Figure S1

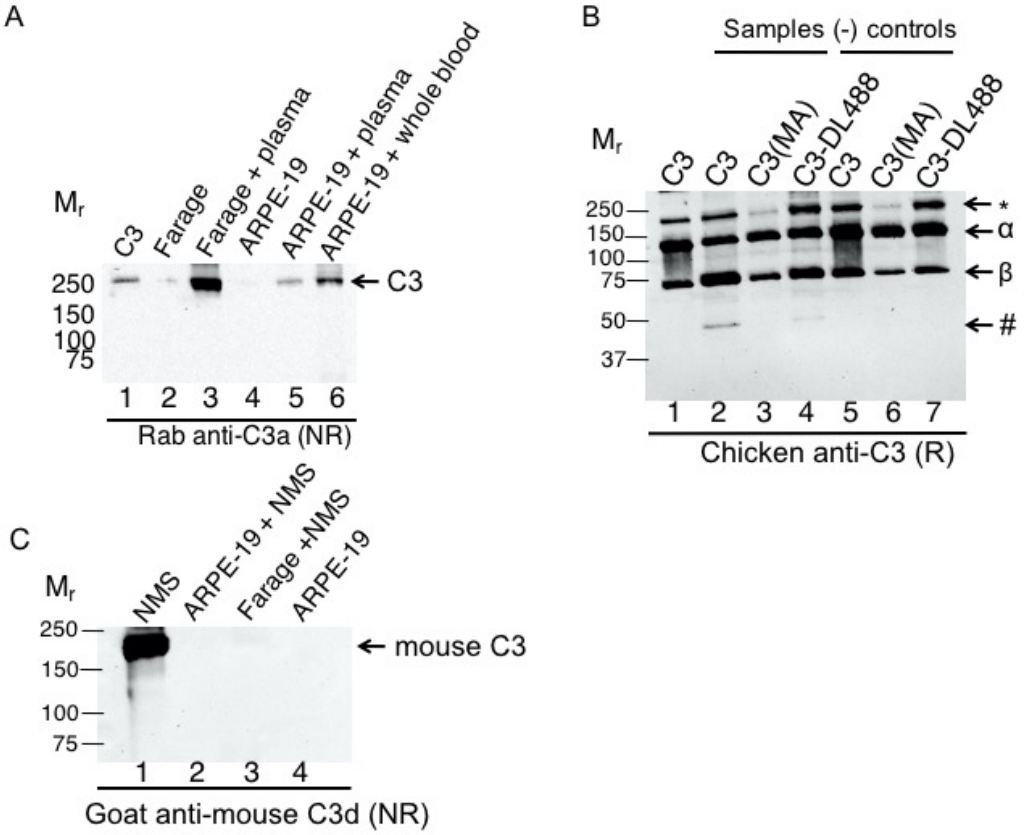

Figure S1. Specificity of C3(H_2O) uptake. (A) Whole cell lysates from Farage cells and ARPE-19 incubated with 10% human plasma and ARPE-19 incubated with EDTA treated human whole blood were evaluated for C3 uptake by WB with a rabbit anti-C3a pAb under NR conditions. Farage, $2.4x10^5$ cell equivalents; ARPE-19, $1.5x10^5$ cell equivalents. (B) Autolytic cleavage was performed to determine if the thioester bond is intact or hydrolyzed in C3-DL488. The resulting reaction was analyzed by WB with a chicken anti-C3 pAb. #, autolytic cleavage fragment at 46 kD is detected in native C3 (positive control) but not in C3(MA) (negative control); *, bands represent dimers of C3 that occur in purified preps due to the instability of the thioester bond. (C) The human cell lines Farage and ARPE-19 were incubated in 10% normal mouse serum (NMS) and the resulting cell lysates analyzed for mouse C3 uptake by WB with a goat anti-mouse C3d pAb. 1:10 diluted NMS used as control. NR conditions. $2.4x10^5$ cell equivalents loaded. (A) and (C) representative of two independent experiments. (B) representative of three independent experiments.

Figure S2

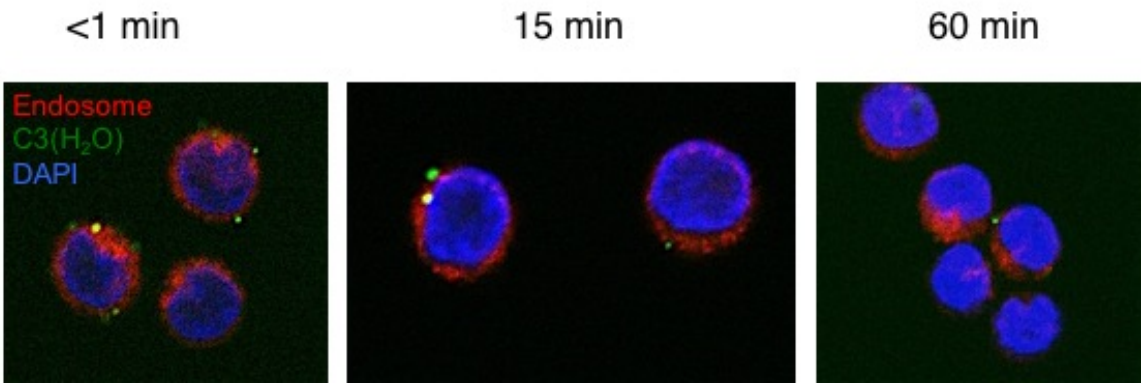

Figure S2. C3 is localized to an intracellular compartment following rapid uptake. Representative confocal microscopy images showing exogenously added C3 co-localizes to early endosomes. $CD4_+$ T cells were incubated with C3-DL488 (50 µg/ml, green) for 5 min, washed and then incubated for an additional 1, 15 or 60 min in normal growth media. C3-DL488 loaded cells were washed, fixed and permeabilized at the indicated time points and then stained for early endosomes (Rab5, red). Blue, DAPI. Images acquired with 60X objective. Representative images are from two independent experiments.

Figure S3

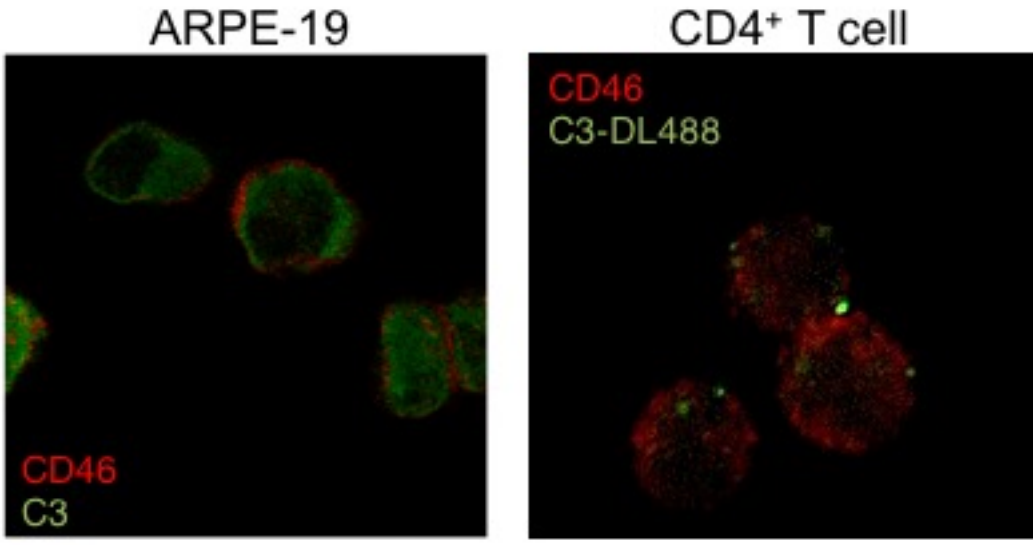

Figure S3. C3 is not on the plasma membrane 15 min after loading.
Representative confocal microscopy image showing that loaded C3 does not colocalize to the plasma membrane. ARPE-19 cells were incubated with 10 µg/ml C3(H_2O) (left panel) or $CD4^+$ T cells with C3-DL488 (right panel, green) for 15 min. Loaded cells were then washed, fixed and permeabilized and stained for C3a (left panel, green) and CD46 (plasma membrane marker, red). Images acquired with 60X objective. Image representative of two independent experiments.

Figure S4

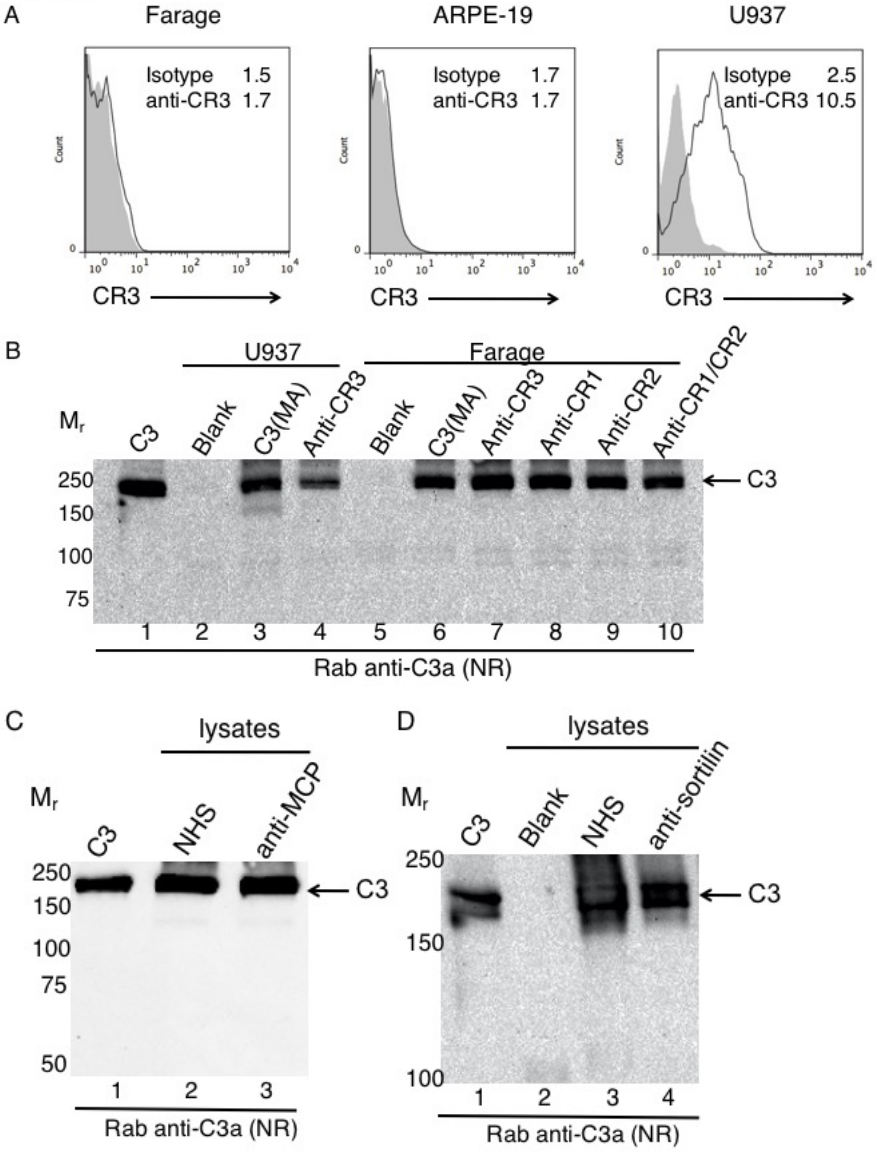

Figure S4. Known C3b binding partners do not mediate C3(H_2O) uptake.
(A) Membrane expression of CR3 on Farage, ARPE-19 and U937 cells was assessed by flow cytometry with an anti-human CR3 mAb. Results representative of 2 independent experiments. Function blocking mAbs to CR3, CR1 and CR2 (B), MCP (C), and sortilin (D) were pre-incubated with U937 (B) or Farage (B-D) prior to exposure to NHS. The resulting cell lysates were assessed for inhibition of C3(H_2O) uptake by WB with rab anti-C3a pAb under NR conditions. Results representative of three independent experiments.

Figure S5

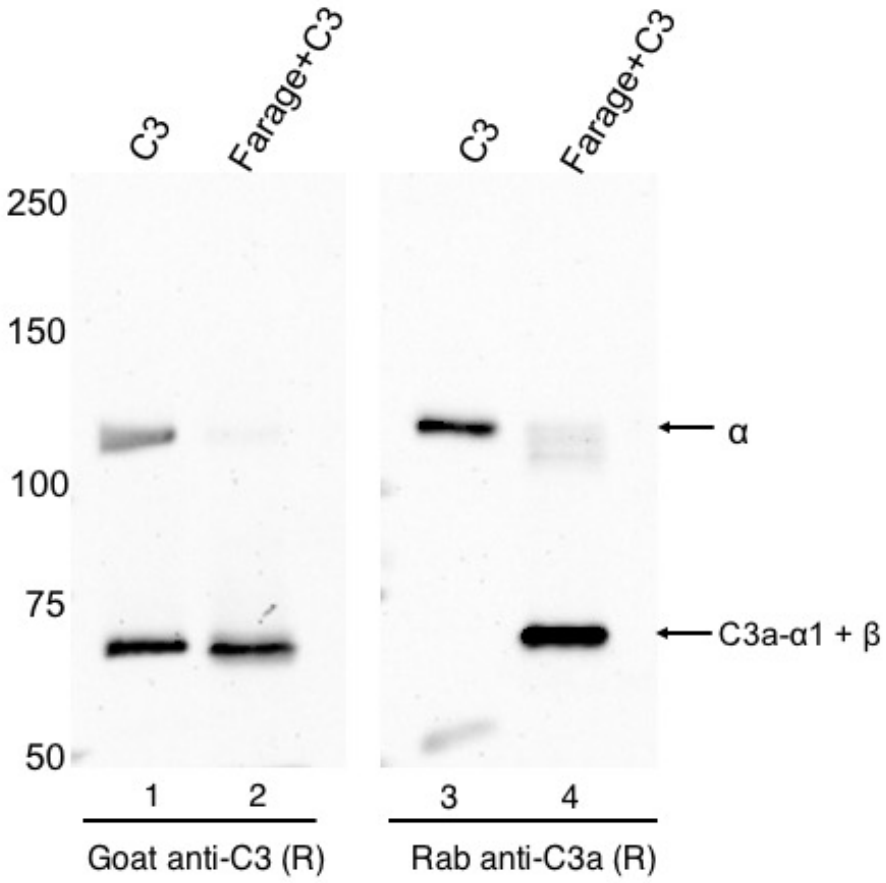

Figure S5. Loaded C3(H_2O) is subject to CA in the presence of serum factors. Farage cells were incubated with purified C3(H_2O) diluted in 10% C3-depleted serum for 1h. The resulting cell lysate was analyzed by WB with a rabbit anti-C3a pAb and a goat anti-C3 pAb under R conditions. C3, 50 ng; 2.4 x 10^5 cell equivalents. Results representative of two independent experiments.

Figure S6

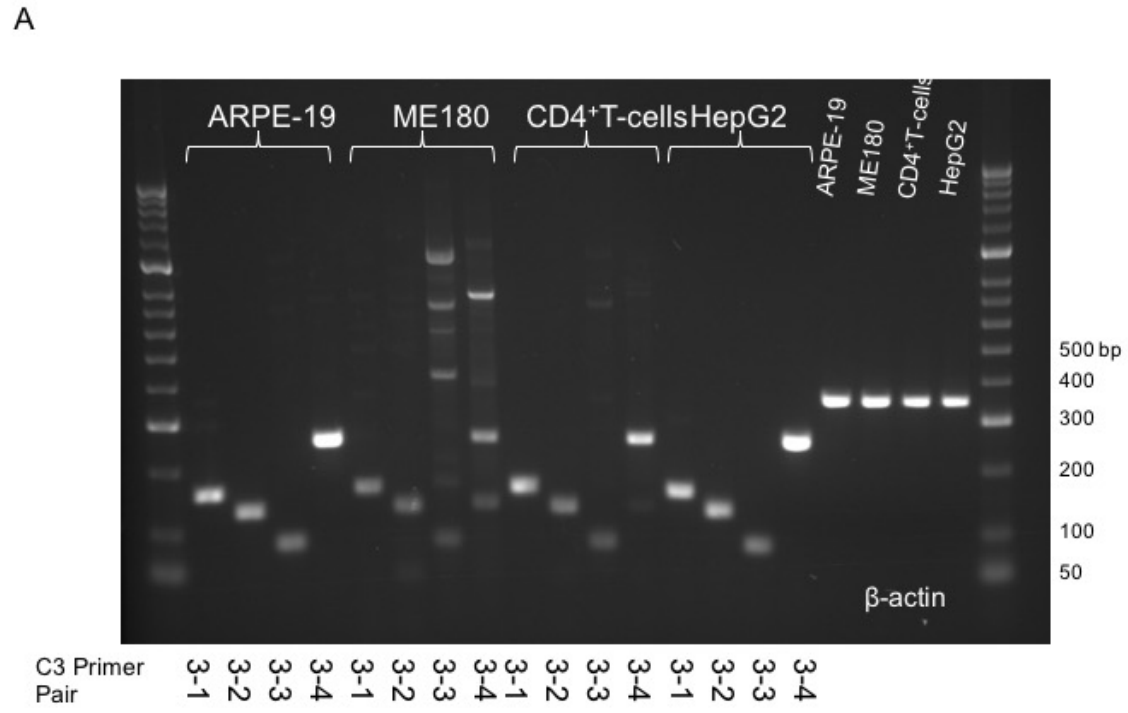

Figure S6. C3 mRNA in cell lines and $CD4^+$ T cells. Human cell lines ARPE-19 and ME180, and peripheral blood $CD4^+$ T cells contain mRNA coding for C3 (A). The location of the primers in the C3 protein are indicated in (B).

Figure S7

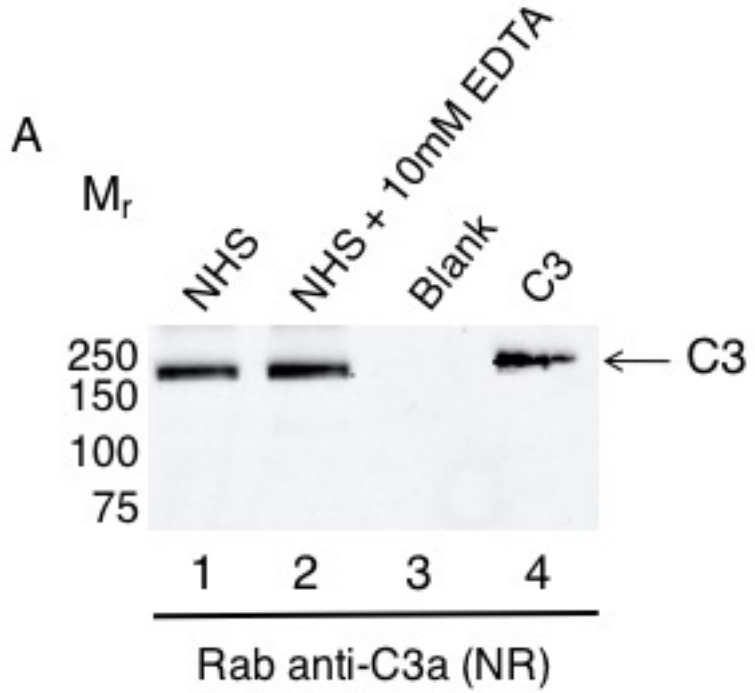

Figure S7. $C3(H_2O)$ uptake is not dependent on convertase activity. Farage cells were incubated in 10% NHS in the presence of absence of 10mM EDTA for 15 min. The resulting cell lysates were analyzed for $C3(H_2O)$ uptake by WB with a rabbit anti-C3a pAb. C3, 20 ng; Farage 2.4 x 10^5 cell equivalents. Blot is representative of two independent experiments.

Figure S8

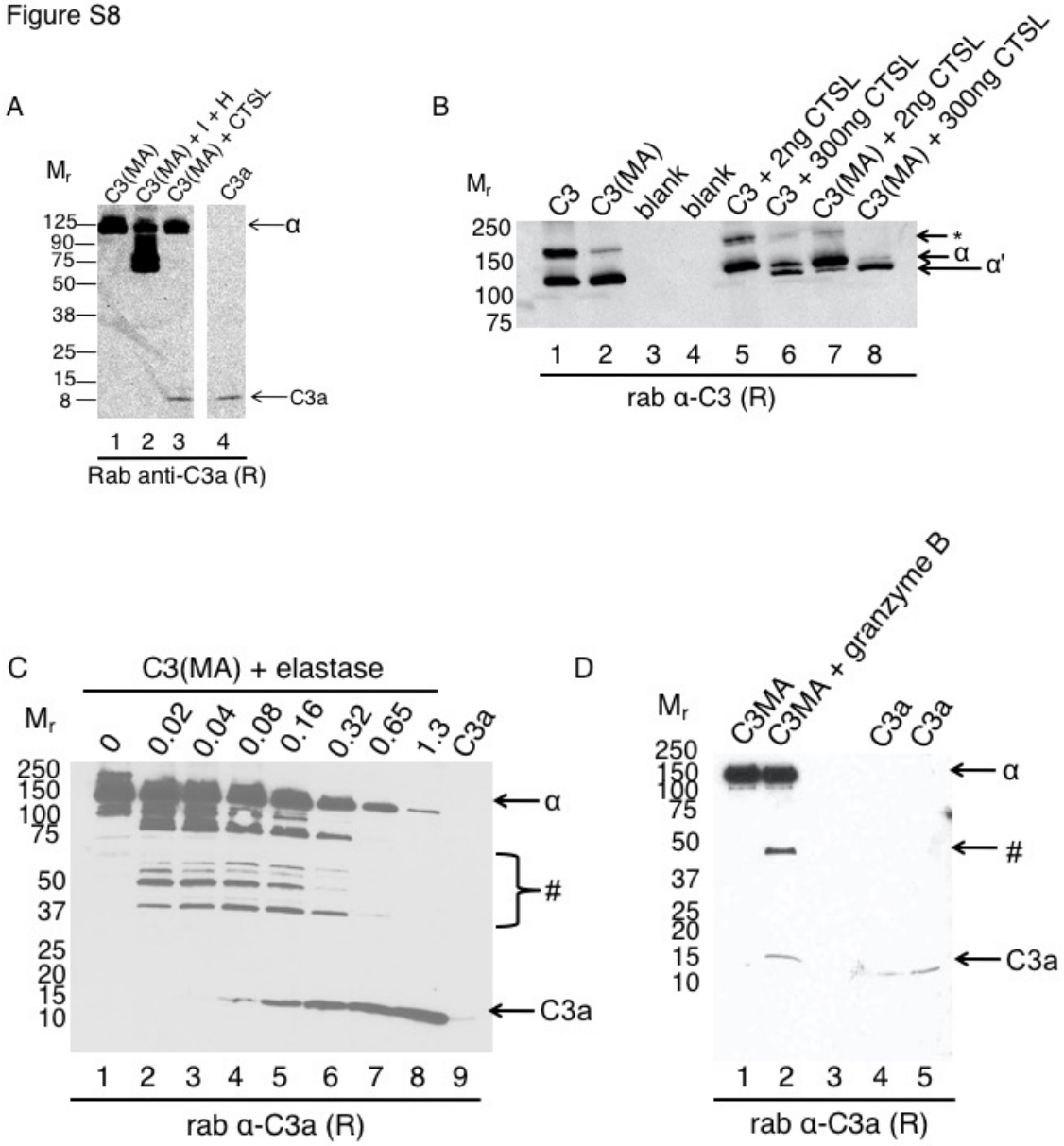

Figure S8. Multiple proteases cleave C3(H_2O) to liberate C3a. Purified C3(MA) (A-D) or C3 (B) were assessed for C3a liberation (A, C and D) and cleavage (B) by CTSL (A and B), elastase (C), and granzyme B (D). The resulting reactions were analyzed by WB with a rab anti-C3a pAb (A, C, and D) or a rab anti-C3 pAb (B) under R conditions. In (A), lane 4 was run on the same gel but was noncontiguous. In (B), C3 cleavage does not occur with 2 ng CTSL but cleavage of C3(MA) is detectable. Additionally, ~ 50% of the C3 but 100% of the C3(MA) was cleaved by 300 ng CTSL. *, bands represent dimers of C3 that occur in purified preps due to the instability of the thioester bond. #, similar C3a containing proteolytic fragments were identified between 50 and 37 M_r when C3(MA) is cleaved with elastase or granzyme B. Results are representative of at least two independent experiments.

Figure S9

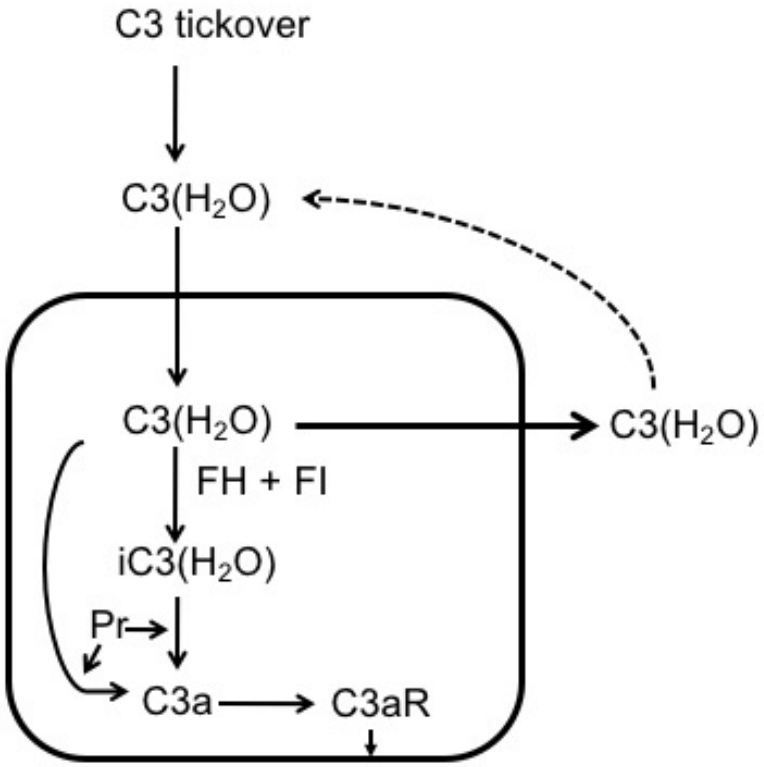

Figure S9. C3(H_2O) recycling pathway
The C3(H_2O) generated in plasma is continuously internalized by cells and a majority returned to the extracellular milieu. Under steady state conditions, ~20% of the C3(H_2O) is retained in the cell. Multiple intracellular proteases can cleave the C3(H_2O) releasing C3a and likely other active fragments. The C3a and C3aR could become membrane associated and/or the C3a secreted (4). Additionally, C3(H_2O) can engage the AP feedback loop. Pr, protease