CD46 Plays a Key Role in Tailoring Innate Immune Recognition of Apoptotic and Necrotic Cells*

Kristina Elward†, Mark Griffiths§, Masashi Mizuno¶, Claire L. Harris†, Jim W. Neal†, B. Paul Morgan†, and Philippe Gasque†

From the †Brain Inflammation and Immunity Group (BIPG) and §Complement Biology Group, Department of Medical Biochemistry and Immunology, ‡Neuropathology Laboratory, Department of Pathology, School of Medicine, Heath Park Campus, Cardiff University, Cardiff CF14 4XN, United Kingdom

Complement is the canonical innate immune system involved in host defense and tissue repair with the clearance of cell debris. In contrast to the robust armory mounted against microbial nonself-pathogens, complement is selectively activated on altered self (i.e. apoptotic and necrotic cells) to instruct the safe demise by poorly characterized mechanisms. Our data shed new light on the role of complement C1q in sensing nucleic acids (NA) rapidly exposed on apoptotic Jurkat T cell membranes and in C3 opsonization but without the lytic membrane attack complex. DNA/RNase-treated apoptotic cells failed to activate complement. We found that several other apoptotic cell models, including senescent keratinocytes, ionophore-treated sperm cells, and CMK-derived platelets, stained for cleaved caspase 3 were rapidly losing the key complement regulator CD46. CD46 from nuclear and membrane stores was found to cluster into blebs and shed into microparticles together with NA, phosphatidylserine, C1q, and factor H. Classical and alternative pathways of complement were involved in the recognition of H$_2$O$_2$-treated necrotic cells. Membrane attack complex was detected on necrotic cells possibly as a result of CD46 and CD59 shedding into soluble forms. Our data highlight a novel and universal paradigm whereby the complement innate immune system is using two synergistic strategies with the recognition of altered self-NA and missing self-CD46 signals to instruct and tailor the efficient removal of apoptotic and necrotic cells in immunoprivileged sites.

In analogy to the specificity of the adaptive immune response, innate immunity is extremely selective and has divided the universe into innocuous “self” and potentially noxious (danger) substances ranging from toxic cell debris (“altered self,” e.g. apoptotic cells, necrotic cells, amyloid fibrils, and prion infectious agent) to nonself-microorganisms.

Hence, selective innate immune recognition is established essentially according to particular molecular patterns (i.e. apoptotic cell-associated molecular patterns (ACAMPs)), pathogenic protein-associated molecular patterns (PPAMPs), and pathogen-associated molecular patterns (PAMPs) (1–4). Ultimately, “professional” and “amateur” phagocytes recognize ACAMP/PPAMP/PAMPs “eat me” signals through specific soluble and membrane-bound pattern-recognition receptors (e.g. complement collectins and phagocytic receptors), which lead to clearance of the different target cells (4–7).

The complement system is the canonical innate immune system capable of recognizing a plethora of PAMPs, PPAMPs, and ACAMPs essentially through mannan-binding lectin (MBL) and C1q to initiate the activation of the complement cascade (7–9). Historically, C1q was first shown to bind to antigen-antibody complexes, but it is increasingly evident that C1q multimeric structure is key to the selective recognition of apoptotic cells, toxic amyloid fibrils, and the pathogenic prion agent by mechanisms that remain poorly understood (10–18). These events will lead to the opsonization of the target with C1q, C4, and C3 opsonins recognized by macrophages bearing complement pattern-recognition receptors such as CR1, CR3, and CR4 (where CR indicates complement receptor) (7, 19, 20).

Furthermore, innate immune recognition is based also on recognition of molecular markers specific for self to prevent uncontrolled phagocytosis (3). These markers are gene products expressed only on the surface of normal uninfected cells of the host but not on microbial cells. They are lost from the membrane of infected cells or abnormal cells such as senescent, apoptotic, necrotic, and transformed cells. Recognition of these “don’t eat me” signals (also known as self-associated molecular patterns (SAMPs)) (4, 7) by different soluble and membrane-bound pattern-recognition receptors is coupled to the inhibition of the innate immune response (3, 21). The best characterized example of the marker of self is the recognition of the major histocompatibility complex class I molecules by various inhibitory receptors expressed on natural killer (NK) cells (22). As major histocompatibility complex I is constitutively expressed on all cells (apart from erythrocytes) and is often down-regulated as a result of viral infection or cellular transformation, recognition by NK cells of a missing self-ligand allows them to selectively eliminate infected and transformed cells and spare normal, healthy self-cells.

The missing self-strategy is not unique to NK cell function and is widely used by the innate immune system (for review see Refs. 3, 4, 7, and 21). CD47, CD200, and sialic acids have been recognized recently as classical SAMPs involved in the control of phagocytosis by macrophages (23–28). Another well known example is the regulation of the complement system by membrane-bound complement regulatory proteins (CRregs, e.g. CD46, CD55, and CD59) (for comprehensive review see Refs. 29–31). As cells of “nonself” origin, including pathogens, lack these host gene products, activation of the complement cascade can proceed uninhibited resulting in lysis or phagocytosis of the target cells.

Uniquely, complement plays a critical role in the scavenging of toxic cell debris for safe and efficient removal by phagocytes before...
engaging the pro-inflammatory response (mediated by C5a) and secondary necrosis (because of the membrane attack complex (MAC)). Neighboring cells can express a full complement system on demand (32), and C1q will flag target cells through its capacity to bind to apoptotic blebs and necrotic cells. The mechanisms for selective C1q binding to abnormal cells remain poorly characterized, although natural IgM antibodies have been shown to be required for C1q-dependent complement opsonization of late apoptotic/necrotic cells (33–35). Of note, in immunoprivileged sites such as the testis and brain, which lack an adaptive immune response (hence IgM), it is plausible that other signals are involved to initiate the activation of the complement classical pathway (36).

We recently argued that the expression of CRegs could be modulated when the cells undergo programmed cell death (4). It has already been reported that CRegs are reduced on dying cells (37–39); however, it is unknown how CRegs are lost from apoptotic/necrotic cells and to what extent the loss of one or more CRegs provide key signals to promote the recognition and clearance of these dying cells and to ensure the anti-inflammatory/pro-inflammatory responses, respectively.

This study describes for the first time the molecular mechanisms and routes involved in the recognition and decoding of apoptotic and necrotic cells by the canonical innate immune complement system. Unique knowledge of these pathways will be discussed in the context of physiological and pathological settings.

MATERIALS AND METHODS

**Cells and Reagents**—Human Jurkat E6 T lymphocyte cell line obtained from the European Collection of Cell Cultures (ECACC) (Porton Down, Salisbury, UK) was cultured at 37°C, 5% CO₂ in RPMI 1640 containing 5% heat-inactivated fetal bovine serum, penicillin/streptomycin (50 μg/ml), L-glutamine (2 mg/ml), sodium pyruvate (1 mg/ml), and fungizone (2.5 μg/ml) (Invitrogen) at a density of 1 × 10⁶/ml in 75-cm² tissue culture flasks (Greiner Bio-One Ltd., Stonehouse, Gloucestershire, UK). Human cell lines originally sourced from the ECACC were cultured in the laboratory as described previously (40) (epithelial cells: HeLa, MCF7, ECV304, SW13, HepG2, and A549; glial cells: T98G; myeloid cells: THP1, HL60, and CMK). The human oligodendrocyte cell line (HOG) was from Dr. G. Dawson (Department of Biochemistry, University of Chicago). HMC1 mast cell line and the EAHy929 endothelial cell line were kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) and Dr. C. J. S. Edgell (University of North Carolina), respectively. Primary cultures of human keratinocytes and serum-free media were obtained from Lonza (RTM), for 1 h at room temperature. Heat inactivation of NHS was carried out at 56–60°C for 30 min. In some experiments, apoptotic or necrotic cells were treated with a mixture of DNHase I/RNase (Sigma, 10 μg/ml in RPMI, 30 min at 37°C) before incubation with NHS. Anti-complement antibodies were either from commercial sources (see TABLE ONE) or were kindly provided by Dr. M. Fontaine (INSERM U519, Rouen, France), Dr. P. W Taylor (CIBA Geigy Pharmaceuticals, Sussex, UK), and Professor O. Gotze (University of Gottingen, Germany). Cells were analyzed by dual fluorescence flow cytometry and by microscopy analysis essentially as described (40).

**Expression Profile of Complement Regulators and Other Surface Markers on Apoptotic/Necrotic Cells**—In order to define complement regulators (CD46, CD55, and CD59) and other surface markers (i.e. annexin I and CD43) with a modulated expression during the course of apoptosis/necrosis, the profile of surface molecule expression was assessed by flow cytometry, essentially as described (40). In some experiments, Jurkat cells were incubated with a selective inhibitor of the Src family of protein-tyrosine kinases (4-amino-5-(4-chlorophenyl)-7-[(t-butyl)pyrazololo][3,4-d] pyrimidine, 1 μM), for 15 min prior to apoptosis induction. Casein kinase II inhibitor (TBBT) and protein kinase C inhibitor (Myr-N-FARKGALRQ) were from Calbiochem and were used at 10 μM for 1 h. Mouse antibodies against human CD59 (clone BRIC 229G2) and human DAF (CD55, clone BRIC 216G1) were from the International Blood Group Reference Laboratory (Bristol, UK). We used different mouse monoclonal antibodies against human CD46 as follows: clone 14.48G1 against the SCR1 domain from Serotec (Oxford, UK); clone Tra 2.1G1 against SCR1; clone GB24G1 against SCR3/4 (from Prof. J. Atkinson, Washington University, St. Louis, MO); clone MEM258G1 (from Dr. V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic); or clone 11C5G1 (from Dr. E. Rubinstein, INSERM U268, Paris, France) (44). The rabbit polyclonal anti-human CD46 and anti-human CD55 were produced in-house. The mouse anti-CD47 (clone BRIC 126G2) was from International Blood Group Reference Laboratory. Mouse monoclonal anti-CD43 and anti-annexin I antibodies were from Pharmingen.
Preparation of Lysates from Total Cells and Nuclear, Cytoplasmic, and Bleb-enriched Fractions Using Normal, Apoptotic, and Necrotic Jurkat—Cell lysates of normal, apoptotic, and necrotic Jurkat cells were prepared from 1 × 10^7 cells using lysis buffer (500 µl of 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 µg/ml pepstatin A, 1 µg/ml leupeptin in phosphate-buffered saline). Nuclear and cytoplasmic fractions were prepared according to the manufacturer’s instructions (NE-PER, 78833 kit from Pierce). The fetal calf serum-free medium bathing treated cells (10 ml) was dialyzed, freeze-dried, and resolubilized in Laemmli loading buffer (500 µl in total) to detect the soluble and vesicle forms of proteins shed from the cells. To isolate specifically microparticles released by apoptotic/necrotic cells, 10 ml of culture supernatants were collected from the initial spin at 2000 rpm for 10 min and ultracentrifuged (100,000 g, 1 h, room temperature) in a Beckman TL-100 ultracentrifuge with a TLA 100.3 rotor. Bleb/vesicle-enriched pellets were finally resolubilized in Laemmli loading buffer (500 µl in total) (45). The remaining 10 ml of supernatant containing soluble proteins was dialyzed overnight in phosphate-buffered saline, freeze-dried, and resuspended in 500 µl of Laemmli loading buffer. Samples were separated on SDS-PAGE, and Western blotting was performed using rabbit polyclonal antibodies against CD55, CD46, cleaved caspase 3, and cleaved PARP. Precast gels (4–20% acrylamide) and prestained protein markers were from Invitrogen. Double immunoblots were also performed using mouse anti-CD46 (MEM258, V. Horejsi), mouse anti-CD43 (MEM59 V. Horejsi), and mouse anti-CD16 (MEM65 V. Horejsi). The mouse anti-B2-tubulin was used to test for equal protein loading (Sigma).

| Treatment | Camptothecin-treated cells | H_2O_2-treated cells |
|-----------|-----------------------------|-----------------------|
| Trypan blue | Negative | ~90–95 |
| PI | Weak (mem) | ~40–50 |
| Annexin V-FITC | Strong (mem) | ~30–35 |

| Immunostainings | Camptothecin (mean FL2) | H_2O_2 (mean FL2) |
|-----------------|-------------------------|--------------------|
| Mo α-C1q (12A5B7, ATCC) | 4.3 | 19.7 |
| Rb α-C1q (Dako) | 37 | 183.5 |
| Gt α-C1q (Calbiochem) | 47.3 | 605.9 |
| Gt α-C4 (ATAB, Dr. M. Fontaine) | 104.3 | 1031.8 |
| Gt α-C4 (ICL, Dr. M. Fontaine) | 71.2 | 1187.8 |
| Rb α-C2 (Nordic) | 25.6 | 251.3 |
| Gt α-C2 (Calbiochem) | 73.4 | 371.2 |
| Mo α-C3a H1C3 (Dr. M. Fontaine) | 26.6 | 183.8 |
| Rb α-C3c (L440, Dr. M. Fontaine) | 17.13 | 230.7 |
| Gt α-C3 (Calbiochem) | 11.7 | 1297.9 |
| Mo α-C3b neo (C3/30, Dr. Taylor) | 62.8 | 1345 |
| Mo α-iC3b neo (Quidel) | 72.4 | 1030 |
| Mo α-C3d (BGR111, IBGRL) | 4.30 | 56.9 |
| Rb α-C3d (Dako) | 25.50 | 712.6 |
| Rb α-C5 (Dr. M. Fontaine) | 2.31 | 56.6 |
| Sh α-C9 (binding site) | 3.6 | 158.7 |
| Mo α-C9 neo (B7, in-house) | 12.1 | 42.5 |
| Rb α-Bb (Prof. O. Gotze) | 5.5 | 149.1 |
| Rb α-IH (L740, Dr. M. Fontaine) | 26.5 | 81 |
**Statistical Analysis**—Statistical analyses were performed using Sigmaplot and Sigmastat software package. Differences between parameters were analyzed using unpaired Student’s t tests. The p values were considered statistically significant at p less than 0.05.

**RESULTS**

NA Exposure Precedes Phosphatidylserine (PS) “Flip-flop” on Apoptotic Cells—First, Jurkat T lymphocytes were induced to undergo apoptosis or necrosis by chemical treatments using either camptothecin, an inhibitor of topoisomerase I, or the oxidant H2O2, respectively. Second, three more apoptotic triggers were tested by exposure of Jurkat to anti-Fas IgM antibody, ionizing γ-irradiation or UV light treatment. The 4-h time point was selected to give ~30% of apoptotic cells with intact plasma membranes. These cells were weakly positive for PI (membrane staining) and excluded trypan blue (TABLE ONE), whereas the necrotic cells were membrane-permeable and strongly stained with trypan blue and PIbright (TABLE ONE). When the camptothecin-treated cells were analyzed by FACS, we clearly identified three distinct cell populations as follows: viable cells, cells stained with PI but not with annexin V (called apoptotic I), and cells stained with PI and annexin V (apoptotic II cells).

**FIGURE 1.** Tailoring complement activation on apoptotic and necrotic cells. A, P, and annexin V-FITC cell scatter histograms of camptothecin-treated cells clearly depict the presence of the following: 1) viable cells; 2) a subset of very early apoptotic cells (apoptotic I) expressing nucleic acid at the cell surface (PI dim); and 3) a subset of apoptotic cells (apoptotic II) with nucleic acid and phosphatidylserine exposure at the cell surface (PI dim and AnV dim). Necrotic cells were strongly stained with PI (nuclear staining) and annexin V-FITC. B, camptothecin-treated cells (4 h) were seeded onto glass slides, fixed with acetone, and stained with DAPI. Apoptotic cells (Ap) with condensed and fragmented nuclei were also staining for DAPI (†). Magnification ×1000. C, camptothecin-treated Jurkat cells incubated with NHS (1:4, 15 min) were stained for C1q (rabbit) or double-stained for C3b (mouse) and C2 (rabbit) before being spotted onto glass slides. Viable cells with intact nuclei failed to stained for C1q, C3b, and C2 (data not shown).

**D,** Jurkat cells were either untreated (control) or induced to undergo apoptosis (4 h, 5 μg/ml camptothecin) and necrosis (4 h, H2O22), then incubated with 25% NHS, and analyzed by two-color flow cytometry for complement opsonization and annexin V FITC. Data are from one experiment and with reproducible findings (n = 4).
CD46 Controls Innate Immune Recognition of Apoptotic Cells

The key hallmarks of cell death and the overall capacity to activate and control the complement system were ascertained in two models of apoptosis (camptothecin and UV-irradiated cells) and were compared with one model of necrosis (H$_2$O$_2$-treated cells). Cells were either untreated (control) or treated for various times (30 min to overnight (O/N)). Immunostainings for complement activation products (C1q, C3bneo, and MACneo), complement regulatory proteins (CD46, CD55, and CD59), and other cell surface markers (CD43, CD47, and annexin I) were carried out as described in TABLE ONE (n = 3 for camptothecin and H$_2$O$_2$ experiments; n = 2 for UV-treated cells). Data are expressed as mean of FL2 intensity.

| Table ONE | Camptothecin, inhibitor topoisomerase I, -treated cells |
|-----------|------------------------------------------------------|
| **Time course** | Co | 30min | 1h | 2h | 4h | 8h | O/N |
| PI $^{high}$ cells % | - | 12 | 20 | 24 | 45 | 42 | - |
| PI $^{low}$ cells % | - | <1 | <1 | <1 | <2 | 15 | >95 |
| PS $^{high}$ cells % | - | - | - | 5 | 35 | 60 | >95 |
| Mo cC3b neo | - | 26 | 48 | 57 | 62 | 123 | 172 |
| Mo cC5b neo | - | 20 | 15 | 34 | 42 | 185 | 314 | 760 |
| Annexin I | - | 5 | 10 | 18 | 367 |
| CD46 | 110 | 110 | 95 | 87 | 42 | 35 | 32 |
| CD55 | 68 | 61 | 58 | 58 | 59 | 55 | 46 |
| CD59 | 364 | 354 | 345 | 360 | 325 | 329 | 330 |
| CD43 | 90 | 84 | 54 | 16 | 7 | 4 | 3 |
| CD47 | 479 | 411 | 459 | 489 | 511 | 554 | 527 |
| Annexin I | <5 | <5 | 8 | 10 | 37 | 56 | 111 |

| Table TWO | Uv-treated cells |
|-----------|------------------------------------------------------|
| **Time course** | Co | 30min | 1h | 2h | 4h | 8h | O/N |
| PI $^{high}$ cells % | - | 5 | 23 | 54 | 50 | 70 | - |
| PI $^{low}$ cells % | - | <1 | <1 | <1 | <5 | 13 | >95 |
| PS $^{high}$ cells % | - | - | 5 | 63 | 90 | >95 |
| Mo cC3b neo | - | 13 | 25 | 37 | 47 | nd | nd |
| Mo cC5b neo | - | 20 | 37 | 40 | 221 | nd | nd |
| Mo cMAC neo | - | 24 | 62 | 11 | 52 | 11 | 17 |
| CD46 | 127 | 114 | 106 | 99 | 27 | 126 |
| CD55 | 72 | 67 | 65 | 68 | 60 | 56 | 48 |
| CD59 | 360 | 325 | 314 | 310 | 304 | 355 | 340 |
| CD43 | 139 | 132 | 82 | 41 | 35 | 9 | 5 |
| CD47 | 369 | 419 | 442 | 465 | 478 | 480 | 475 |
| Annexin I | <5 | <5 | 12 | 23 | 57 | 44 | 77 |

| Table THREE | Oxidative Necrosis: H$_2$O$_2$ treated cells |
|--------------|------------------------------------------------------|
| **Time course** | Co | 30min | 1h | 2h | 4h | 8h | O/N |
| PI $^{high}$ cells % | - | - | <10 | - | - | - |
| PI $^{low}$ cells % | - | 21 | 62 | 76 | 82 | >95 | >95 |
| PS $^{high}$ cells % | - | 19 | 64 | 65 | 76 | >95 | >95 |
| Mo cC3b neo | - | 45 | 234 | 560 | 754 | nd | nd |
| Mo cC5b neo | - | 200 | 347 | 871 | 1560 | nd | nd |
| Mo cMAC neo | - | 34 | 30 | 45 | 87 | 121 | 232 |
| CD46 | 101 | 86 | 47 | 28 | 22 | 25 | 21 |
| CD55 | 71 | 72 | 70 | 60 | 48 | 45 | 34 |
| CD59 | 362 | 321 | 305 | 287 | 240 | 210 | 167 |
| CD43 | 84 | 84 | 16 | 7 | 4 | 4 | 4 |
| CD47 | 355 | 368 | 417 | 492 | 554 | 569 | 420 |
| Annexin I | 9 | 16 | 25 | 44 | 120 | 280 | 9 |

The exposure of NA in the outer leaflet of the plasma membrane promotes C1q binding and drives complement activation on apoptotic Jurkat cells; FACS analysis

Camptothecin-treated Jurkat cells (4 h) were incubated in either RPMI alone or RPMI with 10 μg/ml DNase I and 10 μg/ml RNase (30 min at 37 °C, Sigma). 90–95% of the cells excluded trypan blue (data not shown) and confirmed membrane integrity. Nucleic acid exposure was determined by propidium iodide (PI) staining, and the level of complement opsonization (C1q and C3b staining) was performed as described (TABLE ONE) followed by FACS analysis. Data are expressed as mean of FL2 ± S.D. The p value from the t test was calculated using Sigmaplot software (n = 4).

Innate Immune Recognition and Tailoring of Complement Activation on Altered Self (Apoptotic Cells Versus Necrotic Cells) While Preserving Normal Self-cells—Complement activation was tested by incubation of treated cells with NHS, and the level of complement proteins on target cells was analyzed by double microscopy and FACS analysis using several anti-complement antibodies and annexin V FITC (Fig. 1, C and D, and TABLES ONE and TWO). Control cells failed to activate the complement system when exposed to NHS for 30–60 min (Fig. 1, C and D, and TABLE TWO). Immunofluorescent microscopy further indicated that complement C1q opsonized at the surface of apoptotic cells but not viable cells (Fig. 1C) was capping in vesicle-like structures blebbing out from the cell surface (Fig. 1C, arrowhead) together with C3b and C2. We found that C1q staining was most prominent when cells were incubated only for 10–15 min with NHS. Complement activation was taking place through the classical pathway as confirmed by the lack of staining when we used EDTA serum and C1q-depleted serum (data not shown). Hence, we found prominent staining of annexin V-positive cells for C1q, C3b, and C3b following camptothecin treatment, whereas the monoclonal anti-C5b9 neoepitope did not detect the MAC on apoptotic cells. This is in sharp contrast to necrotic cells that displayed strong staining for complement opsonins and MAC (Fig. 1D, and TABLES ONE and TWO). FACS analysis using different monoclonal and polyclonal antibodies confirmed that the classical pathway was the main route of complement activation on cells undergoing apoptosis. Similar results were obtained when Jurkat cells were induced to undergo apoptosis by treatment with anti-Fas antibody, UV treatment, or following γ-irradiation (TABLE TWO and data not shown). Although necrotic cells bound C1q, activation of the alternative pathway by necrotic cells was also observed, with only a weak Bb staining on apoptotic cells compared with strong staining on necrotic cells (TABLE ONE, Part b). The Exposure of NA in the Outer Leaflet of the Plasma Membrane Drives Complement Activation on Apoptotic Cells—The C1q staining clearly identified two populations of camptothecin-treated cells double-stained or not for PS exposure (Fig. 1D). Most interestingly, this pattern was reminiscent of the PI/annexin V staining pattern (Fig. 1A). We hypothesized that NA exposure at the cell membrane may contribute to the initiation of the classical pathway given that DNA has long been known as a C1q-binding molecule (46). To test this, we compared the level of C1q binding and C3 opsonization prior to and after DNase/RNase treatment of camptothecin-treated Jurkat cells. Treated cells remained viable and displayed markedly reduced levels of PI staining at the cell membrane (TABLE THREE). The removal of NA dramatically affected the activation of the classical pathway on apoptotic cells with reduced C1q binding and C3b levels at the cell surface (p < 0.005). In contrast, only a 15–20% reduction of C1q binding was observed when necrotic cells were treated with DNase/RNase (data not shown), suggesting that other mechanisms (e.g. cardiolipin exposed on mitochondria) are driving C1q binding and complement activation on necrotic cells (47).

Apopotic and Necrotic Cells Control Differentially the Complement System—Previous studies have reported a reduction in the expression of CRegs in response to apoptosis (37–39). To confirm these findings and
FIGURE 2. Expression of complement regulatory proteins on apoptotic versus necrotic Jurkat T cells: dramatic loss of CD46 on apoptotic cells is caspase-dependent but Src kinase-independent. A, control cells (thin line) or cells treated for 4 h with camptothecin (filled histogram) and H2O2 (solid line) were stained for the membrane complement regulators (CD46, CD55, and CD59) and other cytoplasmic/cell surface proteins (annexin I, CD43, and CD47) and analyzed by single flow cytometry using mouse anti-CD antigens and R-phycocerythin-conjugated goat antimouse antibodies (FL2 channel). Of note, the cytoplasmic annexin I protein has been reported to translocate to the cell surface on apoptotic cells and was used as an internal control in our experiments (48). CD43 is a highly sialylated membrane protein that is known to be rapidly shed from apoptotic cells (49). B, the expression of CD46, CD55, and CD59 was also analyzed when Jurkat cells were exposed to different apoptotic paradigms using the anti-Fas antibody (4 h) or following γ-irradiation and 8 h in culture. Both treatments induced more than 40% annexin V-positive cells with intact cell membrane as confirmed by trypan blue exclusion (data not shown). C, Jurkat cells were incubated with either a general caspase (Z-VAD-FMK) or an Src kinase (PP2 (4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d] pyrimidine)) inhibitor (69) for 1 h prior to apoptosis induction (camptothecin or anti-Fas for 4 h). D, Western blot analysis of camptothecin-treated cell lysates following cell sorting in annexin V-negative and annexin V-positive cell fractions. Equivalent cell numbers were used, and we confirmed equal protein gel loading using the anti-tubulin antibody.
CD46 Controls Innate Immune Recognition of Apoptotic Cells

Ascertaining the contribution of CRegs to the selective regulation of complement on apoptotic cells and necrotic cells, flow cytometry analyses were performed using monoclonal antibodies to the key membrane CRegs, CD46, CD55, and CD59, alongside other antibodies against cytosolic and membrane proteins (annexin I, CD43, and CD47). Jurkat control cells (Fig. 2A) expressed high levels of all CRegs together with CD43 and CD47. The annexin I staining was used as an internal control and was detected at the cell surface only after apoptosis and necrosis as reported previously (48). We confirmed that CD43 was rapidly lost from apoptotic and necrotic cells with a distinct bimodal distribution observed on apoptotic induced cells (49). A high loss of CD46 expression was observed on camptothecin- and H2O2-treated cells, with a distinct bimodal distribution of expression apparent on apoptotic induced cells (49). We observed the distinct bimodal distribution of CD46 expression as assessed by FACS analysis with a population of decreased expression compared with normal in these three new models (Fig. 2B and TABLE TWO). Kinetics analyses indicated that CD46 was rapidly lost from the apoptotic cell surface as early as 2 h post-treatment (TABLE TWO). Moreover, we found that the rate of CD46 loss was identical whether the apoptotic cells were incubated in the presence or absence of complement, suggesting that C3b, the natural CD46 ligand, is not implicated in CD46 fate on apoptotic cells (data not shown).

The Decreased Expression of CD46 Correlates with Caspase-dependent Apoptotic Cell Death but Does Not Require Caspase 3 or CD46-mediated Protein Phosphorylation—To ascertain whether the loss of CD46 membrane antigen staining was restricted to apoptotic cells, dual fluorescence analysis was performed, whereby monoclonal antibody staining was followed by annexin V FITC and (R)-phycocerythrin-conjugated goat anti-mouse immunoglobulin (Fig. 2B). Camptothecin and αFas-treated Jurkat cells showed a clear population of annexin +ve cells with a remarkable 10-fold decreased expression of CD46. Most interestingly, the loss of CD46 from Jurkat cells was totally abrogated when cells were incubated with a broad caspase inhibitor (Z-VAD-FMK) prior to apoptosis induction (Fig. 2B). However, a caspase 3 inhibitor (N-acetyl-Glu-Ser-Met-Asp-al) failed to reduce significantly the loss of CD46 from the apoptotic Jurkat cells (data not shown), and this is in agreement with our findings that CD46 loss is still taking place in a caspase 3-deficient cell line, the MCF7 breast cancer cell line treated with UV light (TABLE FOUR) (42). Cytoplasmic tails of CD46 (CYT-1 and CYT-2) encode putative signals for phosphorylation by kinases, which play pivotal roles in CD46 trafficking with intracellular processing and basolateral localization. Inhibitors of kinases controlling the phosphorylation status of CD46 tails, i.e. 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine for Src kinases, TBBT for casein kinase II, and Myr-N-FARKGALRQ for protein kinase C, failed to control CD46 loss on apoptotic cells (data not shown).

To obtain pure cell populations of normal and apoptotic Jurkat, cells were sorted on the basis of expression of PI/annexin V staining using the

**TABLE FOUR**

| (MEAN of FL2) for: | CD46 | CD65 | CD59 | CD47 |
|-------------------|------|------|------|------|
| (Epithelial cells): |      |      |      |      |
| Hela cervix       | 643  | 161  | 1458 | 965  |
| MCF7 breast       | 429  | 193  | 68   | 58   |
| ECV304 bladder    | 88   | 34   | 68   | 67   |
| SW13 adrenal gland| 88   | 23   | 4.5  | 7    |
| HepG2 liver       | 147  | 59   | 64   | 51   |
| A549 lung         | 228  | 94   | 300  | 279  |

**Primary (ATCC) (Endothelial cells):**

| E4hy922 peas. 52 | 1866 | 675  | 1441 | 1290 | 8149 | 7990 |
|-------------------|------|------|------|------|------|------|
| HL60 neutrophils  | 203  | 54   | 109  | 94   | 217  | 234  |
| HMC1 mast cells   | 328  | 156  | 244  | 210  | 649  | 1011 |
| CMK megakaryoblast| 304  | 89   | 145  | 122  | 118  | 155  |
| T93G astrocyte    | 207  | 89   | 145  | 122  | 118  | 155  |
| HOG oligodendrocyte| 232 | 89   | 189  | 123  | 1375 | 1527 |

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36348 JOURNAL OF BIOLOGICAL CHEMISTRY

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cytomation MoFlo high speed cell sorter. Western blot analysis of the cell lysates indicated that total CD46 expression was reduced on apoptotic (annexin−ve) by a factor of 3–4-fold compared with normal (annexin+ve) cells, whereas CD55 levels remained the same (Fig. 2D).

Different Mechanisms Control the Loss of CD46 from Apoptotic Cells and Necrotic Cells—Several Western blot experiments were performed to clarify further the mechanism involved in the loss of Crreg expression by apoptotic versus necrotic cells. Treated Jurkat cells were prepared and analyzed by double Western blot immunostaining (Fig. 3, n = 4).

Analysis of total cell lysate preparations confirmed CD55 expression to be unaffected after induction of apoptosis and necrosis. CD59 expression was reduced only when cells were treated with H2O2, whereas CD46 expression showed a profound difference between control and treated cells, with a greater than 50% loss of expression from apoptotic and necrotic cell lysates (Fig. 3A). CD43 expression was confirmed to be dramatically reduced from apoptotic cells, whereas necrotic cells were largely CD43-free. Of note, the loss of this anti-adhesive molecule at the cell surface is thought to be involved as a signal for macrophage recognition and removal of the dying cell.

To shed further light on the mechanism of CD46 trafficking in apoptotic and necrotic cells, nuclear and cytoplasmic/membrane cell extracts were prepared using the NE-PER kit from Pierce. Western blotting was used to detect the presence of CD46 along with established markers of apoptosis (cleaved caspase 3neo and cleaved PARPneo), CD43, and lamin, a known nuclear component and substrate of activated caspase 3 (Fig. 3C).
experiments, we used the polyclonal and MEM258 monoclonal anti-CD46 antibodies with identical findings. CD46 was first shown to reside in the nuclear and cytoplasmic/membrane fractions in control cells. The validity of the extraction protocol was confirmed with the expected distribution of CD43 and lamin in the cytoplasmic/membrane and nuclear fractions, respectively. Remarkably, a dramatic loss of CD46 signal was evident from necrotic cells, although without involving the cleavage of PARP and caspase 3. In contrast, robust PARP and caspase 3 signals coincided with the loss of CD46 in the nuclear and cytoplasmic/membrane fractions of two different apoptotic cell models. As expected, apoptotic Jurkat cells displayed weak CD43 and lamin signals.

Identification of Soluble CD46 Shed from Necrotic Cells and CD46-enriched Microparticles Budding from Apoptotic Cells—To investigate the mechanism of release of CD46, supernatants from four separate experiments were collected, filtered using a 0.2-μm filter, and concentrated by lyophilization before being analyzed by Western blot (Fig. 4A). Most surprisingly, we found that control cells (incubated in fetal calf serum-free medium) released soluble (s) forms of CD46 and CD55 but not CD59. Moreover, necrotic cell supernatants were highly enriched for sCD46, sCD55, and sCD59. We were surprised not to find high levels of sCD46 in apoptotic cell supernatants compared with control cells, given the early indication by FACS and Western blot that CD46 was dramatically lost from apoptotic cells. We suspected that CD46 might be released from apoptotic cells in the form of microparticles as part of the membrane blebbing. Hence, we lyophilized the supernatants without further filtering and analyzed by Western blot. Kinetics studies clearly indicated a strong CD46 signal accumulating during the course of apoptosis. CD46 accumulating in the supernatants of apoptotic cells did not undergo proteolysis, although the accumulation of a band at 35 kDa was evident particularly in 8 h late and overnight samples (late apoptotic cells) (Fig. 4B). Most interestingly, CD46 was detected along with complement regulator factor H (fH) and CD55.

CD46 Is Present in Apoptotic Blebs Where PS and Complement Opsonins Are Prevalent before Being Released from Surface Membranes—Double immunofluorescent microscopy of normal and apoptotic unpermeabilized/unfixed Jurkat cells was performed using monoclonal anti-CD46 antibody and annexin V FITC. Fluorescent images confirmed that CD46 was evenly distributed (albeit distributed in small clusters) on control cells with no staining for exposed PS. In contrast, strong PS exposure as indicated by the annexin V staining was found to colocalize with CD46 staining (Fig. 5A). To confirm further that CD46 capping into PS-enriched blebs was shed from the surface of apoptotic cells, microparticles were isolated by ultracentrifugation and analyzed by Western blot. We confirmed that CD46 was reduced in apoptotic total cell lysates \( n = 2 \) and as a result of being shed in the form of microparticles from the apoptotic cells (Fig. 5B). When the apoptotic cells were incubated with NHS as a source of complement (for 1 h), we found a strong C1q signal associated with the microparticle preparation. To ascertain whether there was a correlation between complement activation and loss of cell surface CD46, dual fluorescence flow cytometry analysis for expression of CD46 and C3b was performed. The loss of CD46 revealed stronger C3b opsonization of cells when exposed to complement (as indicated by a monoclonal anti-C3b neo-epitope) (Fig. 5C).

The Loss of CD46 Is a Universal Hallmark of Caspase-dependent Cell Activation—Several more model cell lines were UV light-treated to ascertain whether the rapid loss of CD46 at the cell surface is a unique and universal feature of caspase-dependent cell activation. Without exception, we found a robust loss of CD46 staining on 13 human cell lines from different tissue sources (TABLE FOUR; \( n = 2 \)). The loss of CD46 correlated with the level of apoptosis as indicated by the annexin V staining analyzed by FACS and the PARP staining on fixed cells (data not shown).

To confirm the observation that clustering of CD46 into microparticles is a caspase-dependent mechanism engaged in dying cells and not an artifact observed for the human Jurkat T lymphocyte cell line, a number of cells were stained for CD46 and caspase 3 by immunofluorescent microscopy (Fig. 6). In Jurkat cells treated with camptothecin, we confirmed that CD46 expression was dramatically reduced in cells stained for cleaved caspase 3 and PARP (Fig. 6, a–f). Although viable cells displayed a strong uniformed CD46 staining (Fig. 6a, arrowhead), only a weak cytoplasmic staining was observed on apoptotic cells (Fig. 6d, arrowhead). The apoptotic machinery is also utilized for a wide variety of tasks during development and, for instance, during the “individualization” process of maturing male germ cells, removal of senescent keratinocytes, and the differentiation of megakaryocytes into platelets (50–52). First, human spermatozoa treated with the A23187 ionophore to induce acrosomal reaction were shown to lose CD46 at their acrosomal surface, which correlated with activated caspase 3 and cleaved PARP stainings (Fig. 6, g–i). This observation was confirmed by Western blot analysis, which further indicated that CD46 in microparticles was released in the supernatant of treated cells (data not shown).

Following growth factor withdrawal, we found that primary cultures of human keratinocytes produced caspase 3-positive particles that were highly enriched for CD46. No staining for cleaved caspase 3 was observed on viable keratinocytes strongly stained for CD46. Most inter-
estingly, a unique CD46 distribution was observed on keratinocytes with cytoplasmic and cell membrane distribution particularly at a point of contact between cells (Fig. 6, arrowhead). This distribution is a canonical fingerprint of adhesion molecules generally involved in homotypic interaction (e.g. PECAM1, alias CD31). Unfortunately, no CD31 staining was detected on keratinocytes to confirm the possible colocalization with CD46 (data not shown). Megakaryocyte cell lines have been shown recently (52) to produce platelets by a novel compartmentalized form of caspase-directed cell differentiation. The CMK-derived platelets stained for cleaved caspase 3 were highly enriched with CD46 (Fig. 6, m–p). These platelets were also weakly stained for NA as indicated by DAPI staining.

**DISCUSSION**

Cells undergoing apoptosis display a number of eat me flags. Some are relatively well characterized, such as the exposure of PS normally restricted to the inner membrane leaflet or poorly characterized, such as changes in surface sugars detected by phagocyte lectins. Other eat me signals have also been defined for their capacity to bind adhesive bridging molecules, such as innate immune defense collagen molecules MBL, surfactant proteins SP-A and SP-D, and complement C1q (53). The key role of C1q has been confirmed by comprehensive studies demonstrating that C1q-deficient animals were severely impaired in the clearance of apoptotic cells (11, 54). Our data further argue for a central role of C1q in the early detection of membrane-bound NA on apoptotic cells long before they expose the canonical PS eat me signal. DNA has long been known as a nonimmune activator of the complement pathway through its interaction with both the collagen-like region and the globular region of C1q (46). The precise region of C1q involved in DNA binding might be a matter of controversy (12, 15, 55), but a more recent and elegant study by Palaniyar et al. (56) highlights that all major defense collagens (SP-D, MBL, and C1q but not SP-A) are involved in binding to carbohydrate (pentose sugar-based anionic phosphate) polymers such as free DNA and RNA. Our paradigm does not exclude the newly described role of IgM in engaging the activation of the complement classical pathway on apoptotic cells (33–35).

C1q is one of the rare complement components not to be synthesized by hepatocytes, but its scavenging function should be credited to other cell

**FIGURE 5.** CD46 is clustered to apoptotic blebs where phosphatidylserine exposure and complement activation are prevalent. A, double immunofluorescence staining of unpermeabilized Jurkat cells using the mouse anti-CD46 (11C5 clone) and annexin V-FITC prior to (control) and after camptothecin-treatment (2 h). B, total cell lysates and bleb cell lysates were prepared from control and apoptotic cells (camptothecin (Camptothen) 4 h followed by incubation with NHS 1:4 for 1 h) and analyzed by Western blot for CD46 and complement C1q. C, double FACS analysis of camptothecin-treated cells (30 min and 4 h post-treatment) incubated with NHS and stained using mouse anti-C3b neo antibody and rabbit anti-CD46.
CD46 Controls Innate Immune Recognition of Apoptotic Cells

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types such as macrophages, fibroblasts, epithelial cells, glial cells, and neurons (36). In immunoprivileged tissues that lack an adaptive immune response (hence IgM), we argue that C1q may be the driving force in the recognition and the demise of apoptotic and necrotic cells. C1q has a unique multimeric structure and has been shown to bind to several PAMPs and PPAMPs such as the prion agent and amyloid fibrils accumulating in the brain of demented patients (18, 36, 57). Our report stresses that C1q is also involved in the selective recognition of ACAMPs. C1q may have a dual role in the clearance of apoptotic cells: activator of the classical complement pathway to promote opsonization by C3 fragments and as a complement receptor-like molecule. Macrophages were shown to synthesize and also display C1q as a type II cell surface molecule (58), and it will be important to ascertain the direct contribution of C1q as a receptor binding to NA.

Recent data suggest that uptake of apoptotic cells involves two sequential steps: an initial tethering event followed by macrophagocytosis (59). Macrophages “tether” apoptotic cells using several redundant innate immune receptors such as CD14, CD36, and scavenger receptors, and perhaps C1q expressed at the cell membrane may be involved in this scenario (14, 59). The second step that involves the engulfment of the apoptotic cells has also been linked to C1q but this time through its capacity to bind to surface calreticulin (also known as the collagen-tail C1q receptor, cC1qR) complexed to the endocytic receptor protein CD91.

Our data stress for the first time the role of CD46 as a don’t eat me signal involved in innate immune recognition. During blebbing or zeiosis of cells dying by apoptosis, we found that CD46 was rapidly translocating from cytoplasmic/nuclear and membrane stores into membrane-bound bodies (apoptotic blebs) budded individually from the cell surface. As expected, the reduction in CD46 resulted in increased C3b deposition on apoptotic cells, which ultimately would make them more appetizing to macrophages. In this context and given that CD46 is ubiquitously expressed on all nucleated cells, we would argue that CD46 is a key and novel example of a SAMP to control the demise of the dying cell by the innate immune system. We showed that more than 15 different human cell lines and primary human cell cultures induced to undergo apoptosis were rapidly losing CD46 at the cell surface; furthermore, Crry, the CD46-like protein in rodents, was also found to be dramatically reduced in several apoptotic cell models and possibly through capping into blebs. Is CD46/Crry a universal SAMP to control the innate immune recognition by professional and amateur phagocytes in analogy to major histocompatibility complex class I inhibiting NK cell killing? Regrettably, we don’t know, and in vivo experiments along these lines are now highly warranted. CD46 genetic defects in humans or Crry experimental deficiencies are associated with severe pathologies, including hemolytic uremic syndrome and embryonic lethality (60, 61).

We do not know whether the immunoregulatory properties of CD46/Crry are exclusively dependent on their capacity to control complement opsonization. Crry can protect from innate immune recognition in a complement-independent manner, stressing the plausible role of a CD46/Crry counterreceptor (CD46R) yet to be identified (62). In analogy to the CD47/SIRPα or the CD200/CD200R paradigms, it will be important to ascertain whether canonical innate immune cells (macrophages, neutrophils, mast cells, and dendritic cells) express the putative immunoregulatory CD46R. Preliminary FACS experiments using a CD46-human IgG4Fc fusion protein indicated a strong specific binding to THP1, HL60, and PMN cells. No specific staining above background was obtained with the human IgG4 control protein. Affinity chromatography experiments will help to delineate the nature of the binding molecule(s) and whether CD46R has classical ITIM motifs.

Consistently, CD46 capping into blebs was (i) associated with a caspase-dependent cell maturation processes of sperm and platelet precursor cells; (ii) taking place on senescent keratinocytes; (iii) independent of complement opsonization at the cell surface; (iv) relying on caspase-dependent activation events upstream of caspase 3; and finally (v) released from the cell surface as part of a microparticle together with NA, PS, C1q, iC3b, and opsonins, and factor H.

We would argue that these microparticles could diffuse out and provide “appetizers” to the surrounding macrophages to prepare for a bigger meal, the apoptotic corpses. Several eat me signals are carefully arrayed and highly enriched on these microparticles such as NA, PS, C1q, and iC3b, but interestingly, they could engage immunosuppressive pathways as recently described for iC3b docking onto CR3 (63, 64). The role of CD46 (and factor H) in these microparticles remains unclear apart from ensuring that C3b is converted to iC3b. The clearance of these microparticles by the neighboring cells is of paramount importance before they are captured by antigen-presenting cells (e.g., dendritic cells). Failure to do so could be associated with systemic lupus erythematosus pathologies that are linked to autoantibodies against several entities present in these microparticles (NA, C1q, and iC3b) (65–67).
Most surprisingly, CD46 capping into blebs and budding from the apoptotic cells was not affected by the level of complement opsonization at the cell surface but was a caspase-dependent apoptotic event. Researchers in the spermatogenesis field have known for some time that apoptosis plays an important role in removing abnormal sperm (51), and perhaps the loss of CD46 will promote complement-dependent removal by neighboring Sertoli cells or even by epithelial cells of the vagina/uterus to control possible pro-inflammatory response against this natural foreign particle. Recent data in Drosophila led to the idea that developing spermatozoa use the apoptotic machinery to selectively dissipate unneeded portions of their cytoplasm (membrane blebbing), a process termed individualization (50). Our human sperm and megakaryocyte data clearly indicate that CD46 capping to caspase-positive blebs is part of a physiological process independent of complement activation and highlight a more general (and perhaps primary/ancestral) role of CD46/Crry in membrane blebbing and budding of microparticles from the cell surface.

In conclusion, the role of complement promoting the safe disposal of altered self has received bad publicity with the wisdom that it would do more harm than good. Complement is perhaps the only canonical innate immune system with the intrinsic capacity to handle eat me and don't eat me signals as well as “come and get me” signals in the form of complement C3a and C5a anaphylatoxins. Our original findings support the role of novel and universal eat me signal (i.e. NA interacting with C1q) which will drive and tailor the level of complement opsonization on apoptotic and necrotic cells (Fig. 7). Furthermore, our data stress the emerging role of a novel and universal don't eat me signal (i.e. CD46/Crry). Ongoing experiments in our laboratory will help also to delineate the role of C3a in the nonphlogistic removal of apoptotic cells.

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FIGURE 7. Innate immune recognition and the safe demise of altered self; the NA and CD46 universal model. Although apoptosis is initiated by many physiologic and pathologic stimuli, all apoptotic cells undergo a similar sequence of morphological and biochemical events. Our data underlines the role of two novel and universal key signals: membrane-bound nucleic acids as an eat me or altered self-signal, and CD46 as a don't eat me or missing self-signal. Proposed sequence of events to initiate the decoding of altered self-phenotype. a, viable cells express CD46 (found abundantly on all nucleated cells) and other regulators of the innate immune complement system as well as retain PS on the inner leaflet of the plasma membrane. b, in sharp contrast, apoptotic cells rapidly expose NA in the outer leaflet of the plasma membrane long before PS exposure and promote activation of the complement system (C1q selective binding to NA). Critically, we found that apoptotic cells retained high levels of glycosylphosphatidylinositol-anchored CD55 and CD59, hence preventing MAC formation. However, because CD46 is rapidly shed from the cell surface into microparticles, the apoptotic cell will gradually lose the capacity to control complement C3b opsonization (“missing self”-paradigm). The microparticles (enriched for CD46, NA, and C1q/C3b, and factor H) are likely to diffuse out and dock onto professional and amateur phagocytes to instruct the safe demise of the apoptotic corpses by mechanisms that remain poorly characterized. Phagocyte recognition could involve different receptors for PS, C1q (CD91/calreticulin, CRT), C3b/iC3b (complement receptor type 3, CR3), and other possible receptors for CD46 and NA, yet to be identified. Membrane-bound C1q could act as an NA receptor. Furthermore, Toll-like receptors have been reported to decode nonself-NA, but it is unknown whether they are also involved in sensing altered self-NA.
CD46 Plays a Key Role in Tailoring Innate Immune Recognition of Apoptotic and Necrotic Cells
Kristina Elward, Mark Griffiths, Masashi Mizuno, Claire L. Harris, Jim W. Neal, B. Paul Morgan and Philippe Gasque

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