Supporting Information

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SI Methods

Media, Cells, Cell Lines, Antibodies, and Proteins. RPMI medium 1640 (Cellgro; Mediatech) with the appropriate supplements was used for all cells and cell lines used. Cell lines and supplements were obtained from the tissue culture facility at Washington University School of Medicine. T cells, monocytes, macrophages, or DCs were maintained as previously published (1). Wild-type CHO-K1 cells and CHO mutant lines (CHO-745 and CHO-M1) have been described previously and were cultured as per those protocols (2, 3). The mAbs to human CD3 (HIT3a), CD28 (CD28.2), CD4, CD14, CD80, CD86, CD220, and HLA-DR as well as annexin V and streptavidin were purchased from BD Biosciences. Anti-C1q was purchased from Quidel, and anti-MBL was purchased from Assay Design. Fluorochrome-conjugated anti-C3, made by Cappel, and the biotinylated mouse anti-properdin antibodies, obtained from Assay Design, were used in all FACS analyses except the CHO experiments of Fig. 4 *a* and *b* and the Jurkat experiments of Fig. S5. In those cases, anti-properdin mAb (catalog no. A235; Quidel) and/or anti-C3 mAb (HAV 003-05; Assay Design) were used together with secondary antibody FITC-labeled goat anti-mouse IgG (Sigma–Aldrich). C1q and properdin (purified from human serum) were obtained from CompTech, and purified mannose-binding lectin (MBL) was a gift from Wilhelm Schwaeble (University of Leicester, Leicester, UK). Heparin, heparan sulfate, chondroitin sulfate-A (CS-A; GlcA-GalNAc4S), CS-B (dermatan sulfate; IdoA-GalNAc), CS-C (GlcA-GalNAc6S), CS-D (GlcA2S-GalNAc6S), and CS-E (GlcA-GalNAc4, 6S) were purchased from Seikagaku/ACC and used at a concentration of 20 μg/ml unless specified. The compositions of the Mg^{2+}-EGTA buffer and the EDTA buffer are described in ref. 4.

Sera. Human serum was heat-inactivated at 56°C for 30 min. This treatment deactivates the complement components C1 and C2 (5) and factor B (6), thus blocking the complement activation pathways. Properdin-deficient human serum was obtained from a properdin-deficient patient and was a generous gift from Peter Densen. Properdin-depleted human serum was purchased from CompTech, and the absence of properdin was confirmed by Western blot analysis (data not shown). C3-depleted human serum was obtained from CompTech, and the absence of C3 and the presence of properdin were confirmed by Western blot analysis (data not shown).

Biotinylated Anti-C1q. Seventeen micrograms of sulfosuccinimidyl-6-(biotinamido)hexanoate (EZ-link sulfo-NHS-LC-Biotin; Pierce) was mixed with 125 μg of anti-C1q in 150 μl of water and incubated for 5 min at 25°C. The reactants were treated with 250 μl of PBS, and the resulting mixture spun in a Millipore 500-μl concentrator with a Biomax 10K NMWL membrane. After two washes with 500 μl of PBS, the biotinylated product was recovered from the concentrator with 100 μl of PBS and stored in 5-μl aliquots at −70°C until use.

Phagocytosis assay II. Macrophages were grown to confluence on Lab-Tek Chambered Slides (Nalge Nunc). Apoptotic T cells, preincubated with purified properdin and then washed or left untreated, were added to the phagocytes at 0.5 to 1×10^6 per well in prewarmed RPMI medium 1640, medium containing 15% NHS, 15% heat-inactivated human serum (ΔHS), or 15% properdin-depleted serum (ΔP). Cell mixtures were then incubated at 37°C for up to 1 h. Slides were washed several times with cold PBS to remove noningested T cells. Washed cells were stained with Harris hematoxylin solution (Sigma) according to the manufacturer's protocol and then analyzed for T cell uptake by optical microscopy.

Sodium Chlorate Treatment. Glycosaminoglycan (GAG) sulfation was inhibited by sodium chlorate treatment (7). Purified T cells were activated for 72 h in sulfate-free Ham's F12 medium supplemented with 25 units/ml recombinant human IL-2, 10% dialyzed FBS, and 12.5 mM sodium chlorate. In some cases, media were supplemented for the last 24 h of culture with 10 mM sodium sulfate to reinduce GAG sulfation. Apoptosis was then induced, and T cells were analyzed for properdin binding capacity. CHO cells were cultured overnight in sulfate-free Ham's F12 medium supplemented with 10% dialyzed FBS and 25 mM sodium chlorate. In some cases, media were also supplemented with 10 mM sodium sulfate.

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3. Esko JD, Stewart TE, Taylor WH (1985) Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc Natl Acad Sci USA* 82:3197–3201.
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5. Rother K, Till, GO (1988) in *The Complement System*, eds Rother K, Till GO (Springer, New York), pp 1–4.
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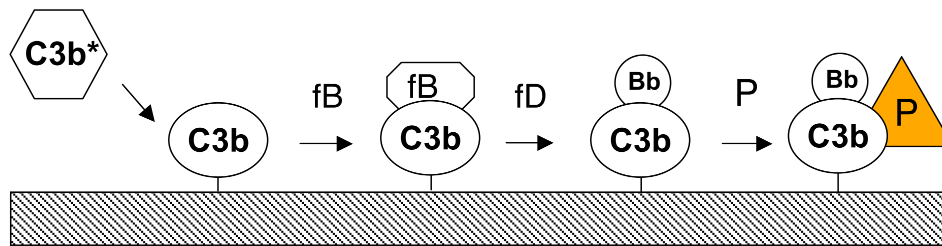
a. Properdin stabilizes assembled C3 convertases



b. Properdin recognizes activating surfaces and initiates C3 convertase formation

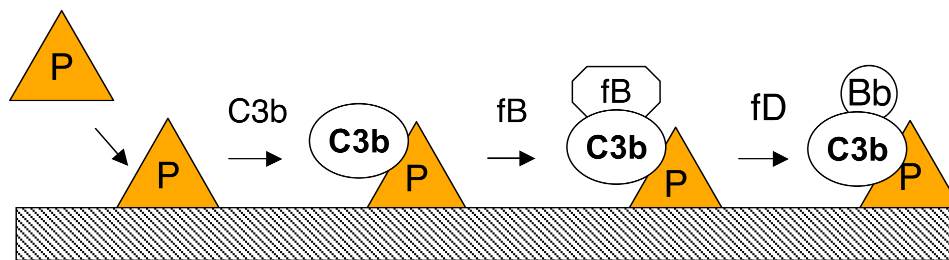


Fig. S1. A novel role for properdin in the initiation of AP activation on a target surface. (*a*) Properdin's ''classic'' role as convertase stabilizer. AP C3 convertase assembly is initiated via nascent C3b(C3b*), which is generated by nonspecific fluid-phase activation of C3 and then covalently binds to nearby surfaces. Target-bound C3b then associates with factor B, and C3bB is cleaved in the factor B subunit by factor D. The resulting complex, C3bBb, is an unstable C3 convertase. The binding of properdin stabilizes the AP convertase. (*b*) Properdin's novel role in the direct recognition of target surfaces. Properdin binds directly to activating surfaces and initiates AP assembly by recruiting fluid phase C3b (nascent C3b is not required). This figure is adapted from Spitzer *et al.* (8). [Reproduced with permission from Spitzer *et al*. (8) (Copyright 2007, The American Association of Immunologists, Inc.).]

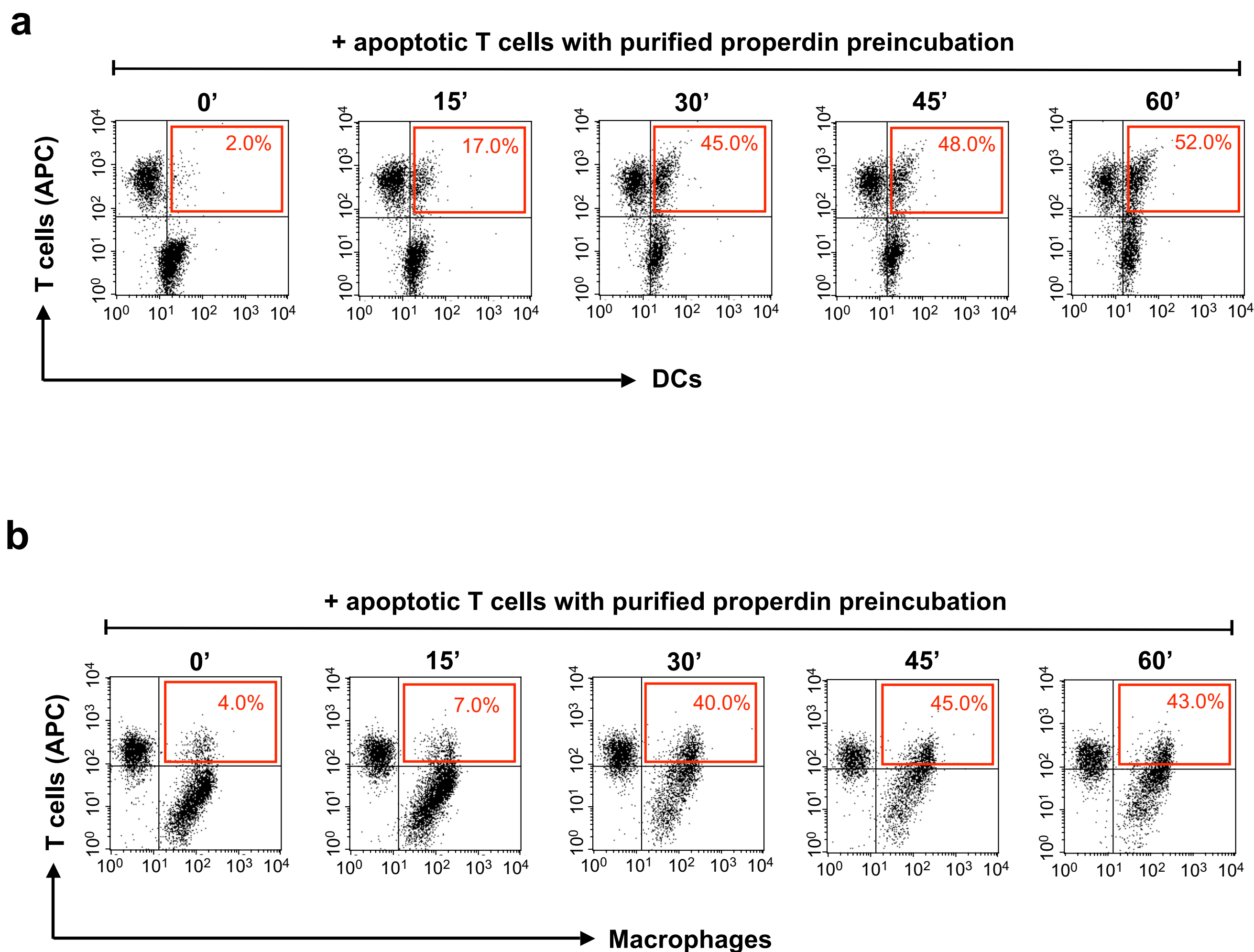


Fig. S2. Properdin-mediated uptake of apoptotic T cells by macrophages and dendritic cells (DCs) reaches maximal levels between 15 and 30 min of cell incubation/interaction. (*a*) Time course of properdin-mediated uptake of apoptotic T cells by DCs. DCs were grown to confluence in 24-well plates and labeled with CFSE. Apoptotic T cells were labeled with an APC-conjugated anti-CD4 mAb and either incubated with 5 μg/ml purified properdin and then washed (as per *Methods*) or left untreated. T cells were added to DCs at 0.5–1 $\times$ 10^6 per well in prewarmed RPMI medium 1640, and cell mixtures were incubated. At indicated time points, unbound/noningested T cells were aspirated and DCs were detached. The percentage of CFSE/APC-positive DCs (CFSE-labeled DCs that ingested apoptotic APC-labeled T cells during the incubation period) was determined by FACS analysis and is shown within the boxes. (*b*) Time course of properdin-mediated uptake of apoptotic T cells by macrophages. Experiments were performed as in *a* but with macrophages in place of DCs. Shown is a representative FACS analysis of four similarly performed experiments.

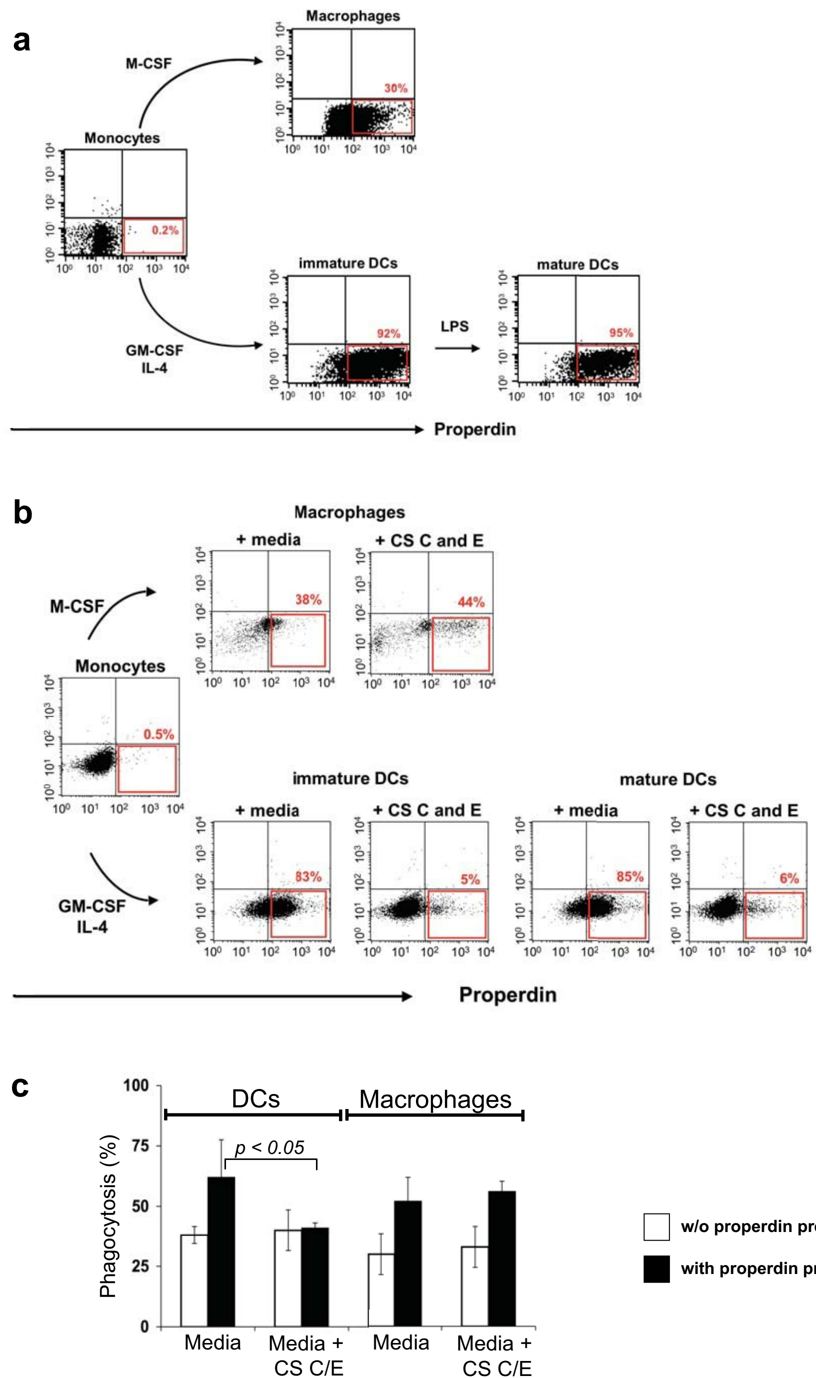


Fig. S3. Macrophages, immature DCs, and mature DCs bind properdin. (*a*) Maturation of monocytes into DCs or macrophages confers the capacity to bind properdin. Macrophages and immature and mature DCs were generated from monocytes as per *Methods* and incubated with purified properdin (10 μg/ml in RPMI medium 1640) for 15 min at 37°C. Bound properdin was measured by FACS analysis. Numbers adjacent to boxes indicate the percentage of properdin-positive cells within a given population. (*b*) Properdin binding to DCs but not to macrophages is inhibited by GAGs. Experiments were performed as in *a* but with preincubation of cells with a mixture of 20 μg/ml of each CS-C (GlcA-GalNAc6S) and CS-E (GlcA-GalNAc4, 6S) in media for 10 min before the addition of 10 μg/ml purified properdin. Preincubating cells with 20 μg/ml either CS-C or CS-E alone resulted in similar data (data not shown). Quadrant borders in the FACS plots were set based on the isotype control staining (data not shown). Shown is one representative result of four similarly performed experiments. (*c*) GAGs inhibit properdin-mediated ingestion of apoptotic T cells by DCs but not by macrophages. Apoptotic T cells were either left untreated or preincubated with 5 μg/ml purified properdin. After washing, T cells were incubated with DCs or macrophages in the presence or absence of CS-C and CS-E (20 μg/ml each) in media for 60 min at 37°C, and the percentage of ingested apoptotic T cells was determined by quantitative FACS analysis (see *Methods*). Shown is the mean ± SD of two independently performed experiments. Statistical significance between samples was determined by the Student *t* test.

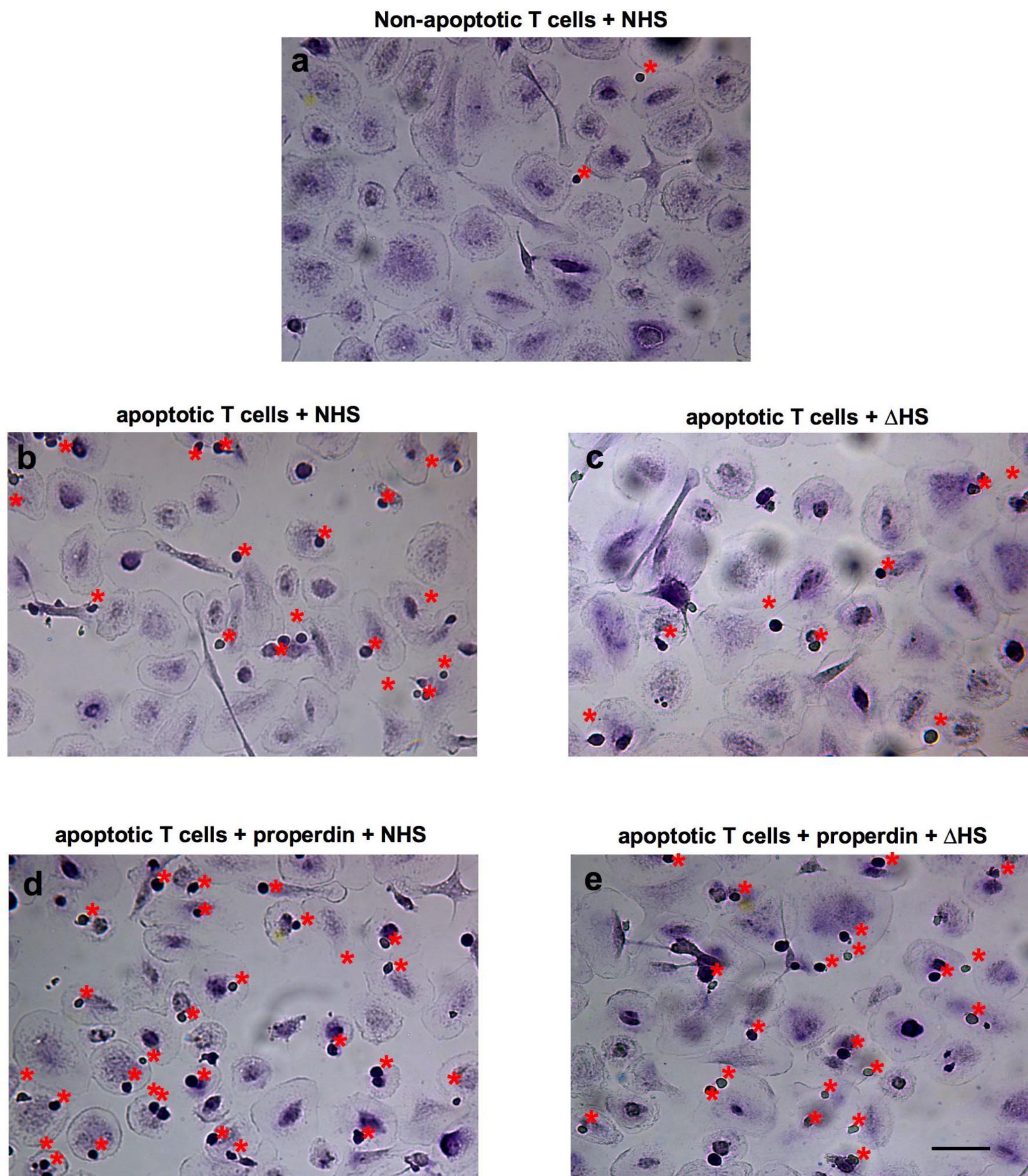


Fig. S4. Properdin bound to apoptotic T cells promotes their phagocytosis by macrophages. Human macrophages were generated and grown to confluence on chamber slides (see *SI Methods*). Apoptotic T cells were generated and either preincubated with 5 μg/ml purified properdin (*d* and *e*) or left untreated (*a*–*c*). T cells were washed and then added to the macrophages in media containing 15% of normal human serum (NHS) or heat-inactivated human serum (ΔHS). Slides with cell mixtures were incubated for 1 h, washed, stained with hematoxylin, fixed, and analyzed microscopically. As seen, macrophages ingested apoptotic T cells more readily than nonapoptotic control cells (compare *a* and *b*), and the uptake of apoptotic T cells by macrophages was increased by $\approx$100 $\pm$ 30% in the presence of normal human serum (compare *b* and *c*). Uptake was greatest when apoptotic T cells were first pretreated with properdin (compare *b* and *d*). As observed in the FACS experiments, pretreatment of apoptotic T cells with properdin alone increased their ingestion (80 $\pm$ 25%) even in the absence of complement activation substantially (compare *c* and *e*). (Original magnification: $\times$100. Scale bar: 50 μm.) Results shown are representative of four independent experiments. A red asterisk indicates ingested T cells.

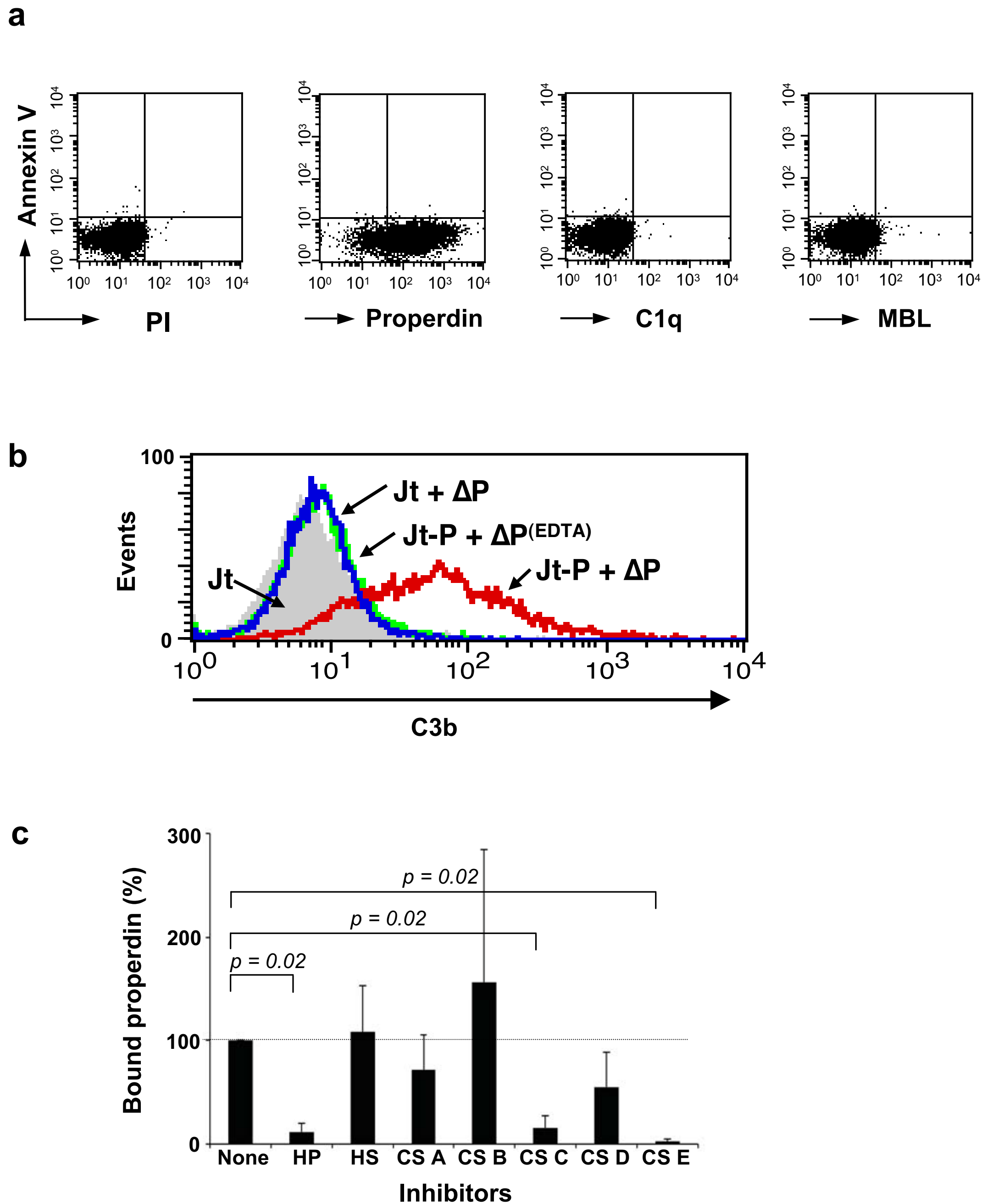


Fig. S5. Properdin recognizes malignant $CD4^+$ T cells via GAGs. (*a*) Properdin binds to nonapoptotic Jurkat T cells. Nonmanipulated Jurkat T cells (clone E6.2 from American Type Culture Collection) were incubated for 15 min with purified properdin, C1q, or MBL (each at 5 μg/ml concentration) in media at 37°C. Binding of these proteins to Jurkat T cells was assessed by FACS analysis using the appropriate mAbs. Shown is one representative result of four similarly performed experiments. (*b*) Properdin binding to nonapoptotic Jurkat T cells initiates C3b deposition. Jurkat T cells were either left untreated (Jt) or preincubated with purified properdin (10 μg/ml) for 15 min at 37°C (Jt-P). Cells were washed and then incubated for 15 min with 15% properdin-depleted (ΔP) serum diluted in Mg^{2+} EGTA buffer or EDTA buffer. Deposition of C3b on apoptotic T cells was measured by FACS analysis. Results shown are representative of three independent experiments. Pretreatment of cells with properdin followed by incubation in $ΔP/Mg^{2+}$ EGTA buffer led to 23-fold greater deposition than for cells pretreated in buffer alone and 18-fold greater deposition than for properdin-pretreated cells incubated in ΔP/EDTA buffer. (*c*) Properdin binds to Jurkat T cells via GAGs. Nonmanipulated Jurkat T cells were preincubated with heparin, heparan sulfate, or the indicated chondroitin sulfates (10 μg/ml buffer/media) for 10 min. Purified properdin (5 μg/ml) was then added to all samples, and properdin deposition on the Jurkat T cell surface was assessed. Heparin (HP; a highly sulfated GAG composed predominantly by alternating IdoA2S-GlnNS6S), heparan sulfate (HS; an undersulfated form of heparin), chondroitin sulfate-A (CS-A; GlcA-GalNAc4S), CS-B (dermatan sulfate; IdoA-GalNAc), CS-C (GlcA-GalNAc6S), CS-D (GlcA2S-GalNAc6S), and CS-E (GlcA-GalNAc4, 6S). Statistical significance between samples was determined by the paired Student *t* test.

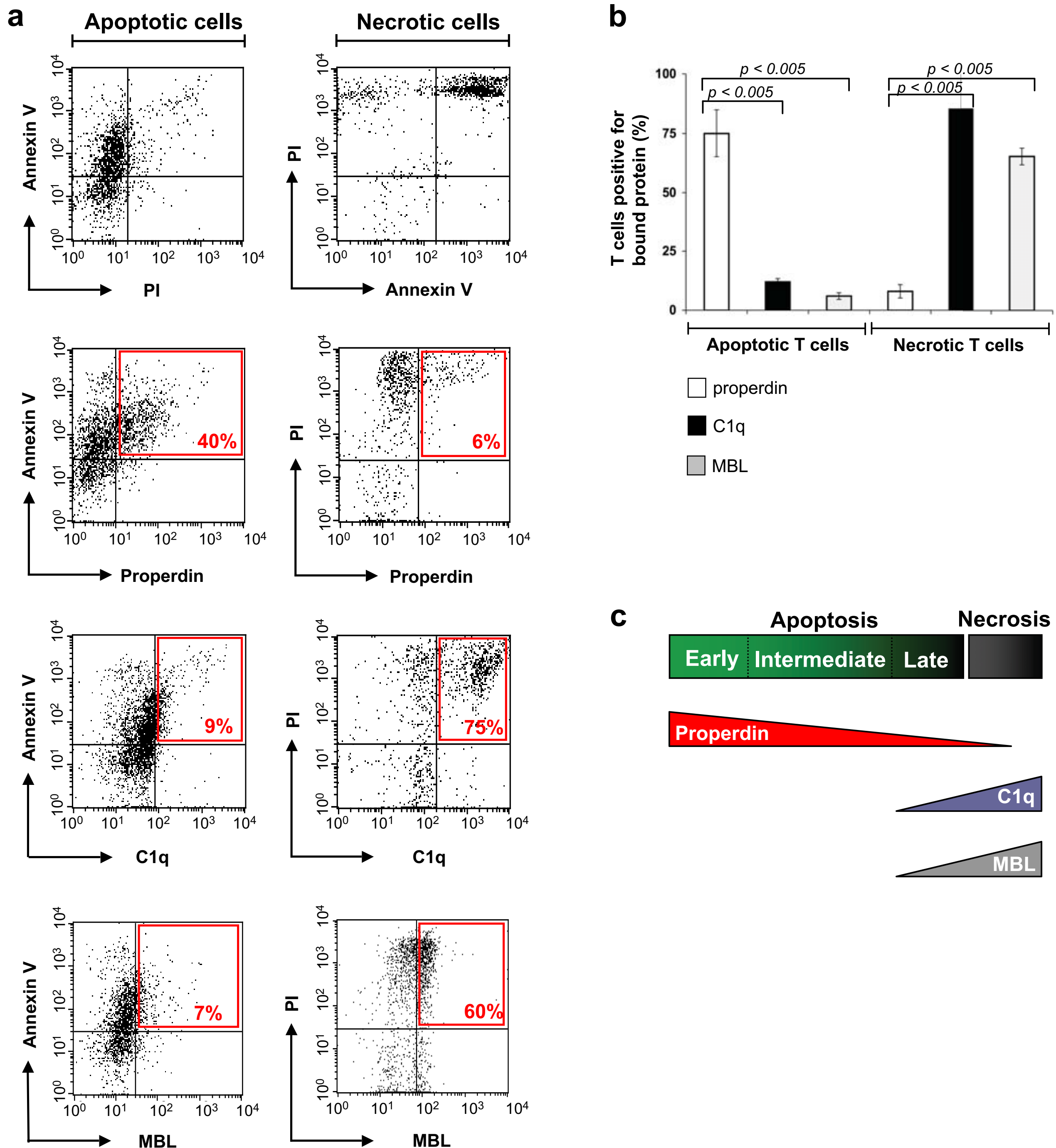


Fig. S6. Properdin, C1q, and MBL recognize distinct target cell populations. (*a*) Purified CD4$^+$ T cells were activated for 3 days. Apoptosis was induced through addition of 10 μg/ml anti-Fas mAbs to the culture (*Left*; cells are annexin V$^+$/PI$^-$), necrosis through addition of H_2O_2 (0.01 M) for 4 h (*Right*; cells are annexin V$^+$/PI$^+$). Apoptotic and necrotic cells were incubated with 5 μg/ml of purified properdin, C1q, or MBL for 15 min at 37°C. Binding of these proteins to the two distinct T cell populations was measured by FACS analysis using the appropriate mAbs. Shown is one representative result of three similarly performed experiments. (*b*) Statistical analysis of properdin, C1q, and MBL binding to either apoptotic or necrotic T cells (shown as mean ± SD) of three separate experiments. Statistical difference between the binding of properdin and C1q and properdin and MBL to either apoptotic or necrotic T cells was $P < 0.005$ in all cases as determined by the paired Student *t* test. (*c*) Summary/model of the contributions of the three complement activation pathways in the recognition of human apoptotic and necrotic primary CD4$^+$ T cells.