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Dendritic Cell Surface Calreticulin Is a Receptor for NY-ESO-1: Direct Interactions between Tumor-Associated Antigen and the Innate Immune System¹

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How the immune system recognizes endogenously arising tumors and elicits adaptive immune responses against nonmutated tumor-associated Ags is poorly understood. In search of intrinsic factors contributing to the immunogenicity of the tumor-associated Ag NY-ESO-1, we found that the NY-ESO-1 protein binds to the surface of immature dendritic cells (DC), macrophages, and monocytes, but not to that of B cells or T cells. Using immunoprecipitation coupled with tandem mass spectrometry, we isolated DC surface calreticulin as the receptor for NY-ESO-1. Calreticulin Abs blocked NY-ESO-1 binding on immature DC and its cross-presentation to CD8⁺ T cells in vitro. Calreticulin/NY-ESO-1 interactions provide a direct link between NY-ESO-1, the innate immune system, and, potentially, the adaptive immune response against NY-ESO-1. *The Journal of Immunology*, 2006, 177: 3582–3589.

Spontaneous immune responses against tumor-associated Ag (TAA)³ induced by progressive tumors in an unmanipulated host are readily detectable. It is speculated that the initiation of anti-TAA responses resembles that against bacteria and viral products in which the innate immune cells such as immature dendritic cells (DC) and macrophages sense “danger signals” (1). Instead of pathogen-associated molecular patterns (2), cancer cell death releases endogenous danger signals such as heat shock proteins (3), apoptotic bodies (4), and uric acid (5), to alert the innate immune system. TAA are generally perceived to be associated with the above danger signals or endogenous adjuvants. According to this paradigm, “spontaneous” anti-tumor immune responses may preferentially recognize products resulting from genetic alternations within cancer cells, against which the host keeps less stringent immune tolerance. However, the human TAA identified to date are mostly nonmutated self-Ags (6). We hypothesize that intrinsic factors from some TAA may directly interact with the innate immune system. In search of intrinsic factors from TAA, we focused on NY-ESO-1, a nonmutated cancer/testis Ag with dis-

tinctively strong immunogenicity (7, 8). Approximately 40% of cancer patients with NY-ESO-1-expressing tumors naturally develop Abs against the protein without immunization (8). “Spontaneous” class I- and class II-restricted T cells are present in patients with NY-ESO-1-positive tumors (9, 10). The “spontaneous” immune response to NY-ESO-1 is not due to levels of expression higher than those of other TAA. Indeed, the expression of NY-ESO-1 is much lower than that of melanocyte differentiation Ags or other cancer/testis Ags based on quantitative real-time PCR analysis of primary tumor samples and early tumor cultures (11). The involvement of NY-ESO-1-specific CD4⁺ T cells in patients with Ab against the protein has been described (10). Although gene-specific CD4⁺ T cells are necessary, they are not sufficient to account for the immunogenicity of NY-ESO-1. Further elucidating the mechanisms responsible for the superior immunogenicity of NY-ESO-1 may illustrate important principles regarding cancer and immune system interactions.

Materials and Methods

Cell lines and reagents

Human leukopheresis was performed by the Surgery Branch of the National Cancer Institute (Bethesda, MD) and the virology core at the University of California Los Angeles Jonsson Comprehensive Cancer Center (Los Angeles, CA). PBMCs from leukopheresis were positively selected for CD14⁺ cells using magnetic beads (Miltenyi Biotec). CD14⁺ cells were then cultured in Iscove's medium supplemented with 10% human serum in the presence of GM-CSF and IL-4, each at 1000 U/ml, for 6 days to generate immature DC. When required, immature DC were cultured with 500 ng/ml CD40L trimers to obtain mature DC. B cells were expanded using CD40L (500 ng/ml, Amgen) plus IL-4 (500 u/ml). The murine immature DC line DC2.4 was obtained from Dr. K. Rock of the University of Massachusetts (Worcester, MA).

The purification of His-tagged NY-ESO-1 and gp100 proteins was as previously reported (12). The purification schema was scaled up at Novavax to obtain clinical, good manufacturing practice-grade proteins for vaccine clinical trials at the National Cancer Institute Surgery Branch. A good manufacturing practice-grade NY-ESO-1 recombinant protein was provided at 1 mg/ml with LPS content <10 enzyme units/mg/ml, and the gp100 protein was at 800 μg/ml with LPS content below 10 enzyme units/mg/ml. A truncated NY-ESO-1 protein, ESO1-74, which contained only the first 74-aa residues, was purified as previously described (12). Murine

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³ Abbreviations used in this paper: TAA, tumor-associated Ag; CRT, calreticulin; CD40L-B, CD40L-activated B cell; DC, dendritic cell; ER, endoplasmic reticulum; GAMF, FITC-labeled goat anti-mouse secondary Ab; KLH, keyhole limpet hemocyanin.

mAb against NY-ESO-1, termed mAb 131 and mAb 132, were generated at the National Cancer Institute Frederick Development Center (Frederick, MD). Alexa 488-labeled OVA and keyhole limpet hemocyanin (KLH) proteins were gifts from Dr. J. Timmerman at University of California Los Angeles. Purified rabbit calreticulin (CRT) and monoclonal and polyclonal Ab from rabbit against bovine CRT were as previously described (13).

Ab conjugation, DC binding assay, and confocal microscopic analysis

Direct conjugation of FITC with the NY-ESO-1 protein failed due to the low solubility of recombinant NY-ESO-1 at the concentration (>2 mg/ml) required for carrying out the reaction. mAb specific for NY-ESO-1, mAb 131, and mAb 132, were labeled with FITC following the manufacturer's instruction (Sigma-Aldrich). The molecular ratios of FITC to both mAb were determined as between 4 and 6 according to a procedure recommended by the manufacturer. The labeled mAb was diluted to 0.5 μ g/ml, and 0.5 μ l of the stock was used for staining 10^5 cells pulsed with the proper amount of proteins.

All protein-DC binding experiments were conducted on ice in 50 μ l and in the presence of 5% FCS to block nonspecific interactions. Unless specified, the protein concentrations used were 3, 10, and 1 μ g/ml for NY-ESO-1, gp100, and ESO1-74, respectively. After a 20-min pulse, cells were washed extensively using cold PBS supplemented with 5% FCS. FITC-labeled NY-ESO-1-specific mAb or a first Ab plus a FITC-labeled secondary Ab were used to stain cells on ice followed by flow cytometry analysis on a FACSCalibur (BD Pharmingen) machine at the University of California Los Angeles Jonsson Comprehensive Cancer Center.

For confocal microscopic analysis of the localization of NY-ESO-1 protein acquired by immature DC, pulsed cells were switched to 37°C for 15 min in the presence of 10 μ g/ml neutral red (Molecular Probe/Invitrogen Life Technologies). Cells were then fixed and intracellularly stained with FITC-labeled mAb 132. To visualize the relative positional distribution of two fluorochromes, the images in two different channels were merged and photographed using a laser scanning system attached to a microscope (Zeiss).

Immunoprecipitation and mass spectrometry analysis

Cell lysates from 2×10^8 DC2.4 and the control EL4 line were incubated on ice with the NY-ESO-1 or gp100 protein at 1 and 3 μ g/ml, respectively. Pull-down was achieved by using an agarose-conjugated mAb (Sigma-Aldrich) against the polyhistidine tag present on the N termini of both proteins. Proteins were analyzed on a SDS-gel followed by staining with Imperial protein stain (Pierce/Endogen). Sections of SDS-gel were sliced and sent to ProtTech for the identification of specific proteins. In brief, each slice was in-gel digested with modified sequencing grade trypsin (Promega), and the resulting peptide mixture was subjected to peptide sequencing by tandem mass spectrometry. A Finnigan ion trap mass spectrometer LCQ coupled with a HPLC system running a 75 - μ m internal diameter C18 column was used. Data were acquired in a data-dependent mode. Tandem mass spectrometry spectra were used to search the most recent nonredundant protein database from GenBank with the ProtQuest software suite (ProtTech). The output from the database search was manually analyzed and validated at ProtTech.

Surface plasmon resonance analysis

Surface plasmon resonance analysis was conducted with a BIAcore 3000 system in the laboratory of Dr. R. Lehrer (University of California Los Angeles Department of Medicine) with the assistance of Dr. W. Wang (University of California Los Angeles School of Medicine). Purified NY-ESO-1 and control BSA proteins were immobilized on a CM5 sensor chip to $\sim 10,000$ resonance units. Various concentrations of purified rabbit CRT protein and heat shock protein 70 (Calbiochem) in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 2.5 mM CaCl_2 were injected for 1 min at 50 μ l/min. The sensorgrams were obtained after subtracting the resonance values against BSA, which were then analyzed following the 1:1 Langmuir binding interaction model. The association rate constant, k_a , and the dissociation rate constant, k_d , were determined by the average of global fittings of multiple curves. The dissociation constant was determined by $K_d = k_d/k_a$.

In vitro cross-presentation to NY-ESO-1-specific CTL clones

Day 6 immature DC were incubated with NY-ESO-1 (3 μ g/ml), gp100 (10 μ g/ml), and ESO1-74 (1.2 μ g/ml) proteins and peptide fragments in OptiMEM medium (Invitrogen Life Technologies) for 8 h in the presence of β 2-microglobulin (2 μ g/ml). Cells were then washed extensively before being cocultured with 586 CTL clone 10 recognizing the HLA-A31 re-

stricted ESO:53-62 peptide or CTL BL8-1 recognizing the HLA-A2 restricted ESO:157-167 peptide. A CD4^+ helper T cell (CT4-1) recognizing the ESO:161-180 peptide and a gp100:209-217 specific CTL (clone CK3H6) were used as controls. To block NY-ESO-1 cross-presentation, rabbit anti-CRT sera or sera against a control protein were incubated with immature DC for 20 min on ice followed by the addition of NY-ESO-1 protein (6 μ g/ml) or a peptide epitope (0.1 μ g/ml). After incubating at 37°C for 30 min, DC were washed and incubated with equal numbers of effector 586 CTL clone 10 for 24 h before IFN- γ release was determined.

Results

NY-ESO-1 protein directly binds to immature DC

The NY-ESO-1 proteins used in this study are of clinical good manufacturing practice grade as described in *Materials and Methods*. The DC binding experiment was performed on ice with all cells and reagents prechilled. This treatment minimized the macropinocytosis and phagocytosis of immature DC. As shown in Fig. 1A, FITC-labeled Ab strongly stained NY-ESO-1-pulsed DC, but not CD40L-activated B cells (CD40L-B), from the same donor. The staining was not due to nonspecific binding of the Ab to DC, because DC pulsed with gp100 protein were not stained with the NY-ESO-1 Ab. Control Ab conjugated with FITC did not stain NY-ESO-1-pulsed DC, which ruled out the possibility that NY-ESO-1 might nonspecifically bind to FITC (data not shown). In a different experiment, immature DC were pulsed with NY-ESO-1, ESO1-74, and gp100, which were tagged with N-terminal polyhistidine. The protein-pulsed immature DC were then incubated with a mouse mAb to the polyhistidine tag followed by a FITC-labeled goat anti-mouse secondary Ab (GAMF). Only immature DC pulsed with the full-length NY-ESO-1 protein were detected by GAMF (Fig. 1B). NY-ESO-1 purified from baculovirus and bacteria had similar binding to immature DC and slightly less binding to DC matured by CD40L (data not shown). In addition, neither OVA nor the conglomerate KLH protein bound to immature DC, further suggesting that the NY-ESO-1/DC interaction was specific (Fig. 1C).

Confocal microscopic analysis was used to monitor the localization of NY-ESO-1 during and after its interaction with immature DC. Cell surface staining of a bound NY-ESO-1 protein showed a patched pattern (Fig. 1*Div*). Immature DC pulsed with NY-ESO-1 on ice were washed and switched to 37°C for 15 min in the presence of neutral red, which is permeable to DC and accumulates in the acidic compartment. Cells were then fixed and stained intracellularly with FITC-labeled mAb 132 in the presence of saponin, which was subsequently removed to allow the membrane to reseal. The images of green and red staining, which indicated the location of NY-ESO-1 and the lysosomal/endosomal compartments, respectively, were superimposed. We observed colocalized yellow spots (Fig. 1*Dvii*), indicating that NY-ESO-1 entered the lysosomal/endosomal compartment after binding to immature DC.

NY-ESO-1 binding to DC is mediated by a DC surface receptor

Due to the difficulty of directly labeling the NY-ESO-1 protein, we could not show a quantitative binding equilibrium or competitive binding inhibition with unlabeled NY-ESO-1. Instead, we measured the percentage of cells binding to an increasing amount of NY-ESO-1 (Fig. 2A). NY-ESO-1 protein apparently bound to immature DC (both human and mouse) and macrophages in a saturable manner, suggesting the presence of a putative receptor and a receptor homologue in the mouse. In contrast, NY-ESO-1 did not bind to CD40L-B cells and early stages of EBV immortalized B cells from the same donor. NY-ESO-1 binding to immature DC

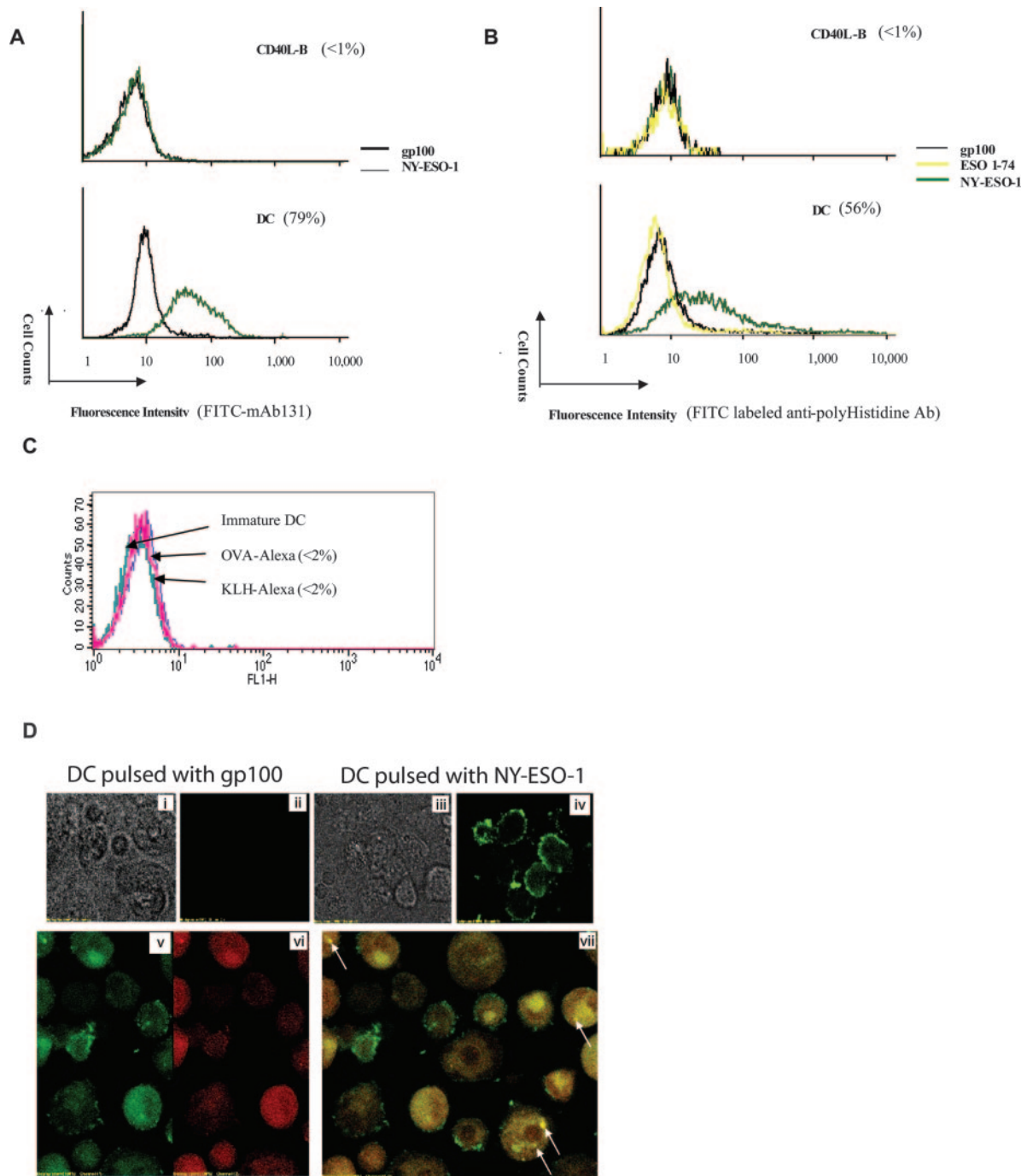


FIGURE 1. NY-ESO-1 protein binds to the surface of monocyte-derived immature DC. *A*, Immature DC and CD40L-B cells were incubated with NY-ESO-1 (green line) or gp100 protein (dark line) in a DC binding assay and stained with mAb 132. DC matured with CD40L had only slightly lower binding to NY-ESO-1 (data not shown). *B*, NY-ESO-1 (green line), ESO1-74 (yellow line), and gp100 (dark line) proteins were pulsed on immature DC and stained with a mAb specific for polyhistidine, which was present on the N termini of all three proteins, followed by staining with GAMF. In parentheses in *A* and *B* are the Abs used (below the graphs) and the percentages of cells stained with the Ab (above in the graphs). *C*, Alexa 488-labeled OVA (purple) and KLH (red) at 10 $\mu\text{g/ml}$ were used for binding to immature DC under the same conditions as in *A* and *B*. DC alone is shown by the green line. All of the above experiments have been repeated with cells from at least three donors. *D*, Confocal microscopic analysis of the NY-ESO-1 protein acquired by immature DC. Day 6 immature DC were incubated with 150 $\mu\text{g/ml}$ gp100 (*i* and *ii*) or 50 $\mu\text{g/ml}$ NY-ESO-1 protein (*iii* and *iv*) in a DC binding assay and stained with FITC-labeled mAb132. The fluorescent images shown in *ii* and *iv* were from the same fields as *i* and *iii*, respectively. In *v*, *vi*, and *vii*, cells were pulsed with NY-ESO-1 on ice and then “chased” for 15 min at 37°C in the presence of neutral red. Cells were washed again before being fixed, followed by staining with FITC-labeled mAb 132 in the presence of 0.01% saponin. Cells were washed again with buffer containing no saponin to allow the cell membrane to reseal. Parts of the green (*v*) and red channels (*vi*) were shown for the localization of NY-ESO-1 and neutral red, respectively. The superimposed image (*vii*) shows the colocalization of NY-ESO-1, and the endosomal/lysosomal compartments are indicated by arrows.

was dependent on divalent cations and significantly inhibited by EDTA (Fig. 2B). In addition, the binding was gradually diminished with increased trypsin treatment, indicating the involvement of a

protein receptor on the DC surface (Fig. 2C). Similarly, pretreatment of immature DC with paraformaldehyde abolished the binding of DC to NY-ESO-1, suggesting that the binding was sensitive

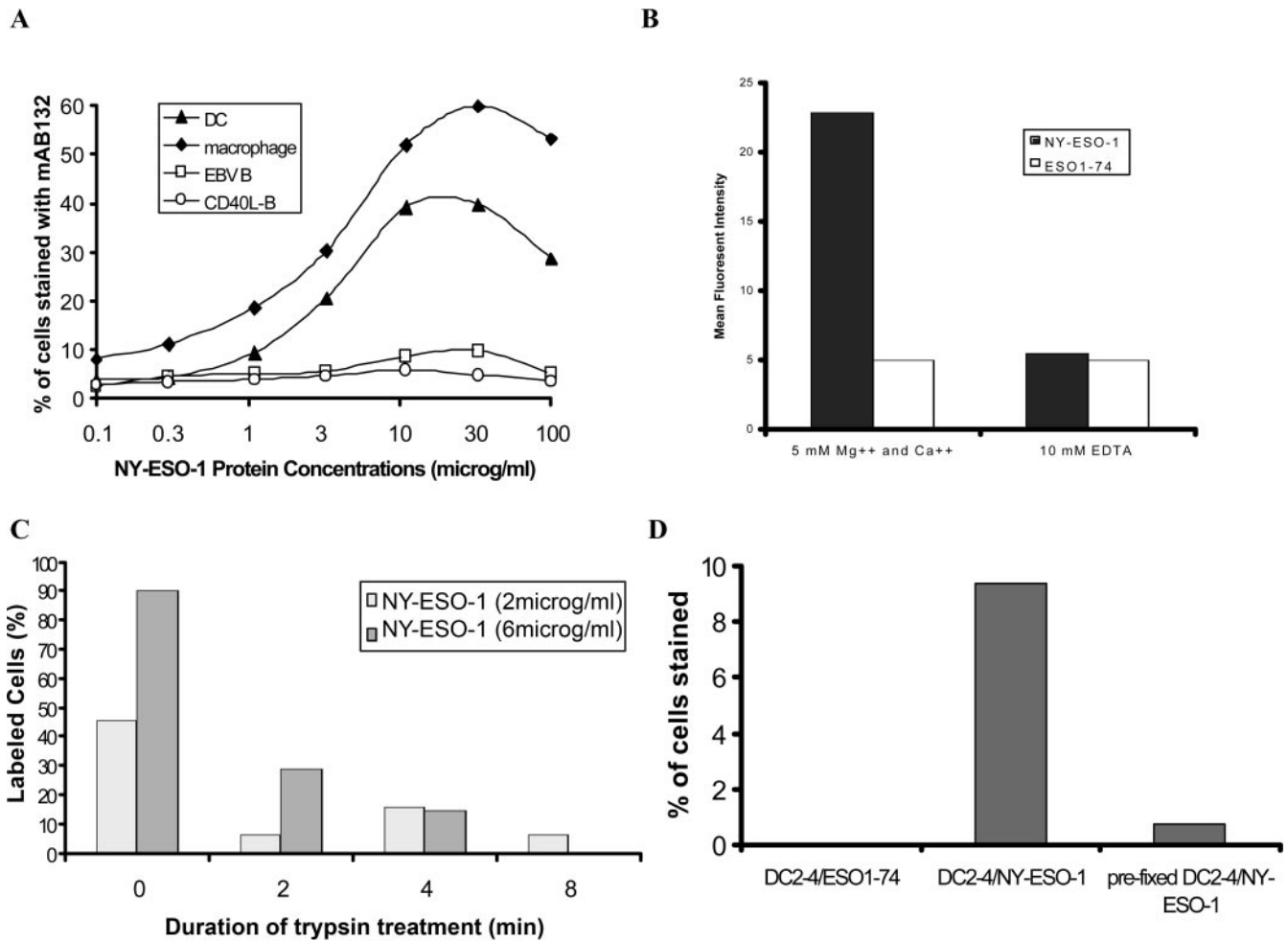


FIGURE 2. Supporting evidence that NY-ESO-1 binds to a DC surface receptor. *A*, Primary cell cultures were incubated on ice with increasing amount of NY-ESO-1 protein in the presence of 100 μ M free arginine to block the negatively charged carbohydrate groups on the cell surface. All cells were from the same donor. *B*, Binding of NY-ESO-1 (3 μ g/ml) to the murine DC2.4 line in the presence of 5 mM MgCl₂ and CaCl₂ or the presence of 10 mM EDTA. To prevent the introduction of divalent cations present in FCS, FCS was reduced from 5 to 1% in the blocking buffer. ESO1-74 was used as a control. *C*, Immature DC were pretreated with trypsin for the indicated time before being used in the binding assay with NY-ESO-1 at the indicated concentrations. *D*, Pretreatment of murine DC2.4 with formaldehyde (1%) for 5 min at room temperature abolished their binding to NY-ESO-1 (3 μ g/ml). In experiments involving DC2.4, 1% mouse serum was used and the gating for flow cytometry was adjusted to exclude the potential Fc receptor-mediated binding to mouse mAb 132-FITC. The absolute percentage of DC binding to NY-ESO-1 varied with the source of DC.

to the native conformation of the putative DC surface receptor (Fig. 2D).

Identification of DC surface complement C1q receptor or CRT as a receptor for NY-ESO-1

The murine DC2.4 cell line was used as the source for immunoprecipitation of the putative NY-ESO-1 receptor, whereas EL4, which did not bind to NY-ESO-1 (not shown), served as the control cell line (Fig. 3A). A total of 72 proteins between 39 and 135 kDa (Fig. 3A, lane 3, boxed area) were identified by a sensitive peptide sequencing approach using tandem mass spectrometry. Candidates such as the NY-ESO-1 receptor were determined based on the following criteria: 1) being present on the cell surface of DC and macrophages; and 2) not being present in the control lanes of the SDS-gel. The murine CRT or complement C1q receptor, which usually resides in the endoplasmic reticulum (ER) of normal cells and was present on the surface of immature DC and macrophages, was the only protein meeting the first criterion among the 72 proteins identified from lane 3 in Fig. 3A. CRT was identified from gel slice no.12 of lane 3 in Fig. 3A and was absent from corresponding control gel slices of lanes 4 and 5 (Table I), which made CRT the

only protein meeting both of the above criteria. Flow cytometry analysis of cell surface CRT in major leukocyte populations from healthy donors revealed cell surface staining of immature DC, macrophages, and monocytes, but not of T cells or B cells, which correlated with their binding to the NY-ESO-1 protein (Fig. 3B). Direct staining of DC surface CRT was also achieved using an anti-CRT mAb followed by a PE-labeled secondary Ab (Fig. 3C).

To validate DC surface CRT as the NY-ESO-1 receptor, monoclonal and polyclonal CRT Ab and the purified rabbit CRT protein were used as competitors in the NY-ESO-1/DC binding experiment. It is noteworthy that CRT is a highly conserved protein with >95% identity at the amino acid level between those from human, rabbit, and bovine. As shown in Fig. 4A, the binding of NY-ESO-1 to human immature DC was inhibited by the presence of soluble CRT, a mAb, and anti-CRT sera for ~50, 75, and 90%, respectively. The inhibition of binding by anti-CRT sera was further evaluated and found to be dose dependent (Fig. 4B). To quantitatively monitor the binding of NY-ESO-1 to CRT, a surface plasmon resonance assay was performed with NY-ESO-1 immobilized on the surface of a CM5 sensor chip. Purified CRT bound to the immobilized NY-ESO-1 in a dose-dependent manner (Fig. 4C),

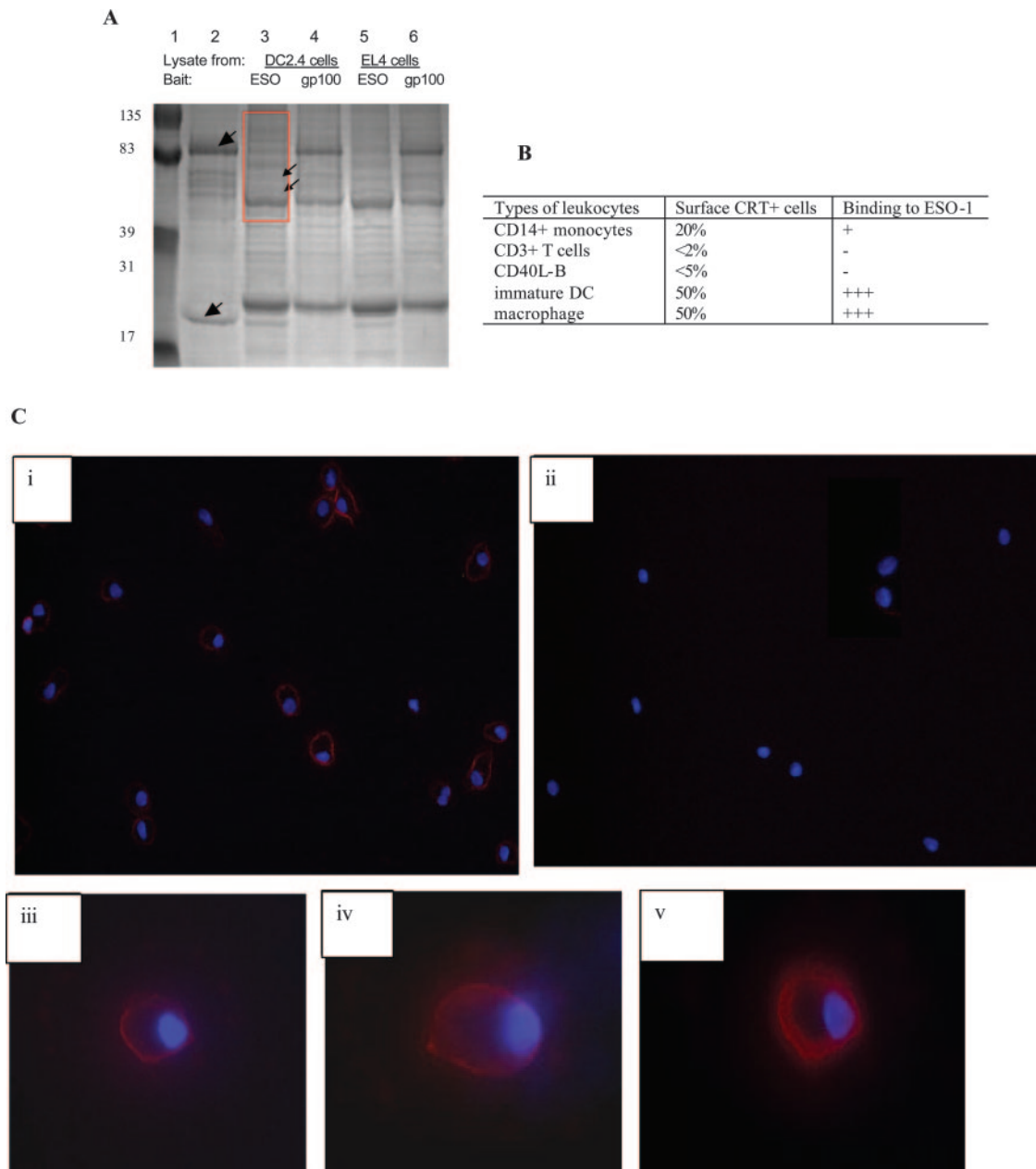


FIGURE 3. Identification of calreticulin as the DC surface receptor for NY-ESO-1. *A*, SDS-PAGE of the immunoprecipitation of NY-ESO-1 receptor. *Lane 1*, molecular mass standard in kDa; *lane 2*, mixture of recombinant NY-ESO-1 (bottom arrow) and gp100 protein (top arrow) (the gp100 protein used in this experiment had some degradation shown as bands below the intact gp100); *lanes 3 and 4*, DC2.4 lysates incubated with the NY-ESO-1 and gp100 protein, respectively, followed by pull-down with anti-polyhistidine Ab conjugated with agarose; *lanes 5 and 6*, EL4 lysates pulled down with NY-ESO-1 and gp100, respectively. The SDS-gel was stained with Imperial Blue. The two arrows in *lane 3* indicated two visible bands only found in DC2.4 lysates pulled down with the NY-ESO-1 protein. The boxed area was sliced and subjected to tandem mass spectrometry analysis. Successful pull-down of the NY-ESO-1 and gp100 protein was apparent in *lanes 3 and 5* as well as *lanes 4 and 6*, respectively. *B*, The presence of cell surface CRT in major leukocyte populations correlated with their binding to NY-ESO-1. CD40L-B cells, CD3⁺ T cells, and CD14⁺ monocytes were all derived from the same donor. This experiment has been repeated using cells from two donors. *C*, Staining of CRT on the surface of immature DC with CRT specific sera (*i*) or control sera (*ii*) followed by PE-labeled goat anti-rabbit Ab. Three enlarged views of individual DC stained with CRT sera are shown in *iii*, *iv*, and *v*. The cell nucleus was counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride, shown in purple.

whereas other molecular chaperones such as heat shock protein 70 did not bind to NY-ESO-1, which further demonstrated the specificity of CRT and NY-ESO-1 interactions. The K_d between CRT and NY-ESO-1 was $0.16 \mu\text{M}$ ($k_a = 2.53 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_d = 3.93 \times 10^{-2} \text{ s}^{-1}$). These experiments provided strong evidence that NY-ESO-1 directly binds to CRT on the surface of DC, macrophages, and monocytes.

CRT/NY-ESO-1 interactions contribute to the cross-presentation of NY-ESO-1.

Immature DC surface CRT is also a critical component of early phagosomes, which are fused with ER-derived membranes soon after phagocytosis (14). Components of these early phagosomes include TAP, tapasin, CRT, and Erp57, which are sufficient for the processing and cross-presentation of exogenous Ags acquired by

Table 1. Results of mass spectrometry analysis of SDS-gel slice no. 12

Fig. 3A, Lane 3 Gel Slice 12	No. of Peptides	Peptide Mass	Sequence Header	Subcellular Localization	Presence in Control Lanes
Protein 1	7	57,878.15	M2-type pyruvate kinase	Cytoplasm	Yes
Protein 2	7	54,506.45	Non-POU domain-containing octamer binding protein	Nucleus	Yes
Protein 3	3	60,302.59	T complex protein 1	Cytoplasm	Yes
Protein 4	3	51,516.66	Ig H chain γ 2a	Contaminating protein?	No
Protein 5	1	59,585.99	Chaperonin subunit 5 (ϵ unit)	Cytoplasm	Yes
Protein 6	1	58,148.23	Chaperonin subunit 6b (ζ unit)	Cytoplasm	Yes
Protein 7	1	50,944.4	Heterogeneous nuclear ribonucleoprotein K	Nucleus	Yes
Protein 8	1	47,964.9	Calreticulin	Cell surface of DC/ macrophage	No

phagocytosis. To investigate whether binding to DC surface CRT is related to the cross-presentation of NY-ESO-1, experiments were conducted to assess the cross-presentation of exogenous NY-ESO-1 protein in vitro. Immature DC pulsed with NY-ESO-1 were able to stimulate NY-ESO-1-specific CD4⁺ and CD8⁺ T cells but not a control CTL CK3H6 specific for gp100:209–217 (Table II). In contrast, immature DC pulsed with a soluble gp100 protein failed to stimulate CTL clone CK3H6. A truncated recombinant protein, ESO1–74, which was not capable of binding to immature DC (Fig. 1B), or a synthetic 41-mer polypeptide, ESO:30–70, did not stimulate 586 CTL clone 10 despite the fact that they both contained the ESO:53–62 epitope. Similarly, DC pulsed with ESO:150–180 failed to stimulate CTL TE8-1 recognizing A2-restricted ESO:157–167, whereas DC pulsed with the same peptide stimulated a CD4⁺ T cell line CT4–1 recognizing HLA-DP4-restricted ESO:161–180. These results demonstrated that only the full-length NY-ESO-1 protein was cross-presented to HLA-A31-restricted and, to a much lesser extent, A2-restricted CD8⁺ CTL. Cross-presentation of class I-restricted epitopes seemed to require sequences beyond the epitope and adjacent regions, because truncated proteins or polypeptides were not cross-presented even though they contained the MHC class I epitopes. This finding implied that structural features promoted efficient uptake and/or cross-presentation of the intact NY-ESO-1 protein, which correlated with their binding to the CRT receptor on immature DC. Blocking of CRT on the surface of immature DC by anti-CRT sera successfully inhibited cross-presentation of NY-ESO-1 protein to a HLA-A31-restricted CTL clone (Fig. 4D). In contrast, presentation of the peptide epitope by DC to the same CTL clone was not affected. This experiment indicated the role of DC surface CRT in cross-presentation of NY-ESO-1 and possibly other tumor- and virus-associated Ags.

Discussion

Molecular mechanisms governing the initiation of “spontaneous” immune responses against endogenously arising tumors are poorly understood, especially in the human system. Major questions remain unanswered, such as the following two. 1) How does the human immune system recognize nascent and established tumor? 2) What makes the adaptive immune system react predominantly to some of the nonmutated self-proteins but not others? NY-ESO-1 represents an attractive TAA for investigating some of the above

issues because of its superior immunogenicity and widely shared expression in human cancers. In this study, we provide evidence that NY-ESO-1 directly engages the innate immune system through the complement C1q receptor or CRT present on DC, macrophages, and monocytes. The apparent dissociation constant between CRT and NY-ESO-1 protein in vitro was ~160 nM, whereas heat shock protein 70 did not bind to NY-ESO-1. Previous studies (15, 16) have shown that CRT normally binds to glycoproteins that carry specific *N*-linked oligosaccharide tags diagnostic of incomplete folding. NY-ESO-1 used in our study is derived from *Escherichia coli* and possesses no glycosylated tags. These lines of evidence directly support our contention that the interaction between NY-ESO-1 and CRT is specific and not an interaction between a chaperone and its client protein. Similar binding affinities are present among other critical molecular interactions known to exert regulatory functions to the innate immune system, such as those between IgGA1/A2 and Fc α RI (17), between the hepatitis B surface Ag and the CD14/TLR4 complex (18), and between heat shock proteins and CD91 (3). Thus, CRT represents an innate immune receptor that directly binds to NY-ESO-1 without mediators.

CRT has been known to be an important immunological player. First, it is a ubiquitous ER-resident chaperone that facilitates MHC class I peptide presentation (19). Second, CRT acts as a macrophage surface receptor for complement C1q to mediate phagocytosis of opsonized pathogens and apoptotic bodies (20, 21). Third, the CRT/CD91 complex on macrophages is responsible for inflammation in response to lung collectins complexed with necrotic bodies (22). Recently, the presence of CRT on the surface of monocyte-derived DC was reported (23). Immature DC selectively express higher levels of surface CRT than mature ones while the total level of CRT protein and RNA remain the same (23), which agrees with our observation that DC matured by certain approaches have a slight lower binding to NY-ESO-1. Immature DC surface CRT is also a critical component of the early phagosome that is fused with ER-derived membranes soon after phagocytosis (14, 24). Components of these early phagosomes include TAP and other loading complexes necessary for MHC class I Ag processing such as tapasin, CRT, and Erp57, which are sufficient for processing and cross-presentation of exogenous Ags acquired by phagocytosis. Our current study identifies DC surface CRT as the receptor for the cancer/testis Ag NY-ESO-1, which may be responsible for the cross-presentation of NY-ESO-1. As shown in

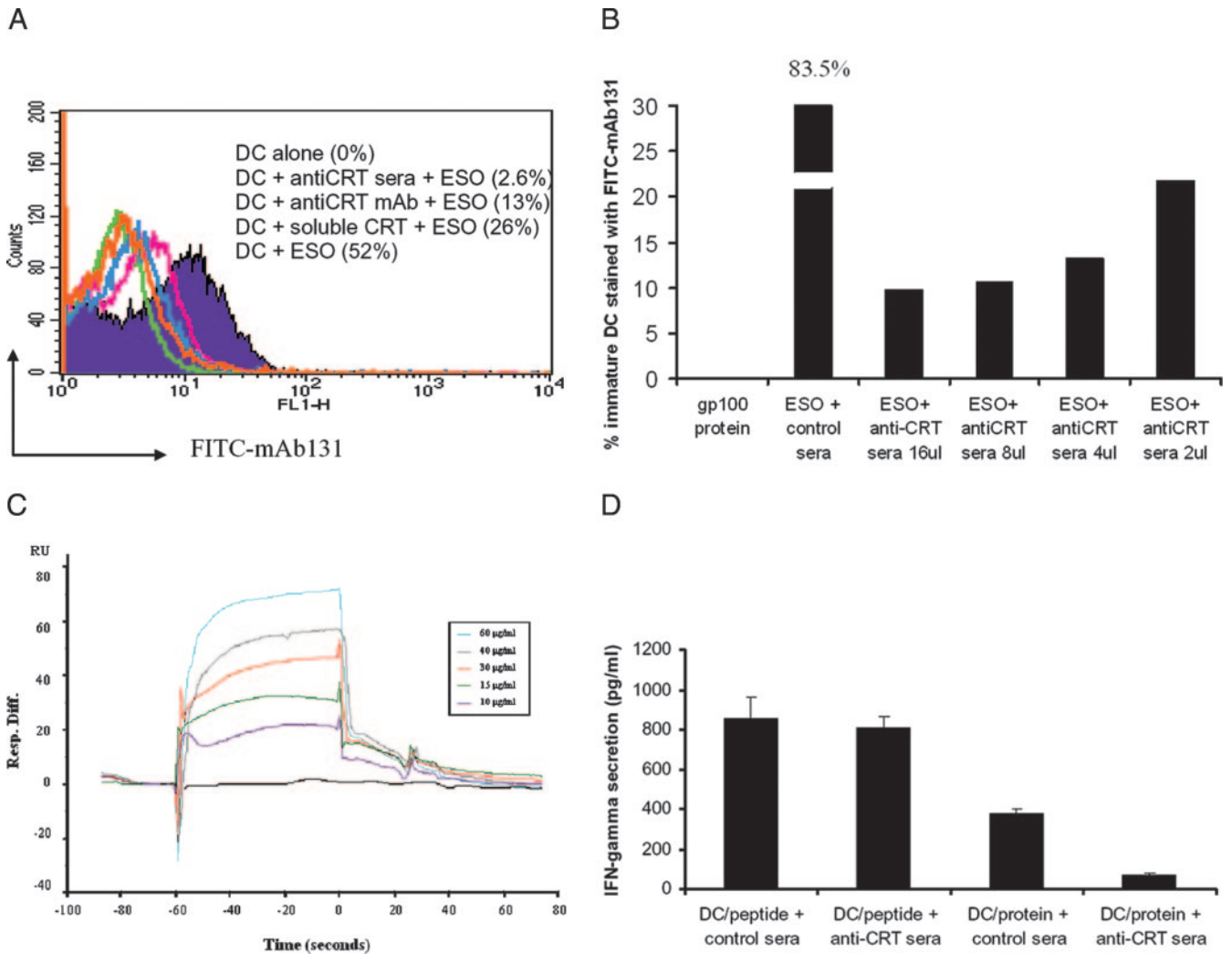


FIGURE 4. A, Blockage of NY-ESO-1 (ESO) (2 $\mu\text{g/ml}$) binding to immature DC by soluble rabbit CRT protein at 100 $\mu\text{g/ml}$ (red line), a mAb against bovine CRT at 100 $\mu\text{g/ml}$ (sky blue line), and polyclonal sera against bovine CRT (10 μl , orange line). Immature DC (green line) and DC binding to NY-ESO-1 (blue histogram) were also shown. Numbers in parentheses are percentages of cells binding to immature DC. B, Dose-dependent inhibition of the NY-ESO-1 (ESO) protein (2 $\mu\text{g/ml}$) binding to immature DC by polyclonal sera against bovine CRT. The number on top of the broken column indicates the percentage of DC binding to NY-ESO-1. C, Surface plasmon resonance experiment showing the interaction between purified CRT at the indicated concentrations and NY-ESO-1 immobilized on a sensor chip. NY-ESO-1 binding to purified heat shock protein 70 (60 $\mu\text{g/ml}$) is shown as the dark line. Resonance units (RU) binding to immobilized BSA were subtracted from each binding curve. Individual binding curve was repeated once. Resp. Diff., response difference. D, Cross-presentation of NY-ESO-1 by immature DC was blocked by the presence of rabbit sera against bovine CRT but not sera against a control protein. Presentation of the HLA-A31 peptide epitope to the effector CTL was not affected under the same conditions. CTL responses against DC only were subtracted from each determination. The DC used in this experiment were from a melanoma patient, AK, and are different from those used in Table II, which probably caused the lower IFN- γ secretion observed in protein-pulsed DC than the peptide-pulsed DC (different from the data in Table II).

Table II. The full-length, but not the truncated, NY-ESO-1 protein was cross-presented by immature DC

Effector Used to Pulse DC	IFN- γ Release (pg/ml)				
	Expt. 1		Expt. 2		
	BL8-1 Line (CD8 $^{+}$)	CT4-1 Line (CD4 $^{+}$)	CK3H6 Clone (CD8 $^{+}$)	586-10 Clone (CD8 $^{+}$)	CT4-1 Line (CD4 $^{+}$)
None	124	47	13	20	167
Defined epitope ^a	>1330	>1000	>1000	1360	846
NY-ESO-1 protein	404	>500	3	2420	>1000
ESO:1-74 protein	51	8	ND	20	90
ESO:30-70 peptide	105	70	ND	20	ND
ESO:150-180 peptide	78	>500	ND	ND	751
gp100 protein	125	100	20	ND	ND

^a Peptide epitopes used were ESO:157-167 for BL8-1, ESO:161-180 for CT4-1, gp100:209-217 for CK3H6, and ESO:53-62 for 586 CTL clone 10. All peptides and proteins were used at 0.3 μM , except for the class II peptides, which were used at 3 μM . DC used in this experiment were generated from patient RC, who was HLA-A31, HLA-A2, and HLA-DP4. Microcultures that secreted >100 pg/ml cytokines and were at least 2-fold above the negative control were defined as positives. All positives are set in boldfaced type.

Table II, cross-presentation was relatively inefficient for generating the HLA-A2-restricted ESO:157–165 epitope comparing to the A31-restricted ESO:53–62 epitope. The cross-presentation of the ESO:157–165 epitope was also studied by other investigators. In one report, cross-presentation of the epitope was only possible through the IgG-NY-ESO-1 immune complex (25). Another study reported cross-presentation of the NY-ESO-1/ISCOMATRIX complex and, to a much lesser extent, the NY-ESO-1 protein alone (26), which indicated that subpopulations of DC were critical for determining whether the protein was cross-presented. Cross-presentation of the NY-ESO-1 protein and the protein/IgG complex was also compared between monocyte-derived DC and Langerhans cells. The cross-presentation of free protein by monocyte-derived DC was more efficient than that by Langerhans cells, even though it was still much less efficient than cross-presentation of the protein/IgG complex (27). These studies plus our current report indicated several pathways for NY-ESO-1 cross-presentation as an immune complex and as a free protein. Direct interactions between NY-ESO-1 and the innate immune system may contribute to the initiation of adaptive immune responses, including Ab responses against NY-ESO-1, which further enhances the uptake and cross-presentation of NY-ESO-1 by forming immune complex with the Ag to bind to higher affinity receptors such as the Fc γ R on APCs.

Overall, NY-ESO-1 binding to the DC and macrophage surface receptor CRT provides evidence for a direct link between NY-ESO-1, the innate immune system, and possibly the adaptive immune response against NY-ESO-1. Such interactions may have implications for future development of more effective cancer immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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