Keratin mediates the recognition of apoptotic and necrotic cells through dendritic cell receptor DEC205/CD205

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Clearance of dead cells is critical for maintaining homeostasis and prevents autoimmunity and inflammation. When cells undergo apoptosis and necrosis, specific markers are exposed and recognized by the receptors on phagocytes. DEC205 (CD205) is an endocytotic receptor on dendritic cells with antigen presentation function and has been widely used in immune therapies for vaccine generation. It has been shown that human DEC205 recognizes apoptotic and necrotic cells in a pH-dependent fashion. However, the natural ligand(s) of DEC205 remains unknown. Here we find that keratins are the cellular ligands of human DEC205. DEC205 binds to keratins specifically at acidic, but not basic, pH through its N-terminal domains. Keratins form intermediate filaments and are important for maintaining the strength of cells and tissues. Our results suggest that keratins also function as cell markers of apoptotic and necrotic cells and mediate a pH-dependent pathway for the immune recognition of dead cells.

DEC205/CD205 | keratin | dead cell recognition | mannose receptor family | cell death

The immune system is responsible for removing self-antigens such as dead cells to maintain tissue homeostasis and prevent autoimmunity (1–3). Dead cells are recognized and engulfed by phagocytes through their cell surface receptors (4), leading to either immune activation or tolerance (5–8). A number of receptors have been found to be able to mediate the clearance of dead cells (3); for example, CD14 (9), CD36 (10), integrin (11), PtdSerR (12), CLEC9A (13–15), and TIM receptor family members (16). Among known dead cell markers, phosphatidylserine (PS) is the most common one that can be recognized by several receptors such as CD36, PtdSerR, and TIM receptors, and acts as an "eat-me" signal for phagocytes mediating dead cell clearance (4). Other than PS, several cellular proteins have also been found to be able to perform the similar function. For example, actin filaments can be recognized by CLEC9A as a damaged cell marker (13, 15). The recognition of different dead cell markers by phagocytes provides an efficient way to remove cell debris completely.

Keratins are important cytoskeletal components and form intermediate filaments in cytoplasm. There are 54 known members in the keratin family that are divided into two types based on their isoelectric points and sequences (17). Keratin monomers can assemble into bundles first and then form fibrous filaments ∼10 nm in diameter, which act as a scaffold and provide mechanical support for maintaining the strength and toughness of cells and tissues (18, 19). Recently, evidence has accumulated showing that keratins play physiological roles in addition to their structural functions, for example, in cell growth, proliferation, mobility, apoptosis, and tumorigenesis (18, 20, 21).

DEC205 (CD205 or Ly75, molecular mass of 205 kDa) is an endocytotic receptor highly expressed on dendritic cells and thymic epithelial cells (8, 22) and capable of inducing either tolerance or immunity in the absence or presence of inflammatory stimuli (23). DEC205 belongs to the mannose receptor family (24), which includes five members: the mannose receptor, DEC205, Endo180, PLA2R, and FcRY (25). Structural results indicate that these receptors share similar structural features (26–28). Their ectodomains begin with a cysteine-rich domain (CysR), followed by a fibronectin type II domain (FNII) and 8 (10 for DEC205) C-type lectin-like domains (CTLDs). Recently, our data indicate that the ectodomain of DEC205 adopts a double-ringed conformation at acidic pH and also show that DEC205 recognizes apoptotic and necrotic cells specifically in acidic environments through its N-terminal smaller ring (27), suggesting that it mediates a different dead cell recognition pathway from the previously identified receptors. However, because the physiological ligand(s) of DEC205 has not been found, the mechanism of this pathway remains unknown.

Here we identified the natural ligand(s) of DEC205 by a series of biochemical and biophysical assays and found that keratins were the cellular ligands of DEC205. DEC205 binds to keratins at acidic pH through its N-terminal smaller ring, suggesting that DEC205 is a pH-dependent keratin receptor mediating the immune recognition of apoptotic and necrotic cells.

Results

DEC205 Recognizes Protein Ligands on Apoptotic and Necrotic Cells. To identify the ligand of DEC205, we treated dead (apoptotic and necrotic) cells with a number of enzymes including protease K, DNase I, and RNase A (Fig. 1*A*). The results showed that only the protease K treatment abolished DEC205 binding to dead cells. In parallel, we also tested the lipid binding activities of DEC205 by dot-blot assays, and no obvious binding was detected (Fig. 1*B*). To verify this result, we treated HEK293 cell lysates with protease K at different concentrations and with different

Significance

DEC205 (CD205) is an endocytotic receptor on dendritic cells that recognizes dead cells in a pH-dependent fashion and has been widely used for vaccine generation in immune therapies. However, the physiological ligand(s) of DEC205 has remained unknown for decades. Here we identify that keratins are the cellular ligands of human DEC205 and DEC205 recognizes keratins specifically at acidic pH. Keratins are structural proteins providing mechanical support for cells and tissues. Our results suggest that keratins act as markers of dead cells in acidic environments. Moreover, since keratins have been used as diagnostic markers for various tumors, the finding of DEC205 as a scavenging receptor of keratins may provide insights for the therapeutic strategies against tumors and other related diseases.

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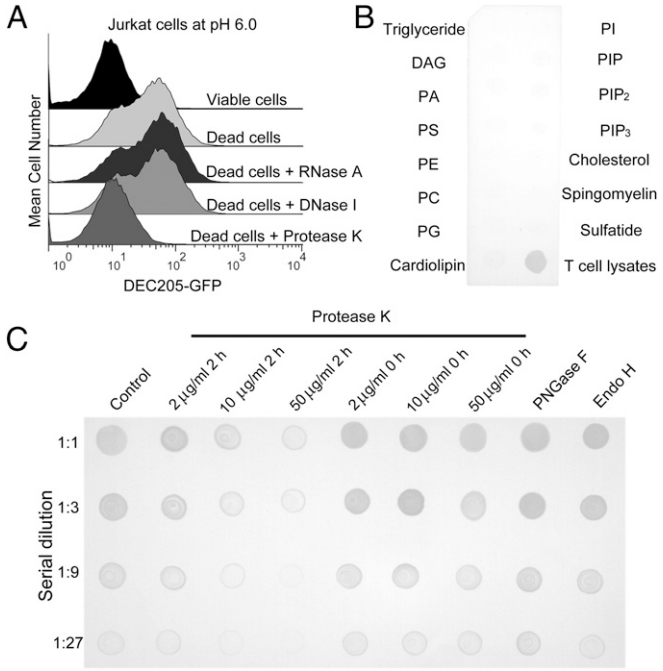

Fig. 1. Human DEC205 recognizes protein ligands on apoptotic and necrotic cells. (*A*) The binding of DEC205 to dead cells was abolished by protease K treatment. (*B*) DEC205 showed no binding activity to the lipids on the lipid strip. T-cell lysates were spotted as a positive control. (*C*) The binding of DEC205-Fc to HEK293 lysates was diminished by protease K treatment in a time- and concentration-dependent manner. PNGase F and Endo H treatments had no effect on the binding of DEC205-Fc to HEK293 lysates.

incubation time in the dot-blot assays (Fig. 1*C*). Similarly, the glycosidases PNGase F and Endo H were applied to cell lysates to evaluate the carbohydrate binding activity of DEC205 (Fig. 1*C* and Fig. S1). The results showed that the binding of DEC205 to dead cells diminished gradually as the protease K concentration and incubation time increased, whereas glycosidase treatments had no effect to the binding. Taken together these results suggest that the DEC205–ligand interaction is protein dependent, rather than lipid or glycan dependent.

Keratins Are the Cellular Ligands of DEC205. To isolate the protein ligands of DEC205, biotinylated DEC205 was incubated with apoptotic HEK293 cells at pH 6.0 and cross-linked by 2% paraformaldehyde. The cells were then washed with PBS at pH 7.4 to remove uncross-linked DEC205. Then cells were lysed and loaded onto streptavidin beads to remove the unbound material. The boiled beads were analyzed by SDS/PAGE showing a high molecular weight band, whereas the control sample without paraformaldehyde cross-linking showed no obvious band (Fig. 2*A*). Mass spectrometry analysis of the band indicated that it contained mostly keratins along with DEC205 (Fig. 2*B*). This result implied that keratins could be the cellular ligands of DEC205. To verify the cross-linking results, we used a different method for ligand isolation. A human DEC205 ectodomain fused with an IgG Fc fragment (DEC205-Fc) was mixed with Jurkat cell lysates at pH 6.0, purified with protein A/G beads, and then eluted at pH 8.0. An IgG Fc fragment alone was used as a control. Mass spectrometry results showed that DEC205-Fc pulled down more keratins than Fc alone and keratins were the major proteins showing large differences over the control (Fig. 2 *C* and *D*), suggesting keratins were the ligands of DEC205 under acidic conditions. The different mass spectrometry counts of identified keratins may correlate with their molecular weights and the binding affinities with DEC205 or the cell lines used in the experiments. In addition, because keratin members are assembled into filaments with each other, some of them may be pulled out without having direct interactions with DEC205. By contrast, the mass spectrometry counts of the proteins previously identified as dead cell markers, such as calreticulin or actin, are only at background level, suggesting they might not have interactions with DEC205.

DEC205 Binds Keratins at Acidic pH Through Its N-Terminal Smaller Ring. To verify the interactions between DEC205 and keratins, we evaluated the binding of DEC205 to keratins in vitro using a series of ELISA experiments. Human keratins derived from epidermis were immobilized on ELISA plates, and DEC205-Fc protein was added at different concentrations and at different pH values. The data showed that the binding of DEC205 to keratins was pH dependent (Fig. 3 *B* and *D*). DEC205 only bound to keratins at acidic pH and lost detectable binding at around pH 6.8 (Fig. 3*D*). A similar binding profile was observed for an N-terminal fragment of DEC205, CysR∼CTLD3 (Fig. 3 *C* and *D*), which adopts a ring-shaped conformation (the smaller ring) at acidic pH (Fig. 3*A*) (27). In contrast, the DEC205 fragment FNII∼CTLD6, which forms a larger ring at acidic pH (Fig. 3*A*), exhibited no binding affinity to keratins at either acidic or basic conditions (Fig. 3 *C* and *D*), suggesting DEC205 recognizes keratins through its N-terminal smaller ring. In our previous studies, we identified a single mutant (H129E) on the smaller ring that abolished the DEC205 binding to apoptotic and necrotic cells (Fig. 3*A*) (27). Similarly, ELISA results showed that this mutant had no binding affinity to keratins either (Fig. 3*C*), thus supporting the specific interactions between DEC205 and keratins. In addition, we tested the binding of keratins with Endo180 and PLA2R, which are also the members of the mannose receptor family. The data showed that neither Endo180 nor PLA2R had keratin binding activities (Fig. 3*B*). Besides ELISA experiments, we also evaluated

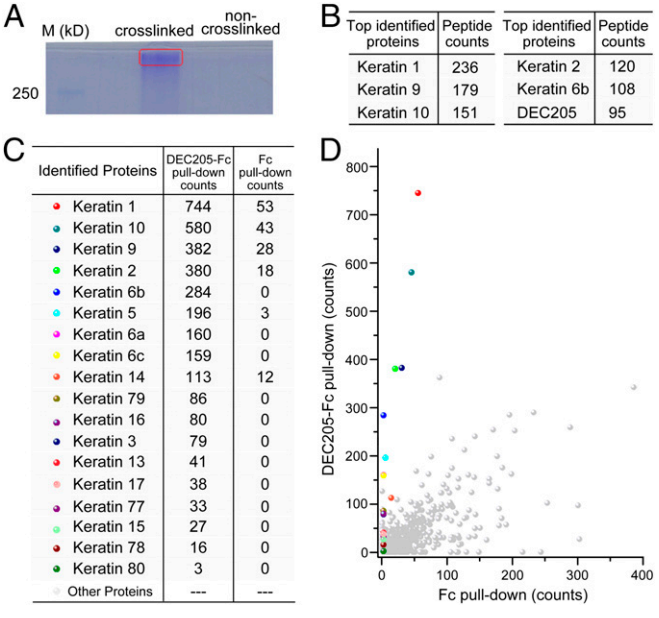

Fig. 2. Keratins are the cellular ligands of human DEC205. (*A*) SDS/PAGE of the ligand isolation by cross-linking. Apoptotic HEK293 cells were incubated with biotinylated DEC205, washed, and cross-linked with paraformaldehyde or mock cross-linked with PBS. The lysates were purified by streptavidin beads and loaded onto SDS/PAGE showing a high molecular weight band (red square). The top hits of mass spectrometry results obtained from the band are shown in *B*. (*C* and *D*) Mass spectrometry results of direct pull-down experiments showed a larger amount of keratins were pulled down by DEC205-Fc than Fc alone. The count of each protein represents the summation of the number of times of its peptides detected by mass spectrometry.

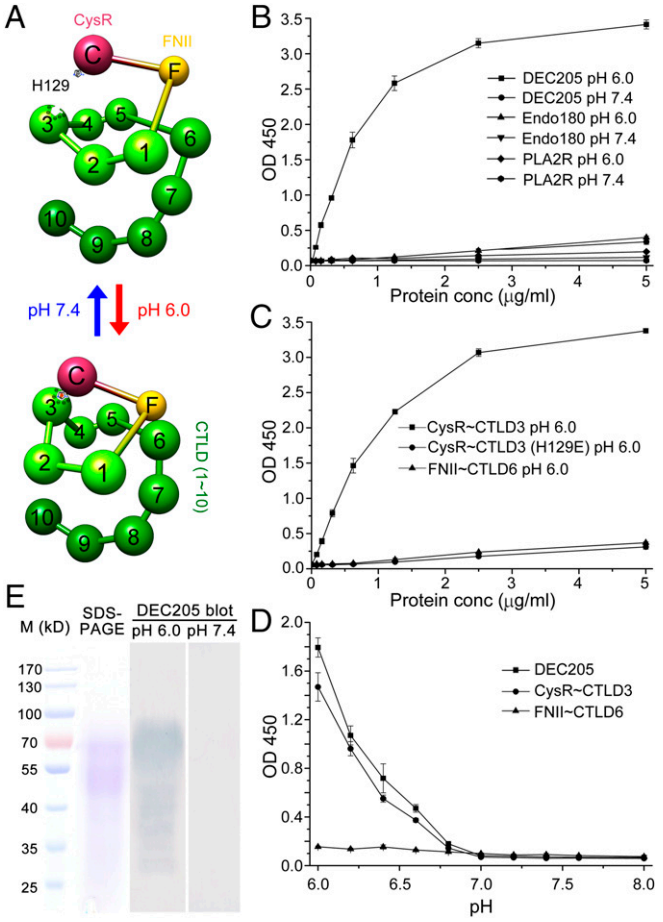

Fig. 3. Human DEC205 binds to keratins at acidic pH with its N-terminal smaller ring. (*A*) Ball-and-stick models of DEC205 showing its domain arrangement and the pH-dependent conformational change. (*B*) DEC205 bound to human keratins at pH 6.0 rather than at pH 7.4. The ectodomains of Endo180 and PLA2R showed no binding activities to keratins at either pH 6.0 or pH 7.4. (*C*) The N-terminal smaller ring (CysR~CTLD3) of DEC205 bound to keratins at pH 6.0, whereas the larger ring (FNII~CTLD6) and the smaller ring mutant (H129E) showed no keratin binding affinities. (*D*) DEC205 and its N-terminal smaller ring bound to keratins in a pH-dependent manner. (*E*) Western blot assays showed that DEC205 bound to human keratins at acidic, rather than basic, pH. The SDS/PAGE of human keratins is shown on the *Left*.

DEC205 interactions with keratins using Western blot assays (Fig. 3*E*). Human keratins were loaded onto SDS/PAGE, transferred to PVDF membranes, and blotted by DEC205-Fc at either pH 6.0 or pH 7.4. Indeed, the bands of keratins were only detected at acidic pH rather than at basic pH (Fig. 3*E*). Because proteins were usually linearized on SDS/PAGE, this result implied that DEC205 may recognize linear sequence motifs on keratins, as had been observed in the cases of bacterial proteins ClfB (29) and Srr-1 (30).

DEC205 Recognizes the C-Terminal Gly-Rich Region of Keratins. To further characterize the interactions of DEC205 with keratins, full-length human keratin 1 and keratin 10 were both expressed in *Escherichia coli* and purified from inclusion bodies. The interactions of DEC205 with purified keratins were investigated by Western blot assays as discussed above. The results showed that DEC205 bound to keratin 1 and keratin 10 only at acidic pH (Fig. 4*B*), confirming the direct interactions between DEC205 and keratins. The structures of keratins usually contain a central α-helical rod domain that is flanked by a nonhelical head and a tail domain (31) (Fig. 4*A*). To narrow down the binding region of DEC205 on keratins, we expressed a series of truncation fragments of keratin 1 for Western blot assays (Fig. 4*A*). The results showed that DEC205 only recognized the C-terminal tail domain of keratin 1 specifically at acidic pH (Fig. 4 *C* and *D*), which contains a high percentage of glycine and serine in sequence, a feature shared by many keratin family members (32). Similarly, two truncation mutants of keratin 10 were also expressed for binding assays (Fig. 4*A*), and results showed that only the tail domain of keratin 10 could be recognized by DEC205 at acidic pH (Fig. 4*E*). The interactions of DEC205 with keratin tails were also evaluated by ELISA (Fig. 5*C*), which validated the pH-dependent binding properties of DEC205 with keratins.

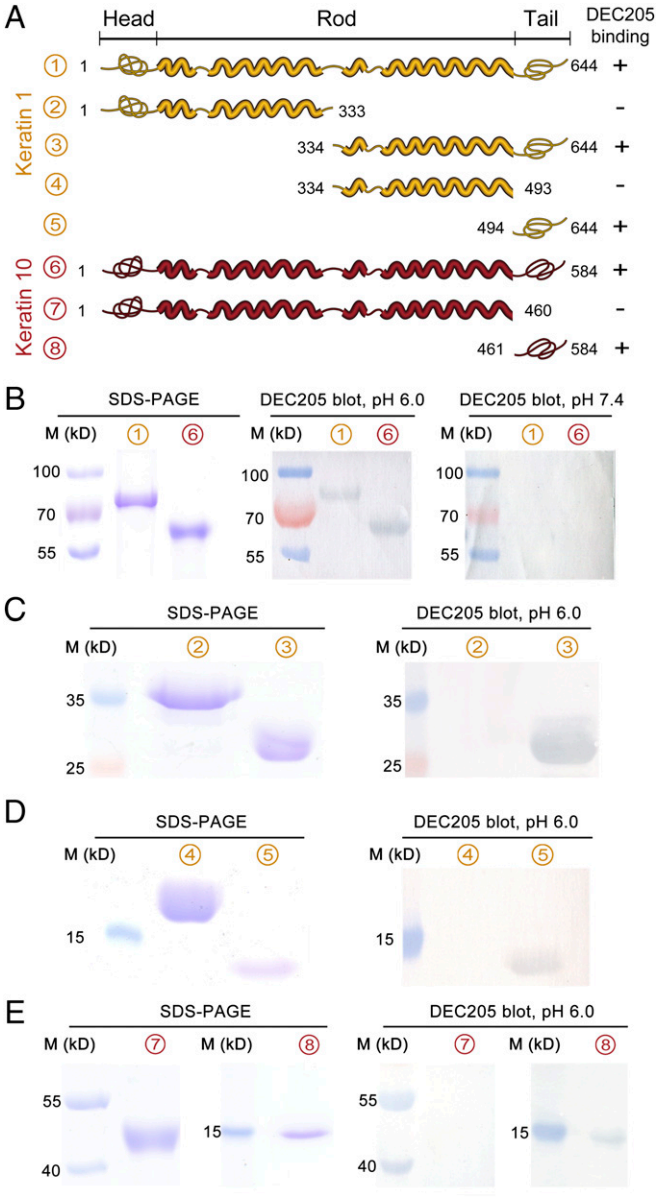

Fig. 4. Human DEC205 binds to the C-terminal tail domains of keratin 1 and keratin 10 at acidic pH. (*A*) Schematic representation of keratin 1 (yellow) and keratin 10 (red) domain arrangement and their truncation mutants expressed for binding assays. (*B*) DEC205 bound to the full-length keratin 1 and 10 at acidic pH. (*C* and *D*) Western blot assays showed that DEC205 bound to the C-terminal tail domain of keratin 1 at acidic pH. (*E*) Western blot assays showed that DEC205 bound to the C-terminal tail domain of keratin 10 at acidic pH.

DEC205 Recognizes Cellular Keratins at Acidic pH. To investigate the interactions of DEC205 with cellular keratins, we stained permeabilized HEK293 cells with both anti-keratin 1 antibody and DEC205-Fc. Fluorescent images showed that keratin 1 and DEC205 were well colocalized at acidic pH (Fig. 5*A* and Fig. S2*A*), suggesting the specific interactions between the two proteins. In parallel, an anti-pan keratin antibody was also used for colocalization assay with DEC205-Fc, and similar results were observed (Fig. 5*B* and Fig. S2*B*), thus confirming the in vitro binding results of DEC205 and keratins. Our previous results indicated that DEC205 could bind to the apoptotic and necrotic cells specifically at acidic pH (27). Therefore, if keratins are the cellular ligands of DEC205, the keratin tail fragments identified above should be able to inhibit the binding of DEC205 to dead cells. Indeed, the FACS results showed that the tail fragments of keratin 1 and keratin 10 could block the binding of DEC205 to dead cells almost completely (Fig. 5*D*), suggesting that keratins are the specific ligands of DEC205 on these cells. Furthermore, we repeated binding/inhibition experiments using primary cells isolated from mouse spleen and obtained similar results (Fig. 5*E*). These results are expected because the C-terminal Gly-rich motif is shared among many mouse keratin members. We also expressed the N-terminal smaller ring of mouse DEC205 (CysR~CTLD3), and found that it could recognize mouse dead cells and exhibited higher binding affinity under acidic pH (Fig. S3*B*). Interestingly, although human DEC205 could bind to mouse dead cells probably through keratins (Fig. 5*E*), the smaller ring of mouse DEC205 did not show binding affinities to human keratins (Fig. S3*A*). These results suggest that mouse DEC205 might also be a receptor for dead cells (33), but its interactions with the ligand may be different from human DEC205. To evaluate the recognition of DEC205 with keratins on cell surface, we stained the ActD-treated Jurkat cells with annexin V and propidium iodide (PI) to differentiate the stages

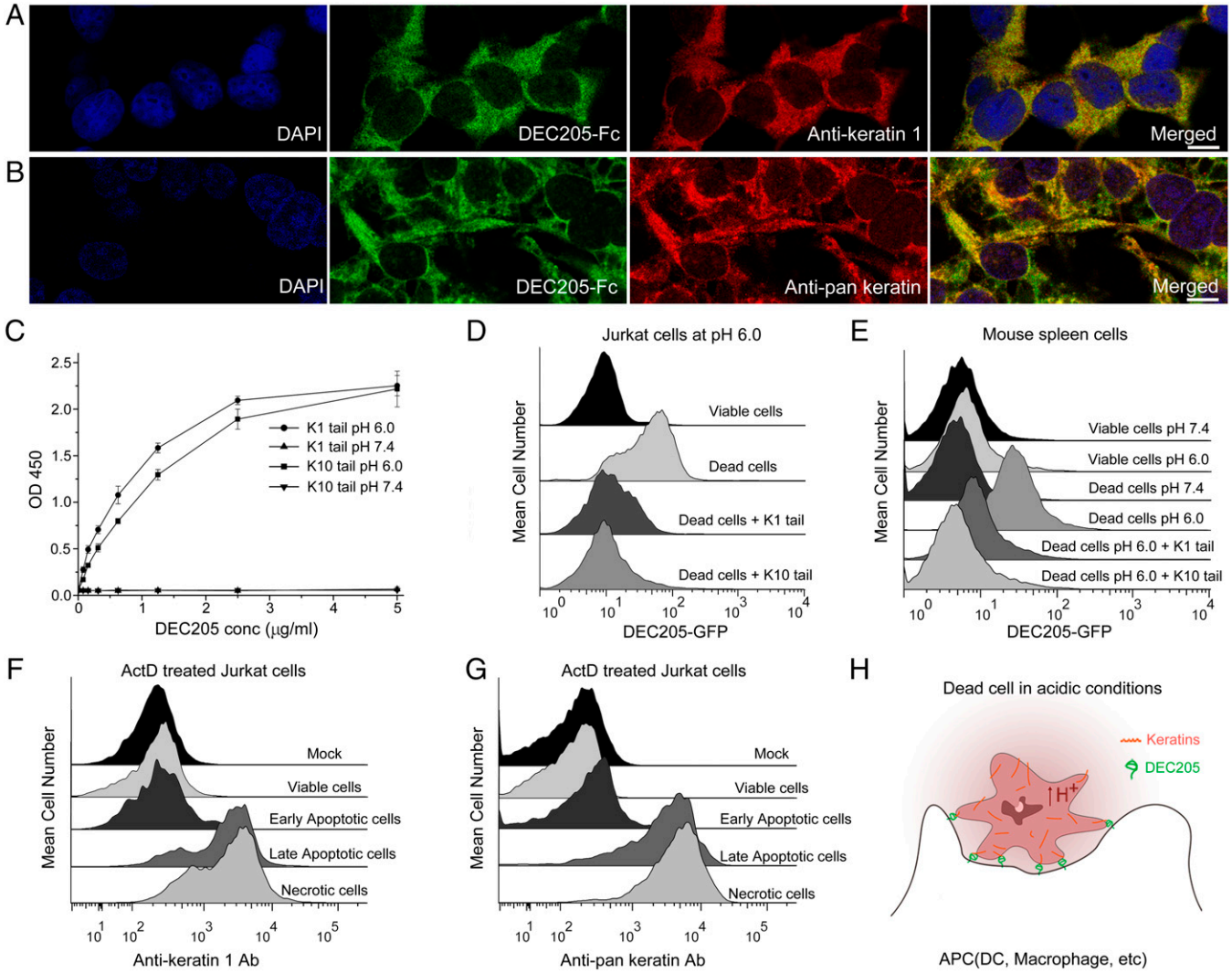

Fig. 5. Human DEC205 binds to cellular keratins at acidic pH. (*A*) DEC205 colocalized with anti-keratin 1 antibody in HEK293 cells at pH 6.0. (Scale bar, 10 μm.) (*B*) DEC205 colocalized with anti-pan keratin antibody in HEK293 cells at pH 6.0. (Scale bar, 10 μm.) (*C*) ELISA experiments showed that DEC205 bound to the C-terminal tail domains of keratin 1 and keratin 10 at acidic pH. (*D*) The tail domains of keratin 1 and keratin 10 blocked the binding of DEC205 to dead cells. (*E*) DEC205 bound to the frozen-thawed mouse spleen cells at acidic pH and the binding was blocked by the tail domains of keratin 1 and keratin 10. (*F* and *G*) ActD-treated Jurkat cells were stained with Annexin V and PI to differentiate the stages of viable cells (Annexin V^-PI^-), early apoptotic cells (Annexin V^+PI^-), late apoptotic/early necrotic cells (Annexin V^+PI^+), and necrotic cells (Annexin V^+PI^{++}). The exposure of keratins on the surface of cells was monitored by anti-keratin 1 antibodies (*F*) and anti-pan keratin antibodies (*G*). Anti-keratin antibodies were not added under mock conditions. (*H*) A cartoon representation of the recognition of apoptotic and necrotic cells by human DEC205 through keratins in acidic conditions.

of apoptosis and necrosis and compared the exposure of keratins on the surface of dying cells using anti-keratin 1 and anti-pan keratin antibodies (Fig. 5 *F* and *G*). The FACS data showed that keratins were marginally exposed at the early stage of apoptosis and largely exposed at and after the late stage of apoptosis, suggesting that DEC205 recognizes dying cells mainly at the stages of late apoptosis and necrosis.

Discussion

As a member of the mannose receptor family, DEC205 has been suggested to be able to bind various targets (33, 34); however, the natural ligands of DEC205 remained a mystery for decades (22). Recently we have shown that DEC205 could recognize apoptotic and necrotic cells specifically in a pH-dependent fashion (27), thus providing the feasibility to identify its cellular ligands. Previous data also imply that the binding of DEC205 to apoptotic cells does not require protein synthesis (27, 33), suggesting the ligands could be existing protein components, consistent with the finding of keratins as the ligands of DEC205. It has been shown that keratins may undergo a variety of post-translational modifications, including phosphorylation and glycosylation (35); our results suggest that these modifications may not affect the recognition between keratins and DEC205. Recent evidence shows that the mannose receptor family members might share similar structural features (26–28, 36), but their natural ligands are different. The finding of keratins as the ligands of DEC205 adds more diversity to the receptor–ligand interactions of this family.

A keratin monomer usually contains three domains: an N-terminal head, a middle rod-forming domain, and a C-terminal tail. The DEC205 binding data show that it only recognizes the C-terminal tail domains of keratin 1 and keratin 10, and notably, the binding tests can be done by Western blot assays, suggesting that the target sequence of DEC205 on keratins adopts a linear conformation. Indeed, the tail domains of keratin 1 and keratin 10 contain a high percentage of glycine and serine in a $X(Y)_n$ format, where X represents an aromatic or a long-chain aliphatic residue and Y is either glycine or serine (32, 37). The $X(Y)_n$ pattern is quasirepetitive and may form "glycine loops" where the Gly-rich peptides have flexible loop-like structures without rigid conformations (37), thus explaining the results of Western blot assays. Recently, it has been shown that a bacterial protein ClfB could recognize the Gly-rich tail domain of keratin 8 and keratin 10 (38, 39), and the crystal structure of ClfB complexed with the keratin 10 tail shows that the Gly-rich sequence also adopts a linear conformation (29). In fact, the Gly-rich sequences are found in the C-terminal tail domains of many keratin members including both type I and type II keratins (32, 37), suggesting that DEC205 might be a generic receptor for keratins targeting to these sequences.

Because keratins form an intermediate filament network and distribute all over the cytoplasm, they could be an efficient marker for dead cells or cell debris. Our results show that keratins are gradually exposed as apoptosis proceeds and become accessible to DEC205. Evidence has also shown that during apoptosis, keratins can be degraded and redistributed and serve as a regulating factor (40, 41), which may increase their association with cell debris and also the accessibility to receptors. This is consistent with the previous observation showing that the binding affinity of DEC205 to dead cells increases as apoptosis proceeds (27). Unlike actin filaments or microtubules, intermediate filaments are more elastic and resistant to strain damage (42), which may give them advantages in removing dead cells and also facilitating "cargo" delivery by DEC205 (43). Moreover, a number of pathogens have been found to be able to interact with keratins during infection (44); therefore, DEC205–keratin interactions may facilitate the antigen delivery and presentation against these pathogens.

Compared with other receptors such as CLEC9A (13, 15), PtdSerR (12), and TIM family members (16), the DEC205–keratin interaction represents a different pathway for dead cell recognition and clearance (Fig. 5*H*). Both keratin exposure and acidification are required for the functional activity of DEC205, which act to verify the eat-me signal on target cells or debris. As acidification is a common feature for apoptosis (45–47), it is not entirely unexpected that DEC205 could recognize keratins on dead cells in a pH-dependent fashion. Unlike other endocytotic receptors, such as mannose receptor or CLEC9A, DEC205 usually targets to late endosomes and lysosomes (14, 43); thus, DEC205 may also bind keratins on cell debris in phagosomes or early endosomes and transport them to late endosomes and lysosomes for more efficient antigen presentation. It is noteworthy that keratins are not only expressed inside cells, they are also found on the surface of some cell types such as carcinoma cells (30, 48–50). Evidence shows that extracellular acidification is usually associated with inflammation and tumorigenesis and is treated as a danger signal by the immune system (51–53). Therefore, DEC205 may be able to recognize nonapoptotic cells in these cases and act as an enhanced version among scavenging receptors in acidified regions (54, 55).

Due to the high efficiency of antigen delivery and presentation, DEC205 has been one of the major receptor targets for vaccination in dendritic-cell–based immune therapies (56, 57), and it is typically done by fusing DEC205-specific antibody with a fragment or intact protein of the antigen as a surrogate ligand (58–60). The finding of keratins as the natural ligands of DEC205 may give opportunities to improve this protocol by using ligand-fused antigens for better presentation. On the other hand, it has been shown that the abnormal expression of keratins is largely associated with tumors and may facilitate their invasion and metastasis (48–50, 61, 62). In fact, the correlation between keratins and cancer has been realized for a long time, and keratins are widely used as diagnostic markers for various tumors (20, 63). Therefore, as a specific scavenging receptor against keratins, DEC205 may have advantages over other receptors in tumor recognition and clearance, and further understanding of this pathway would provide more insights relevant to therapeutic strategies against cancer and other diseases.

Materials and Methods

Constructs encoding the ectodomain of human DEC205 (including the native signal sequence and residues 1–1,668 of the mature protein), CysR~CTLD3 (the smaller ring, residues 1–630), FNII~CTLD6 (the larger ring, residuals 155–1,094) were cloned into the pTT5 expression vectors with a human IgG1 Fc-tag and a C-terminal six-His tag. DEC205-GFP was also constructed with a C-terminal six-His tag into the pTT5 vectors. The supernatants of the transfected HEK293F cells were buffer exchanged with 50 mM Tris, 150 mM NaCl at pH 8.0 by dialysis, then applied to Ni-NTA chromatography (Ni-NTA Superflow, Qiagen). The imidazole eluates were further purified by gel filtration chromatography with a Superdex 200 column. All DEC205 samples were prepared following similar procedures.

Further experimental details can be found in *SI Materials and Methods*.

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