5ar+/− and c5ar−/− neutrophils.

b. Surface and intracellular staining of C5aR by 20/70 in c5l2+/+ and c5l2−/− neutrophils.

c. Surface and intracellular staining of C5aR by 20/70 in c5l2+/+ and c5l2−/− macrophages.

**Supplementary s7.** Comparable C3aR expression on WT and C5L2−/− Neutrophils.

a. Surface staining of C3aR on c3ar+/+ and c3ar−/− macrophages by D20 antibody.

b. Surface and intracellular staining of C3aR by D20 in c5l2+/+ and c5l2−/− neutrophils.

c. Surface staining of C3aR by D20 on c5l2+/+ and c5l2−/− macrophages.

**Supplementary s8.** Surface C3aR (upper) and C5aR (lower) expression on WT and C5L2−/− macrophages after C5a (100ng/ml), C3a (5μg/ml) and LPS (1μg/ml) stimulation.
Supplementary protocols

Radioligand binding assay

Cell membranes of COS cells (30-50 µg) transiently expressing C5L2 was incubated with binding buffer (50 mM HEPES, 1 mM CaCl₂, 5 mM MgCl₂, 0.1% BSA, pH 7.4) containing 0.1 nM [¹²⁵I]hC5a (2200 Ci/mmol; Perkin Elmer) in the presence or absence of test ligands (hC5a, mC5a or hC3a; 100 nM respectively) in a 96-well microplate at 4°C with slow shaking for 1 hour. For competition binding studies, serially diluted mouse C5a (R&D Systems) was incubated with [¹²⁵I]hC5a (0.1 nM). Plates were filtered through GF/B 96-well filters in 96-well Unifilter Harvester (Perkin Elmer), dried, and the bound radioactivity was counted in Topcount (Perkin Elmer).

Generation of c5l2⁻/⁻ mice

The c5l2⁻/⁻ mice were generated by Lexicon Genetics (The Woodlands, TX). The targeting vector was constructed by replacing the entire C5L2 coding region with a LacZ/Neo cassette. Linearized targeting vector was electroporated into 129SvEvBrd-derived Lex1 ES cells. G418-resistant homologous recombinants were identified by PCR and confirmed by Southern blotting. Two ES clones were injected into 3.5-day C57BL/6 blastocysts. Chimeric mice with germline transmission were backcrossed with C57BL/6 mice to produce B6.F8 c5l2⁺/- mice. For the asthma model, mice were also backcrossed with BALB/c mice to produce BALB/c.F7 c5l2⁺/- mice. The heterozygotes were then intercrossed to generate homozygous c5l2⁻/⁻ mice. The primers a and b indicated in Fig. 1a were used to detect the WT C5L2 allele, while primers b and c were used to detect the mutated C5L2 allele. The sequences of primers are: primer a, 5’-ACTGTGCCTTCTGCTGTCTAC-3’; primer b, 5’-ATGCAGCCTGGAGTGACTTG-3’;
primer c, 5’-GCAGCGCATCGCCTCTATC-3’. The BALB/c.F8 c5aR−/− mice were obtained from Jackson laboratory.

RT-PCR

Total RNA was purified from WT and c5L2−/− liver tissue using TRIzol reagent (Gibco BRL). Complementary DNA was reverse-transcribed using SuperscriptIII RT (Invitrogen) and used as a template for PCR analysis of C5aR and C5L2 mRNA expression. The PCR primer sequences are: mC5aR-sense: 5’-ATGGATCCTAACATACCTGCGGATG-3’; mC5aR-antisense: 5’-TCTACACCGCCTGACTCTTCCG-3’; mC5L2-sense: 5’-AACCACACCACCAGCGAGTATTATG-3’; mC5L2-antisense: 5’-AGCCCTCTTTGCCTACACCAGG-3’.

Preparation of neutrophils, macrophages and lung fibroblasts

Neutrophils were purified from bone marrow.22 Mononuclear cells were removed by Histopaque-1083 (Sigma) gradient separation. The cell pellet was mixed with 10ml ACK lysis buffer (8.29g/L NH₄Cl, 1g/L KHCO₃, and 37mg/L Na₂EDTA, pH7.2) to remove RBCs. Cells were washed and counted prior to use in experiments. The purity was confirmed by flow cytometry. For macrophages, mice were intraperitoneally injected with 1ml TG broth (thioglycollate, 2.4% in H₂O, Difco). After 3 days, cells that infiltrated in the peritoneal cavity were harvested and RBCs were lysed. The remaining cells were washed, counted and cultured overnight in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum. Attached macrophages were washed and counted prior to stimulation. Lung fibroblasts were cultured from adult mice lung tissues. Smashed lung tissues were first trypsinized for 30min and then cultured on 10cm tissue culture plates with 10ml 10% FBS DMEM medium for 1 week. Fibroblasts migrated
from lung tissues were attached onto plate and further sub-cultured for 4 to 5 generations for the transfection experiments.

**Assessment of cellular activation**

Stimulated cells (5x10^5 cells/sample) were harvested in PBS/10mM EDTA, washed, and then incubated with 0.5\(\mu\)g Fc-blocker (anti-CD16/CD32, BD-Pharmingen) in staining buffer (PBS/1% FBS/0.1% NaN\(_3\)) for 10min. Cells were then incubated with anti-CD40, anti-CD86, anti-CD11b/Mac-1 (BD-Pharmingen) or anti-Gr1 (eBioscience) for 20min. After 3 washes, marker expression on viable cells staining negatively with 7-aminoactinomycin D (7-AAD, Sigma) was analyzed by flow cytometry.

**Flow cytometry**

For detection of cell surface proteins, cells were washed and then incubated with 0.5\(\mu\)g Fc-blocker (anti-CD16/CD32, BD biosciences) in staining buffer (PBS/1% FBS/0.1% NaN\(_3\)) for 10min. Cells were then incubated with antibodies for 20min. After 3 washes, marker expression on viable cells staining negatively with 7-AAD was analyzed by flow cytometry. For intracellular staining (ICS), cells were washed with PBS and then fixed in CytoFix/CytoPerm buffer (BD biosciences) for 15min. After 1 wash with 1X BD Wash/Perm buffer, cells were incubated with antibodies in Wash/Perm buffer for 20min. Cells were then washed 3X with 1ml Wash/Perm buffer and then re-suspended in staining buffer for flow cytometry analysis.

**Activation of MAP kinases and AKT**

Purified cells (3x10^6/sample) were stimulated with ligands as described above for 5min at 37°C. Cell lysates were fractionated by SDS-PAGE and transferred to PVDF membranes
The blots were first incubated with anti-phospho-p42/p44, anti-phospho-p38 MAPK, anti-phospho-AKT (Cell Signaling) and anti-phospho-JNK (New England Biolabs). Stripped blots were reprobed with anti-p42/p44, anti-p38 MAPK, anti-AKT (Cell signaling) and anti-JNK (Santa Cruz) antibodies to determine total kinase levels. To visualize proteins, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Amersham-Pharmacia), and processed with the ECL system (Amersham-Pharmacia).

**Actin polymerization**

Neutrophils (2x10^5/sample) were stimulated in 80μl RPMI medium with C3a (12.5μg/ml) at 37°C for 1min. The stimulation was then stopped by adding 10μl formaldehyde (37%) and 10μl L-α-lysophosphatidylcholine solution (1mg/ml in PBS; Sigma) for 30min. The samples were washed and then stained with 50μl washing buffer (PBS/0.5% BSA) containing 1U Alexa-Fluro®488 phalloidin and 3μM DAPI (Molecular Probes) on ice for 30min. After washes, cells were cytospinned onto glass slides, and analyzed by confocal microscopy in Advanced Optical Microscopy Facility (AOMF) at UHN. For each treatment, 20 fields per slide were taken, and numbers of total (DAPI positive) and F-actin positive neutrophils were counted.

**Thioglycollate-induced peritonitis**

Acute peritonitis was induced in mice by intraperitoneal injection of 1ml 2.4% TG broth. Peritoneal exudate cells were harvested at 72 hours post-injection with 6ml cold PBS/10mM EDTA. Cells were washed, then counted using a flow cytometer in high-speed mode for 1min. Neutrophil and macrophage percentages were determined by staining cells with anti-Mac-1 and anti-Gr1 antibodies followed by flow cytometry.
Air pouch model

Air pouches were formed by the dorsal subcutaneous injection of 5ml sterile air on day 0 and day 3. On day 6, 100μl TG and 2μg C5a in 1.5 ml 0.5% carboxymethyl cellulose (CMC, medium viscosity; Sigma) were injected into air pouches. At 24 hours post-injection, mice were sacrificed and cells that had infiltrated into the air pouch were recovered with 2ml PBS/10mM EDTA. Collected cells were washed, counted, stained and analyzed.

OVA-induced asthma model

Asthma-like airway hypersensitivity and inflammatory mouse model induced by ovalbumin (OVA, 20μg/mouse, Sigma)-immunization with 2mg/mouse Alum (day 0 and day 7 i.p.) followed by serial aerosol (5% OVA in 10ml normal saline, 40min/day) challenge on day 14, 16, 18 and day 20 to generate allergic airway disease in mice backcrossed 4 to 5 generations against the BALB/c strain. On day 21, Airway hyperresponsiveness (AHR) to inhaled methacholine (0, 2.5, 5, 10, 20 mg/ml) was measured by using barometric whole-body plethysmograph (Buxco) 24 hours after the last aerosol challenge. Tissues were collected and fixed in PBS buffered-formalin (10%) for further histological analysis. The lungs were infused with 10% neutral-buffered formalin to physiologic volume immediately after euthanasia and the lungs further fixed by immersion fixation. Lung tissue was processed, embedded in paraffin, and sectioned at 5μm prior to staining with Haematoxylin and Eosin.

Hematopoietic cell regeneration

PBLs were monitored before, and for 3 weeks after, a sub-lethal dose of γ-irradiation (3Gy). Tail blood (10μl) was collected and immediately mixed with 90μl PBS/10mM
EDTA, and RBCs were lysed. After washing, cell pellets were resuspended in 400μl washing buffer (PB8/1%FBS/0.1% NaN₃) containing 7-AAD (1μg/sample). Total cell number and percentage of viable cells (7-AAD negative) was determined.
**Supplementary s1**

**a**

![Diagram of C5aR and C5L2 genes with restriction sites and probe locations.]

**b**

![XbaI blot with KO and WT samples, showing bands at 9.1 kb and 5.6 kb.]

**c**

![Graphs showing surface C5L2 and intracellular C5aR (P14) in PEC, F4/80 positive Macrophages.]

**d**

![Graphs showing BM, Gr1 positive Neutrophils with C3aR and C5aR in C57B6 and Balb/c strains.]

**e**

<table>
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Supplementary s3
Supplementary s4
a

C5aR WT
52.9
C5aR +/-
30.3
C5aR KO
7.19

Surface C5aR (20/70)

b

Neutrophils
C5L2 WT 63
C5L2 KO 25

Surface C5aR (20/70)

c

Macrophages
C5L2 WT
C5L2 KO
30.7 29.65

Surface C5aR (20/70)

Supplementary s6
Supplementary s7
Supplementary s8