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A Role for Class A Scavenger Receptor in Dendritic Cell Nibbling from Live Cells

Larry A. Harshyne,* Michael I. Zimmer,* Simon C. Watkins,† and Simon M. Barratt-Boyes2*

Monocyte-derived dendritic cells (DC) possess the unique capacity to capture Ag from live cells through intimate cell contact, a process referred to as nibbling. We sought to define the receptor(s) mediating DC nibbling. Uptake of fluorescently labeled plasma membrane from live cells by DC was inhibited by protease treatment and by a panel of polyanionic ligands, implicating scavenger receptors (SR) in this process. Differential expression of SR on DC and macrophages correlated with the capacity to acquire membrane from live cells. Internalized membrane colocalized with SR ligand and entered the endosomal pathway. DC very efficiently acquired and internalized gp100 tumor Ag expressed at the surface of viable adenocarcinoma cells via recombinant adenoviral infection. Cross-presentation of gp100 by DC to MHC class I-restricted T cells was inhibited by polyanionic SR ligand and an Ab to type A SR (SR-A), whereas Ab to the class B SR CD36, which mediates uptake of apoptotic cells, induced no inhibition. DC capture of fluorescently labeled membrane from live cells was partially blocked by SR-A-specific Ab, suggesting that other SR may also be contributing to nibbling. DC maturation resulted in a switch in expression from type II SR-A (SR-AII) to the SR-AI splice variant. Finally, SR-A was identified on interdigitating DC isolated from monkey lymph nodes. These findings define a novel role for SR-A, and suggest that Ag uptake from live cells by DC may be important in the generation of immunity and in the maintenance of peripheral tolerance in vivo. The Journal of Immunology, 2003, 170: 2302–2309.

Dendritic cells (DC) have two separate pathways by which they can present Ag to MHC class I-restricted T lymphocytes in the generation of an immune response. The first pathway consists of processing endogenous Ag following direct infection by a pathogen, common to all cells bearing MHC class I molecules. Alternatively, DC have the unique capacity to present exogenous Ag derived from the extracellular milieu or captured from neighboring cells in the context of MHC class I, a process referred to as cross-presentation (1). Cross-presentation of cell-associated Ag by DC has been shown to be important for generating immunity to viruses that have restricted tissue tropisms (2, 3) and for maintaining peripheral tolerance to self Ag (4).

It is widely assumed that cell death is required before cell-associated Ag can be captured and cross-presented by DC (5). Uptake of apoptotic cells by DC has been well described, and viral Ag acquired from apoptotic cells is readily cross-presented to CTL (6). Phagocytosis of apoptotic debris does not in itself induce DC maturation or promote T cell stimulation, suggesting that uptake of apoptotic cells is a means by which DC induce peripheral tolerance to self (5, 7). Further exposure of DC to microbial products or necrotic cells promotes maturation and provides the stimulus required to activate Ag-specific T cells (7, 8). A subset of DC containing apoptotic cell fragments is constitutively present inafferent lymphatics draining the intestine and in the T cell-rich paracortex of mesenteric lymph nodes in rats (9), providing in vivo support for the role of apoptotic cell uptake by DC in peripheral T cell tolerance (10).

Although uptake of apoptotic and necrotic cells appears to be important in Ag cross-presentation, several lines of evidence suggest that cell death is not an absolute requirement for cross-presentation of Ag by DC. Uptake of apoptotic cells is mediated primarily by CD36 and αβ2 integrin (11); however, studies using knockout mice indicate that an absence of either of these receptors has no effect on cross-presentation of Ag for the maintenance of peripheral tolerance or the activation of Ag-specific T cells in immunity (12, 13). Moreover, while tissue destruction enhances cross-presentation of self Ag in an OVA transgenic mouse model, deliberate tissue damage is not required (14, 15). Finally, cross-presentation by APC is critical in the initiation of T cell responses to viruses that infect nonhemopoietic cells (2); however, there are examples of such viruses that do not induce cytotoxic effects, suggesting that Ag is captured by DC from intact infected cells (16). In support of this hypothesis, we have shown that monkey and human monocyte-derived DC readily acquire Ag in vitro by nibbling on live cells (17). DC uptake of Ag from live cells required sustained cell-cell contact and generated endocytic vesicles, up to 1 μm in size, within the DC (17). Notably, macrophages, which readily phagocytose apoptotic cells, could not acquire fluorescently labeled membrane from living cells. In contrast, DC acquired labeled membrane from all cells tested, including macrophages, B cells, and T cells (17). In the present study, we sought to identify the receptor(s) mediating this process.

Materials and Methods

Cells

APC were generated, as previously described (18). Briefly, immature DC were generated by culturing normal rhesus macaque and human CD14

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3 Abbreviations used in this paper: DC, dendritic cell; AclDL, acetylated low-density lipoprotein; Ad-gp100, recombinant adenovirus encoding gp100; CD40L, CD40 ligand; DIC, differential interference contrast; DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodio-carboxylic perchlorate; poly(C), polycytidylic acid; poly(G), poliguanic acid; SR, scavenger receptor; TMRM, tetramethylrhodamine-5-maleimide; EEA, early endosomal Ag.Copyright © 2003 by The American Association of Immunologists, Inc.
monocytes in GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) for 4 days. To induce maturation, DC were cultured for an additional 48 h with CD40 ligand (CD40L; 1 μg/ml). Macrophages were generated by culturing CD14+ monocytes for 48 h in the absence of cytokines. Apoptotic cells were generated by irradiating DC with UV-B light (240 mJ/cm²), followed by a 4-h overnight culture. A549 is an adenocarcinoma cell line that is highly susceptible to adenovirus infection. The oligodendroglial T cell line TIL620, which recognizes two epitopes of the melanoma Ag gp100, was derived in the laboratory of S. Rosenberg (National Institutes of Health).

Ab labeling and flow cytometric analysis
Cells were stained for flow cytometric analysis, as previously described (18). Briefly, cells were labeled with cross-reactive unconjugated Ab specific for SR-A (L243; BD Biosciences, Mountain View, CA); mannose receptor, DEC-205 (Seventh Workshop on Human Leucocyte Differentiation Ags); or isotype control Ab (X39; BD Biosciences). Alexa488-conjugated goat anti-mouse or rabbit anti-goat Ab (Molecular Probes, Eugene, OR) were used to visualize surface staining. Alternatively, cells were labeled with acetylated low density lipoprotein (AcLDL)-BODIPY (20 μg/ml; Molecular Probes) at 37°C for 30 min. Cells were harvested, washed, and maintained on ice until analyzed on a FACScan equipped with dual lasers (BD Biosciences) or an EPICS XL (Beckman Coulter, Fullerton, CA) flow cytometer. Alternatively, cells were fixed with 2% paraformaldehyde and analyzed within 48 h of labeling. Data files were analyzed utilizing CellQuest software (BD Biosciences) using log10 fluorescence.

Fluorescence labeling and transfer assays
Cells were incubated with the lipophilic probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD, excitation/emission spectra = 644/663 nm) and the thiol-reactive tetramethylrhodamine-5-maleimide (TMRM, 541/569 nm; both from Molecular Probes) at 37°C for 4 h, as previously described (17). Following a 20-min adherence period at 37°C, cell viability was assessed by lack of annexin V and TUNEL staining, two independent measures of cell death (data not shown). Unlabeled cells were dual labeled with the lipophilic fluorophore DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, excitation/emission spectra = 644/663 nm) and the thiol-reactive tetramethylrhodamine-5-maleimide (TMRM, 541/569 nm; both from Molecular Probes) at 37°C for 30 min at 37°C. In all experiments, labeled DC were cocultured with equal numbers of unlabeled DC or macrophages at 37°C for 3–4 h, as previously described (17).

Inhibition and blocking assays
Unlabeled DC were pretreated with dimethyl-amiloride (500 μM), Arg-Gly-Asp (RGD) peptide (10 μg/ml), mannan (10 mg/ml), polygalacturonic acid (polyG); 500 μg/ml), polyctitydilic acid (polyC); 500 μg/ml), poly(vinyl)sulfate (2.5 mg/ml), fucoidan (5 mg/ml; all inhibitors were obtained from Sigma-Aldrich, St. Louis, MO), or various Ab (30 μg/ml) for 40 min at 4°C. In some experiments, unlabeled DC were digested with 4 μg/ml pronase (Calbiochem, La Jolla, CA) for 30 min at 37°C. Labeled or adenovirus-infected DC or A549 cells (see below) were then added and cultured for 3–4 h (fluorescent studies) or 20 h (ELISPOT assays).

Confocal microscopy
DC were labeled with AcLDL-BODIPY or TMRM for 30 min at 37°C. Labeled cells were washed and then cocultured with either or unlabeled DC in equal numbers at 37°C for 3 h. In some cocultures, pepstatin A-BODIPY was added during the final 30 min to define the late-endosomal compartment. Cells were harvested, resuspended in PBS containing 5 μM nyste dye (Molecular Probes) to label nuclei, and settled onto slides. Following a 20-min adherence period at 37°C, slides were washed and cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Some slides were permeabilized with 0.1% saponin and stained with Ab specific for early endosomal Ag (EEA) (14) (BD Biosciences). In other experiments, A549 cells were infected with recombinant adenovirus encoding gp100-transduced monkey DC (Ad-gp100) as described below, and cultured with equal numbers of unlabeled DC for 3 h before permeabilization and labeling with an Ab to gp100 (HMB45; BioGenex, San Ramon, CA). Alexa488-conjugated goat anti-mouse Ab (Molecular Probes) was used to visualize EEA-1 and gp100 staining. Slides were then washed, coverslipped, and analyzed by confocal fluorescence microscopy on a Leica TCS NT confocal scanning microscope (Leica Microsystems, Northvale, NJ). Final compositions were constructed in Adobe Photoshop 6.0 (Adobe, San Jose, CA).

IFN-γ ELISPOT assay
DC, macrophages, and A549 cells were transduced with replication-defective Ad-gp100, as previously described (17). Briefly, virus was added at a multiplicity of infection of 100 directly to wells containing DC and macrophages, or a multiplicity of infection of 20 for A549 cells, incubated at room temperature for 1 h, and then returned to 37°C. Following overnight culture, transduced cells were harvested, washed three times, and returned to culture for an additional 8 h to allow for virus internalization. TIL620 cells (3 × 10⁵) were cultured with the same number of Ad-gp100-transduced human HLA-A2+ DC or macrophages (direct presentation) or Ad-gp100-transduced monkey DC and human HLA-A2+ DC or macrophages (cross-presentation) on multiscreen-96 plates (Millipore, Bedford, MA) coated with human hemagglutinin (HA) (1-D1K; Mattech, Cincinnati, OH). In some cross-presentation experiments, Ad-gp100-transduced A549 cells were cultured with equal numbers of human HLA-A2+ DC. Following 18-h incubation, IFN-γ production was detected by labeling with a second anti-human IFN-γ Ab (7-B6-1; Mattech), as described (17). Digital images of individual wells were captured and analyzed using an Immunospot analyzer (Cellular Technology, Cleveland, OH). Blocking studies were performed, as stated above.

Analysis of SR expression
For Western blot analysis, lysates of equal numbers of immature and mature DC were separated by PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Membranes were stained with Ab to type A scavenger receptor (SR-A), followed by peroxidase-conjugated rabbit anti-goat Ab and chemiluminescent development using ECL detection reagents (Amersham Pharmacia, Piscataway, NJ). For analysis of lymph node cells, single cell suspensions of normal monkey lymph nodes were generated using digestion with collagenase D, as previously described (18). Cells were labeled with pure Ab to SR-A, biotinylated Ab to HLA-DR (BD Biosciences), and PE-conjugated Ab to CD83 (HB15a; Coulter-Immunotech), followed by Alexa 488-conjugated rabbit anti-goat and streptavidin-Cy5. Samples were analyzed by flow cytometry, as described above.

Statistical analysis
Statistical significance was assessed by ANOVA, followed by Tukey test using SAS System software (SAS Institute, Cary, NC).

Results
Ag uptake from live cells is receptor mediated
We focused our initial investigations on immature DC, as these cells are specialized in Ag uptake and are highly effective at nabbling Ag from live cells (17). Monkey monocyte-derived immature DC were dual labeled with the fluorescent dyes TMRM and DiD to monitor the trafficking of plasma membrane proteins and lipids, respectively, between labeled and unlabeled cells. As in our previous work (17), viability of labeled cells and cocultures was confirmed by lack of annexin V and TUNEL staining, two independent measures of cell death (data not shown). Unlabeled DC efficiently acquired fluorescently labeled membrane proteins and lipids from other viable cells, as 66% of the unlabeled DC captured label from fluorescently tagged DC within 4 h (Fig. 1a). Dimethyl amiloride, an inhibitor of Na⁺/H⁺ exchange used as a generalized inhibitor of phagocytosis, abrogated the ability of DC to acquire fluorescent label from live cells (Fig. 1a). Coculture of cells at 4°C also completely inhibited uptake of fluorescent membrane by DC (data not shown) (17). These findings support our previous observations that capture of labeled plasma membrane proteins and lipids from live cells by DC is an active process that does not involve passive binding of dye to unlabeled cells (17).

To determine whether capture of live-cell Ag by DC occurs via a receptor-mediated process, we used pronase, a mixture of bacterial proteases (19), to strip proteins from the cell surface. Digestion with pronase drastically impaired the ability of unlabeled DC to acquire fluorescent label, reducing fluorescence uptake by 84% as compared with untreated DC (Fig. 1b). Thus, acquisition of plasma membrane from live cells by DC was dependent on protein expression at the cell surface, consistent with a receptor-mediated process. To screen potential live-cell Ag acquisition receptors, we studied pronase sensitivity profiles of select receptors known to mediate Ag uptake by DC. Pronase treatment removed the C-type lectins DEC-205 and mannose receptor from the surface of monkey DC, while levels of MHC class II, used as a control cell surface molecule, were unaffected (Fig. 1c). CD36, a class B SR, and...
AcLDL is a chemically modified form of LDL that binds to several classes of SR and has been used as a surrogate marker for SR (22). Binding of AcLDL was high on immature DC and down-regulated slightly upon maturation with CD40L, consistent with a role for SR in Ag capture (Fig. 3a, upper row). Monocytes and macrophages cultured for 2 days in the absence of cytokines had low level expression of SR, consistent with data in murine and human systems (25, 26). Expression of SR on these cells was highly correlated with their capacity to acquire labeled plasma membrane from other live cells (Fig. 3, a aspect of Ag capture by DC). Classical polyanionic SR ligands inhibit acquisition by monkey DC of fluorescently labeled membrane proteins and lipids from live cells. a, Immature monkey DC were cultured with equal numbers of viable or apoptotic DiD- and TMRM-labeled immature monkey DC or FITC-dextran in the presence or absence of inhibitors. Bars indicate reduction in the percentage of unlabeled cells that acquire fluorescence in the presence of inhibitors based on untreated profiles at 0 and 3 h. Error bars delineate SEM of three experiments. b, Dose-dependent inhibition of live-cell Ag uptake by various SR ligands. Equal numbers of unlabeled and DiD- and TMRM-labeled immature monkey DC were cocultured as above. Bars indicate reduction in the percentage of unlabeled cells that acquire fluorescence in the presence of inhibitors based on untreated profiles at 0 and 3 h. Results are representative of three experiments. PVS = poly(vinyl)sulfate.

Correlation between SR expression and live-cell Ag capture

SR were originally defined as macrophage-associated receptors (24). We therefore compared the expression of SR on DC and macrophages using AcLDL as a ligand. Binding of AcLDL was high on immature DC and down-regulated slightly upon maturation with CD40L, consistent with a role for SR in Ag capture (Fig. 3a, upper row). Monocytes and macrophages cultured for 2 days in the absence of cytokines had low level expression of SR, consistent with data in murine and human systems (25, 26). Expression of SR on these cells was highly correlated with their capacity to acquire labeled plasma membrane from other live cells (Fig. 3, a aspect of Ag capture by DC). Classical polyanionic SR ligands inhibit acquisition by monkey DC of fluorescently labeled membrane proteins and lipids from live cells. a, Immature monkey DC were cultured with equal numbers of viable or apoptotic DiD- and TMRM-labeled immature monkey DC or FITC-dextran in the presence or absence of inhibitors. Bars indicate reduction in the percentage of unlabeled cells that acquire fluorescence in the presence of inhibitors based on untreated profiles at 0 and 3 h. Error bars delineate SEM of three experiments. b, Dose-dependent inhibition of live-cell Ag uptake by various SR ligands. Equal numbers of unlabeled and DiD- and TMRM-labeled immature monkey DC were cocultured as above. Bars indicate reduction in the percentage of unlabeled cells that acquire fluorescence in the presence of inhibitors based on untreated profiles at 0 and 3 h. Results are representative of three experiments. PVS = poly(vinyl)sulfate.
Immature DC acquired membrane to a greater extent than mature DC, whereas uptake by monocytes and macrophages was minimal (Fig. 3a, lower row), as we have previously shown (17).

Subcellular localization of captured Ag in DC

We next used confocal microscopy to determine whether SR ligand and membrane proteins captured from live cells are delivered to the same intracellular compartment within monkey DC. DC labeled with AcLDL-BODIPY were cocultured with an equal number of TMRM-labeled DC, and images were taken of DC that had captured membrane from TMRM-labeled cells. After 3-h incubation, multiple vesicles containing AcLDL were present in the cytoplasm of AcLDL-BODIPY-labeled DC (Fig. 4, upper row). Numerous vesicles containing TMRM-labeled protein were also present in the cytoplasm of these cells, which colocalized with AcLDL (Fig. 4, upper row). Similar results were observed when AcLDL-BODIPY was added during the final 30 min of coculture (data not shown). AcLDL did not inhibit the capture of membrane from live cells, as it was only present for a short period for labeling, and was absent for the majority of the coculture. To determine whether membrane proteins captured from live cells enter the endocytic pathway, we used markers of early and late endosomes. Vesicles containing TMRM derived from labeled cells were positive for EEA-1 as determined by Ab labeling (Fig. 4, middle row). In addition, TMRM colocalized with pepstatin A, which binds cathepsin D in late endosomes (27) (Fig. 4, lower row). These findings indicate that vesicles containing labeled membrane proteins derived from live cells traffic with SR through the endosomal pathway and accumulate in late endosomes within DC.

DC uptake of tumor Ag from live cells

To this point, we have used fluorescent dyes to monitor movement of membrane lipids and proteins and have focused on the transfer of label between DC. To demonstrate biological relevance of our finding and to investigate the capacity for DC to acquire Ag from nonhemopoietic cells, we followed the interactions of DC with the...
adenocarcinoma cell line A549 expressing the tumor Ag gp100. Based on sequence analysis, gp100 encodes a putative transmembrane domain and is predicted to localize to the plasma membrane (28). A549 cells were infected with Ad-gp100 (17) and washed to remove free virus, to prevent potential direct infection of DC. All A549 cells expressed gp100 on the cell surface following 24 h of culture, as determined by Ab labeling (Fig. 5, a and b). A549 cells remained viable following transduction with Ad-gp100 due to the low multiplicity of infection used, as measured by exclusion of trypan blue (data not shown). To study uptake of gp100 from live cells by DC, we cocultured infected A549 cells with equal numbers of DC for 3 h and then labeled with gp100-specific Ab. Strikingly, numerous vesicles containing gp100 were identified within multiple DC (Fig. 5c). Ab labeling of untransduced A549 cells and DC cocultures showed no fluorescence (data not shown). We observed a similar capture of MHC class I molecules from viable allogeneic B cells by DC, indicating that the capture of Ag by DC was not the result of overexpression and subsequent release of membrane Ag induced by adenovirus infection (data not shown). These data demonstrate that a virus-encoded protein expressed in a viable cell can be readily captured and internalized by DC. Moreover, the data show that Ag capture is not restricted to transfer between DC or uptake from lymphocytes (17), as DC can also readily capture Ag from epithelial cells.

SR-A-specific Ab inhibits cross-presentation of live-cell Ag

To confirm the immunological significance of our findings, we utilized a T cell stimulation assay that uses Ad-gp100 and TIL620, an HLA-A2-restricted CTL line specific for gp100 (17). We first used gp100-expressing monkey DC, taking advantage of the fact that monkey DC lack HLA-A2 and cannot stimulate TIL620 (17). As expected, HLA-A2+ human DC and macrophages were able to efficiently present gp100 Ag to T cells when directly infected with Ad-gp100, as measured by IFN-γ production (Fig. 6a). However, only HLA-A2+ immature and mature DC, and not macrophages, were able to cross-present gp100 Ag acquired from live Ad-gp100-transduced monkey DC (Fig. 6a). When compared with direct presentation of Ag, cross-presentation by immature and mature DC elicited between 30 and 40% IFN-γ production (Fig. 6a). The finding that mature DC cross-present gp100 as efficiently as immature DC while expressing lower levels of SR (Fig. 3) most likely reflects the increased capacity for these cells to serve as APC (29). Class A and B SR are the best-characterized SR classes and are known to mediate Ag uptake by phagocytes (30). We used Ab known to block function of these two SR to determine whether they played a role in Ag capture and cross-presentation from live cells by DC. Ab specific to SR-A reduced cross-presentation of Ag by immature DC by 50%, while having no effect on direct Ag presentation (p < 0.05, Fig. 6b). Ab to CD36 had no effect on either the direct or cross-presentation of Ag (Fig. 6b). To test whether Ag captured from epithelial cells can be cross-presented by DC in a SR-A-dependent manner, we performed similar experiments using gp100-expressing A549 cells. Ad-gp100-transduced A549 cells could not directly stimulate TIL620, whereas coculture with HLA-A2+ DC resulted in significant IFN-γ release by T cells (Fig. 6c). This was a result of Ag capture by DC, as cocultures with A549 cells that were fixed to eliminate membrane fluidity failed to stimulate TIL620 (Fig. 6c). The presence of SR-A Ab inhibited stimulation of T cells by 70%, similar to that induced by poly(G) (p < 0.05), whereas the presence of poly(C) or Ab to CD36 had no effect on IFN-γ production by TIL620 (Fig. 6c). In the fluorescence transfer assay, Ab specific for SR-A significantly inhibited uptake of membrane from live cells by monkey DC, although inhibition was not complete (p < 0.05, Fig. 6d). Ab to CD36 and αv integrin, both of which are involved in apoptotic cell uptake (11, 20), failed to reduce uptake from live cells (Fig. 6d). Taken

**FIGURE 5.** Visualization of protein Ag acquired from nonhemopoietic cells by DC. a and b, A549 cells were transduced with Ad-gp100 and stained with Ab to gp100 after 24 h. a, Expression of gp100 in untransduced and Ad-gp100-transduced A549 cells. b, Surface expression of gp100 on Ad-gp100-transduced A549 cells, as determined by confocal microscopy. The gp100 staining (green) and nuclear staining (red) were overlaid onto DIC images. c, Viable Ad-gp100-transduced A549 cells were cocultured with equal numbers of monkey DC for 3 h. Cells were then stained with syto dye (red) and Ab to gp100 (green) and analyzed by confocal microscopy. Arrows highlight vesicles containing gp100 captured from viable A549 cells within DC. Overlay includes DIC to highlight DC morphology.
Percentages represent IFN-γ/H9253/H11001 by cocultures of human HLA-A2/gp100-expressing HLA-A2. These data suggest that SR-A and not CD36 or CD40L (Cultured) show moderate expression of SR-A. Numbers indicate median fluorescence intensity of control (upper) and SR-A Ab-labeled cells (lower).

**FIGURE 6.** Ab specific for SR-A inhibits capture and cross-presentation of live-cell Ag by DC. a, Stimulation of gp100-specific T cells by gp100-expressing HLA-A2* human cells (Direct) or by cocultures of human HLA-A2* cells and viable gp100-expressing monkey DC (Cross). Percentages represent IFN-γ spots elicited by cross-presentation as compared with direct presentation. Error bars delineate the SEM of duplicate determinations, and data are representative of two experiments. b, Stimulation of gp100-specific T cells by gp100-expressing HLA-A2* human DC (Direct) or by cocultures of human HLA-A2* DC and viable gp100-expressing monkey DC (Cross) with or without preincubation of human DC with Ab to CD36 or SR-A. Error bars delineate the SEM of duplicate determinations, and data are representative of two experiments. SEM of cross-presentation wells in the presence of SR-A Ab was 0. c, Stimulation of gp100-specific T cells by cocultures of human DC and viable or fixed (A549F) gp100-expressing A549 cells with or without preincubation of human DC with Ab or polyanionic ligand. Error bars delineate the SEM of triplicate determinations. d, Unlabeled monkey DC were preincubated with Ab, as indicated, and then cultured with an equal number of DiD- and TMRM-labeled DC for 3 h before analysis by flow cytometry. Bars indicate reduction in the percentage of unlabeled DC that acquire fluorescence in the presence of Ab based on untreated profiles at 0 and 3 h. Error bars delineate the SEM of three experiments. Where indicated, bars not having the same letter are significantly different (p < 0.05).

**FIGURE 7.** SR-A expression on monocyte-derived and lymph node DC. a, Western blot of lysates of immature and mature human monocyte-derived DC. Immature DC express a 40-kDa protein, whereas mature DC primarily express a 50-kDa variant of SR-A, consistent with SR-AII and SR-AI, respectively. b, Lymph node single-cell suspensions from normal monkeys were stained with Ab to MHC class II, CD83, and SR-A, respectively. Density plot of MHC class II vs CD83 delineates a population of MHC class IIbrightCD83 cells (R1) and SR-AAb-labeled cells (R2) that represent interdigitating DC. Gate R1 represents a population of control cells. Interdigitating DC from lymph node stained immediately after disruption (Fresh), or after overnight culture with CD40L (Cultured) show moderate expression of SR-A. SR-AI expression on monocyte-derived and lymph node DC. SR-A exists in two different isoforms based on alternative splicing of mRNA. To determine which isoform(s) of SR-A is expressed by monocyte-derived DC, we did Western blot analysis using the SR-A-specific Ab. Western blotting of immature human DC revealed a protein of 40 kDa (Fig. 7a), consistent with type II SR-A (SR-AII) (31). Given that DC matured with CD40L cross-present Ag from live cells to a similar degree as immature DC, we also examined SR-A expression on mature DC. Surprisingly, mature human DC expressed low levels of the 40-kDa protein, but prominently expressed a 50-kDa protein (Fig. 7b), consistent with SR-AI (31). These data indicate that maturation induces a change in the isoform of SR-A expressed by DC.

To determine whether live-cell Ag capture by DC may occur in vivo, we looked for SR-A expression on DC isolated directly from tissues. We analyzed interdigitating DC in monkey lymph nodes, which we have previously characterized as having high level expression of MHC class II and CD83 (32). Single cell suspensions were labeled with Ab to MHC class II, CD83, and SR-A, and analyzed by flow cytometry. A discrete population of MHC class IIbrightCD83bright cells was identified that represented 0.4% of total lymph node cells, consistent with interdigitating DC (32) (Fig. 7b). Freshly isolated DC, which are highly autofluorescent due to large size, had 2.4-fold greater fluorescence when labeled with SR-A Ab as compared with control goat serum, indicating moderate SR-A expression (Fig. 7b). SR-A expression on interdigitating DC was maintained following an in vitro culture period in the presence of CD40L (Fig. 7b), consistent with our findings using mature monocyte-derived DC. SR-A and control Ab produced identical low level staining on MHC class II and CD83 cells (Fig. 7b). Similar together, these data suggest that SR-A and not CD36 or αv integrin mediates Ag capture and cross-presentation from live cells. However, the incomplete blocking of DC capture of fluorescently labeled membrane suggests that other SR may also contribute to this process.
results were found when splenic DC from monkeys were analyzed for SR-A expression (data not shown).

Discussion

DC are highly motile cells with long dendritic processes that facilitate interaction with numerous cell types both in tissues and in culture (29). We have previously shown that in the course of these interactions, DC can acquire pieces of membrane and cytoplasm from neighboring cells that are internalized into multiple endocytic vesicles (17), a process referred to as nibbling (1). Ag captured in this manner is cross-presented to MHC class I-restricted T cells (17). This is consistent with earlier reports demonstrating the need for Ag transfer between DC for optimal Ag-specific T cell proliferation (33). In this study, we demonstrate that capture of Ag from live cells is a receptor-mediated process that uses SR, especially SR-A. We found no evidence for involvement of CD36 or αv integrin, the receptors that mediate DC uptake of apoptotic cells (11), in this process.

SR are an expanding family of structurally diverse molecules that exhibit promiscuous binding to polyanionic ligands (24). As a result of their binding properties, SR possess a broad array of functions, including clearance of lipoproteins (34), uptake of pathogens such as bacteria (35, 36), and intercellular adhesion (37). SR-A, CD36, and LOX-1 also play a role in the phagocytosis of apoptotic cells (11, 21, 38, 39). Our results reveal a new function for SR-A, and provide a mechanism whereby DC can capture cellular Ag from live cells (17). SR-A include SR-AI and the splice variants SR-AII and SR-AIII (23). SR-AI and SR-AII are functionally indistinct from one another, both possessing a collagenous domain that is responsible for polyanionic ligand binding, and differ only with regard to the presence or absence of the C-terminal SR cysteine-rich domain that has an unknown function (22, 40). SR-AIII is a nonfunctional splice variant that is trapped in the endoplasmic reticulum and fails to get to the cell surface (41). Our data indicate that immature DC express SR-AII and that maturation induces a switch in isoform expression to SR-AI. Similarly, macrophages undergo a switch in SR-A expression when differentiated from monocytes, with differentiation being associated with a selective increase in SR-AI, although the significance of this switch is not known (42). Consistent with previous reports (25, 26, 42), our findings indicate that primary macrophage lines cultured in the absence of cytokines express minimal levels of SR, providing an explanation for the inability of these cells to capture Ag from live cells (17).

The role of SR in mediating the capture of Ag from live cells by DC in vivo has yet to be defined. We show that MHC class II<sup>bright</sup> CD8<sup>bright</sup> interdigitating DC in monkey lymph nodes express low levels of SR-A, although freshly isolated or cultured Langerhans cells appear to lack expression of SR-A (data not shown). A recent report suggested that tissue expression of SR-A in the human was restricted to macrophages, although the lack of double-label staining in this study makes it impossible to confirm the identity of SR-A-expressing cells (43). In the human tonsil, as many as five distinct populations of DC have been identified using multiple cell surface markers (44), and a critical analysis of all these DC subsets is needed to determine the relative expression of SR-A on DC and macrophages in vivo. The situation in the mouse appears to be complex. SR-A is found primarily on the CD8<sup>+</sup> DC fraction, although it is the CD8<sup>+</sup> DC subset that mediates cross-presentation of cell-associated Ag (12, 13, 45, 46). Furthermore, SR-A knockout mice have no apparent autoimmune dysfunction (47), which might be expected if Ag capture from live cells was mediated exclusively by SR-A. However, CD36 was the only Ag uptake receptor from a large panel found to be expressed at higher levels on CD8<sup>+</sup> as compared with CD8<sup>+</sup> DC (46), but the lack of CD36 does not influence cross-presentation of cell-associated Ag in knockout mice (12, 13). These data suggest that as yet undefined receptors, potentially SR, play an important role in cross-presentation of cell-associated Ag by DC in the mouse. Moreover, our Ab-blocking data raise the possibility that SR other than SR-A may contribute to the process of live-cell nibbling in vitro in the human and monkey, suggesting a similar redundancy of receptors to those mediating apoptotic cell uptake (48). It is also clear that individual SR can have more than one function. LOX-1 mediates uptake of apoptotic bodies as well as the binding of heat-shock proteins in the cross-presentation of tumor Ag by DC (39, 49), and SR-A mediates uptake of apoptotic cells (21) as well as the capture of Ag from live cells, as we have shown. This most likely reflects the promiscuous nature of SR binding, with the only ligand crossting existence of polyanionic charges (24). The potential role for other SR, particularly LOX-1, in DC nibbling from live cells is currently being examined.

Our data using the putative type I membrane protein gp100 clearly demonstrate that cell surface Ag can be captured from live cells by DC in an SR-specific manner. Amazingly, capture of endogenous protein Ag by DC from live cells was readily detected after only 3 h of coculture when a ratio of 1 donor cell to 1 DC was used, suggesting that the process is highly efficient. Our previous studies using real-time microscopy demonstrated that uptake of membrane-associated molecules by DC was the result of membrane nibbling during extended and intimate contact with other live cells that often occurred repeatedly (17). It is interesting to speculate that the prominent T cell response to gp100 and other tumor-associated Ag, such as MART-1, observed in many melanoma patients may be due in part to the fact that these are putative transmembrane proteins (28, 50), and thus may readily be acquired by tumor-associated DC from live tumor cells for cross-presentation to T cells in draining lymph nodes. Internalization of peptide-MHC complexes from viable APCs by T cells has previously been demonstrated (51). However, our findings are significantly different in that DC did not require Ag-specific engagement with donor cells for capture of membrane Ag to occur. We propose that DC are constantly interrogating other cells and internalizing surface Ag acquired from these cells. Whether cross-presentation ultimately results in T cell tolerance or immunity most likely is a function of the microenvironment in which Ag was encountered and the resulting effect on DC maturation and activation (10).

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