Polymeric Structure and Host Toll-like Receptor 4 Dictate Immunogenicity of NY-ESO-1 Antigen in Vivo*

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In search of intrinsic factors that contribute to the distinctively strong immunogenicity of a non-mutated cancer/testis antigen, we found that NY-ESO-1 forms polymeric structures through disulfide bonds. NY-ESO-1 binding to immature dendritic cells was dependent on its polymeric structure and involved Toll-like receptor-4 (TLR4) on the surface of immature dendritic cells in mouse and human. Gene gun-delivered plasmid encoding the wild-type NY-ESO-1 readily induced T cell-dependent antibody (Ab) responses in wild-type C57BL/10 mice but not TLR4-knock-out C57BL/10ScJN mice. Disrupting polymeric structures of NY-ESO-1 by cysteine-to-serine (Cys-to-Ser) substitutions lead to diminished immunogenicity and altered TLR4-dependence in the induced Ab response. To demonstrate its adjuvant effect, NY-ESO-1 was fused with a major mugwort pollen allergen Art v 1 and a tumor-associated antigen, carbonic anhydrase 9. Plasmid DNA vaccines encoding the fusion genes generated robust immune responses against otherwise non-immunogenic targets in mice. Polymeric structure and TLR4 may play important roles in rendering NY-ESO-1 immunogenic and thus serve as a potent molecular adjuvant. NY-ESO-1 thus represents the first example of a cancer/testis antigen that is a also damage-associated molecular pattern.

It has been speculated that the initiation of spontaneous adaptive immune responses against tumor-associated antigens (TAA)3 may involve receptors of the innate immune system (1, 2). Necrotic cancer cells release endogenous factors or a damage-associated molecular pattern such as heat shock proteins (3), high mobility group box-1 protein (HMGB-1) (4), and uric acid (5). These damage-associated molecular pattern factors alert the innate immune system through CD91, receptor for advanced glycation end products and possibly TLR2/TLR4, as well as the IL-1 receptor (6), respectively. TAA themselves are generally perceived as by-standers of the above “danger signals,” which serve as adjuvants in the initiation of anti-tumor immune responses. According to this paradigm, spontaneous immune responses may favor neo-peptides resulting from an estimated 11,000 genetic alterations within a cancer cell (7). However, human TAA identified to date are commonly non-mutated self-proteins (8). We thus hypothesize that intrinsic factors from some of the known TAA may also play a role in the initiation of anti-tumor immune responses in vivo.

In search of intrinsic factors derived from the host and the tumor that contribute to anti-tumor immune responses, we focused on NY-ESO-1, a non-mutated cancer/testis antigen with distinctively strong immunogenicity (9). Spontaneous Ab and T cell-based immune response against NY-ESO-1 were readily detectable in a wide spectrum of cancer patients with NY-ESO-1-expressing tumors, including older patients with late stage cancers whose immune systems were known to be less responsive (9). Furthermore, spontaneous NY-ESO-1 antigen loss in melanoma suggested that anti-NY-ESO-1 responses could affect the natural history of cancer (10). The immunogenicity of NY-ESO-1 was not due to its higher level of expression compared with other TAA. Indeed, the expression of NY-ESO-1 at least in melanoma was much lower than that of melanocyte differentiation antigens such as gp100, MART-1, TRP-1, and TRP-2, as well as other cancer/testis antigens, such as MAGE-1 and MAGE-3 based on quantitative real-time PCR analysis of fresh tumor samples (11).

Our recent investigation of the specific interaction between NY-ESO-1 and complement C1q receptor (regarded in the literature as the same as calreticulin or CRT) on the surface of immature DC, macrophages, and monocytes, indicated a unique interaction between NY-ESO-1 and the innate immune system (12). In this study, we provide evidence showing that the immunogenicity of NY-ESO-1 is accounted for by its own polymeric structure as well as the host TLR4 status in mice.

**EXPERIMENTAL PROCEDURES**

Construction of Plasmids and Introduction of Site-directed Mutagenesis—Truncated NY-ESO-1 containing amino acid residues 1–74, termed ES0(1–74), was described previously (13). To introduce site-directed mutagenesis to the NY-ESO-1
cDNA, PCR was carried out to replace cysteines at amino acids 75, 76, and 78 with serines using the following phosphorylated primer pairs: forward primer, 5'-TCTTCGATCCGGGCGCAGGGGCGGGGCGCGAGAAGGATGGCGGGCGGACGTCCTTTCTGCTGCCCGTG and reverse primer, 5'-TCCATTCGACCTGAAGCTCAGGGCAT. Long PCR was performed on a template of a previously described expression vector pET-28-NY-ESO-1 (13) using the high fidelity PCR system (Invitrogen). The resultant PCR product was ligated to generate a new plasmid encoding ESOcs1. The same approach was used to introduce Cys-to-Ser mutations at amino acids 152 and 165, termed ESOcs2, with the following primer pairs: forward primer, 5'-TTGATGGATGTCAGCGCAGTGCTTCTCTTTCCGCTGT and reverse primer, 5'-CAGGGAAACTGCTGAGAGAGGAGCTGATG (mutated nucleotides were underlined). Similarly, a plasmid encoding NY-ESO-1 with all five Cys-to-Ser mutations at amino acids 75, 76, 78, 152, and 165 (termed ESOcs3) was obtained.

To construct eukaryotic expression plasmids that encode ESOcs1, ESOcs2, and ESOcs3, PCR was used to amplify the NY-ESO-1 coding region with the corresponding mutations. The resultant PCR products were subsequently cloned into pcDNA3.1TOPo vectors (Invitrogen). All plasmids were verified by DNA sequencing. The wild-type NY-ESO-1 and its variants, including LAGE-1b, a natural homologue sharing four of the indicated venders.

Antibodies, Cell Lines, Western Blot, Purification of Recombinant Proteins, and Immunoprecipitation—Monoclonal Ab against the c-Myc epitope was purchased from GenScript, Inc. (Piscataway, NJ). The anti–FLAG monoclonal Ab M2 (Sigma-Aldrich), monoclonal Ab against human β-actin (Sigma-Aldrich), CRT (Upstate Biotechnology, Lake Placid, NY), and TLR4 (eBioscience, San Diego, CA) were purchased from the indicated venders.

HEK293 and melanoma cell lines 397, 624, and 2984 (kind gifts from Paul F. Robbins, National Cancer Institute) were maintained in DMEM containing 10% fetal calf serum. Mela-
noma lines M202, F5.1, F5.4, and F5.6 were early cultures of primary tumors and gifts from Ali Jazirehi (UCLA). Transfection of plasmids into 293 cells was carried out using the Lipofectamine 2000 system (Invitrogen). Western blotting was performed and developed using an ECL system (R&D Systems, Minneapolis, MN).

The pET-28 (Novagen, San Diego, CA) based bacterial expression vectors encoding the wild-type NY-ESO-1, ESO(1–74), ESOcs1, ESOcs2, and ESOcs3 were used to transform BL21(DE3) Escherichia coli. Protein purification was conducted following a previously described protocol (13). Notably, the wild-type NY-ESO-1 protein was purified by Novavax, Inc. (Rockville, MD) under good manufacturing practice conditions with the resulting endotoxin below 5 enzyme units/mg/ml. Purified proteins were all dialyzed against 50 mM Tris-HCl (pH 8.0) in the presence of 0.5 M free l-arginine for proper refolding.

Immunoprecipitation was conducted using mouse Myc-CaP cell line (15), which endogenously expresses mouse TLR4 and CRT, a gift of Charles Sawyer (Memorial Sloan Kettering Cancer Institute, New York, NY). Myc-CaP was transduced with a retrovirus encoding NY-ESO-1 and its variants fused with the c-Myc tag. Cells collected from a 10-cm culture dish were lysed with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM MgCl2/ CaCl2 in the presence of protease inhibitors. Cell lysates were incubated overnight with Ab against the c-Myc tag conjugated onto magnetic microbeads (Miltenyi Biotech) followed by magnetic separation and analysis of total proteins by native gel or reducing SDS-PAGE.

Immunization of Animals and Detection of Ab Present in Serum—To exclude any potential effects of LPS associated with protein immunization, plasmid DNA purified using endotoxin-free maxiprep kits (Qiagen, Germantown, MD) was used to immunize 6–8-week-old C57BL/10 and C57BL/10ScNJ (TLR4−/−) mice by gene gun (16). For immunization of Balb/c mice with plasmids encoding CA9 and NY-ESO-1 fused with CA9, ~100 µg plasmid/mouse were injected intramuscularly, followed by one or two injections of 50 µg plasmid/mouse with 2-week intervals. In all cases, blood from immunized mice was collected 2 weeks after immunization, and the serum was obtained using a centrifugation-based serum collection tube (BD Biosciences).

Detection of NY-ESO-1 Ab present in mouse serum was carried out using ELISA as described previously (13). A similar protocol was used to measure Ab against β-galactosidase (β-gal, Sigma/Aldrich) and human HMGB-1 (Genway Biotechnology, Inc., San Diego, CA). Ab titers against the Ab v1 allergen was determined using luminometric ELISA as described previously (17).

Preparation of Human and Mouse DC and DC Binding Assay—A procedure approved by the UCLA Institutional Review Board was used in obtaining human peripheral blood mononuclear cells from the virology core laboratory. Human DC were generated from isolated CD14-positive (Miltenyi Biotech) monocytes in the presence of 1000 unit/ml human GM-CSF (Amgen, Thousand Oaks, CA) and 1000 unit/ml human IL-4 (Schering-Plough, San Francisco, CA) for 6 days. Iscove’s medium (Invitrogen) supplemented with 10% human male serum (BioCheMed, Winchester, VA) was used for DC culture. Mouse DC were generated from bone marrow cells of C57BL/10 mice and corresponding TLR2 and TLR4 knock-out mice (The Jackson Laboratory, Bar Harbor, ME) using a previously described protocol (12).

All DC binding experiments were conducted on ice to prevent active internalization of the protein target by immature DC as described previously (12). Unless specified, the protein concentrations used were 3, 10, and 1 µg/ml for NY-ESO-1 and its variants, gp100, and ESO(1–74), respectively, to maintain equal molarities. About 105 pre-chilled, immature DC from mouse or human were mixed with the above protein in 50 µl of PBS buffer supplemented with 5% fetal bovine serum to block nonspecific interactions. After a 20-min incubation on ice, samples were washed twice followed by adding a FITC-conjugated secondary Ab, either a monoclonal Ab against NY-ESO-1 or against the polyhistidine tag (Sigma/Aldrich). Cells were incubated for another 20 min, washed twice, and subjected to flow cytometric analysis.
FIGURE 1. DC-binding properties of NY-ESO-1 correlated with its polymeric architecture held together by disulfide bonds. A, human 293 cells transfected with a plasmid encoding NY-ESO-1 (lane 1), GFP (lanes 2 and 4), or NY-ESO-1 fused with an N-terminal FLAG tag (FLAG-ESO, lane 3). Cell lysates were run on an SDS-polyacrylamide gel, and immunoblots were reacted with the mAb131 (lanes 1 and 2) and M2 monoclonal Ab (lanes 3 and 4) specific to NY-ESO-1 and the FLAG epitope, respectively. Notably, NY-ESO-1 monomer without a fusion partner (indicated by single arrowheads) was ~18 kDa; whereas its dimers (double arrowheads) and tetramers appeared as 36- and 72-kDa bands, respectively on the Western blot. B, Western blot analysis of partially purified wild-type NY-ESO-1 (lane 4) plus ESOcs1, ESOcs2, and ESOcs3 proteins (lanes 1–3, respectively). All protein samples were subjected to standard SDS-PAGE followed by Western blot using the NY-ESO-1-specific mAb131. Notably, His-tagged monomeric NY-ESO-1 and its variants appeared at ~20 kDa. C, Western blot analysis of NY-ESO-1 present in melanoma lines 397, 624, 2984, HEK293, melanoma line M202, F5.1, F5.4, and F5.6 (from lane 1 to lane 8), respectively. Monomers and dimers were indicated with single and double arrowheads, respectively. HEK293, M202, and F5.1 were control lines with undetectable NY-ESO-1 expression based on PCR. GAPDH were used as positive controls to show equal amount of total proteins loaded in each lane. D, NY-ESO-1 oligomeric variants with Cys-to-Ser mutations had decreased binding to mouse and human DC, comparing to the wild-type polymeric NY-ESO-1 protein. ESO(1–74) had the B cell epitope recognized by monoclonal Ab mAb131 for detection and used as a negative control. NY-ESO-1 protein treated with proteinase K was another control. The gp100 protein at equal molar concentration with NY-ESO-1 was also used as a negative control (data not shown). The experiment was repeated using DC from three different donors. Immature DC that had apparent binding to NY-ESO-1 usually ranged from 8 to 80% in vitro largely due to donor difference and experiment-to-experiment variations. Thus, no S.D. were usually presented in DC binding assays. The dot plot of the NY-ESO-1 binding to human DC was shown in (E), including the scattered plot. MW, molecular weight.
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A.

% of DC binding to NY-ESO-1

[Graph showing binding as a function of NY-ESO-1 protein concentration (microg/ml)]

B.

Relative Binding to NY-ESO-1 (%)

[Bar graph showing relative binding to NY-ESO-1 for different conditions, with significance indicated by asterisks (*) and double asterisks (**)]

C.

Anti-NY-ESO-1

Anti-TLR4

Anti-CRT

[Western blots showing protein bands for different conditions]

D.

Myc-Cap/ NY-ESO-1 ESOcs1 ESOcs2 ESOcs3

IP: Anti-c-Myc

IB: Anti-TLR4

IB: Anti-NY-ESO-1

TLR4: Input

[Western blots showing protein bands for different conditions]
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RESULTS

NY-ESO-1 Binding to Immature DC Is Dependent on Its PolymERIC Structures—NY-ESO-1 proteins derived from bacterial and mammalian cells were found to form polymers even in the presence of regular concentrations of β-mercaptoethanol in the loading buffer (Fig. 1, A and B). To further examine the role of cysteines in the formation of NY-ESO-1 polymers, serines were introduced to substitute cysteines present at amino acid residues 75, 76, 78, 152, and 165. Three NY-ESO-1 variants carrying amino acid substitutions were generated: ESOcs1 containing Cys-to-Ser substitutions at 75, 76, and 78, ESOcs2 at position 152 and 165, and ESOcs3 containing C-to-S substitutions at all five positions. These proteins were purified and compared for oligomerization status using Western blot (Fig. 1B). Although the wild-type protein showed a clear formation of dimers, tetramers, and even polymers above 130 kDa; ESOcs1 and ESOcs2 were mainly present as monomers and dimers. ESOcs3 appeared primarily as monomers. This experiment indicated that the oligomerization of NY-ESO-1 was due to intermolecular disulfide bonds. NY-ESO-1 present in tumor cells was detected primarily as trimers or tetramers with few monomers and dimers using Western blot even in the presence of regular amount of β-mercaptoethanol in the loading buffer (Fig. 1C).

According to our previous report, a unique feature of NY-ESO-1 was its capacity of binding to immature DC, monocytes, and macrophages in vitro. Compared with the wild-type NY-ESO-1, binding to immature DC by NY-ESO-1 variants with Cys-to-Ser mutations was significantly affected in vitro (Fig. 1, D and E). NY-ESO-1 engagement of immature DC of mouse and human was thus a function of its own oligomeric structure in vitro.

DC Surface TLR4 Is Involved in Binding of NY-ESO-1 in Vitro—To investigate the involvement of TLR in mediating NY-ESO-1 binding to DC, bone marrow-derived immature DC from wild-type mice were compared with those from TLR4 and TLR2 knock-out mice for NY-ESO-1 binding affinities. The absence of TLR4 but not TLR2 partially impaired the binding of NY-ESO-1 to bone marrow derived DC in vitro (Fig. 2A). Because NY-ESO-1 also bound to immature DC through CRT in mouse and human, it retained significant binding to DC from TLR4-knock-out mice. In accordance, direct binding of recombinant NY-ESO-1 to human and mouse DC was blocked by anti-TLR4 Ab in a similar manner as did anti-CRT Ab (Fig. 2B); meanwhile, Ab against β-actin served as negative controls with no apparent blocking of NY-ESO-1 and immature DC interac-

FIGURE 2. Mouse and human TLR4 were involved in the binding between NY-ESO-1 and immature DC. A, bone marrow-derived immature DC from the wild-type, TLR2−/−, and TLR4−/− mice were used for binding to NY-ESO-1 at the indicated concentrations, which was subsequently detected by staining with a FITC-labeled mAb131. Results are mean ± S.E. in triplicate, and t test with p value < 0.05 was considered significant. *, p < 0.05; **, p < 0.01. The experiment was repeated two more times with similar results using cells from a total of three donor mice. However, there was significant experiment-to-experiment variation in terms of the absolute percentage of DC binding to NY-ESO-1. B, blockage of NY-ESO-1 binding to human and mouse DC by polyclonal Ab against human TLR4, CRT, but not control Ab against β-actin. A standard DC binding experiment was performed in the presence of 3 μg/ml NY-ESO-1 protein and the indicated Ab separately or in combination. The experiment was repeated once with similar results obtained. *, p < 0.05; **, p < 0.01 were obtained against human DC control; #, p < 0.05; ##, p < 0.01 against mouse DC control. B, blockage of NY-ESO-1 binding to human and mouse DC by polyclonal Ab against human TLR4, CRT, but not control Ab against β-actin. A standard DC binding experiment was performed in the presence of 3 μg/ml NY-ESO-1 protein and the indicated Ab separately or in combination. The experiment was repeated once with similar results obtained. C, polymeric NY-ESO-1 formed a macromolecular complex with mouse TLR4 and CRT. Lysates from Myc-CaP cells transduced with Myc-tagged NY-ESO-1 were used for the immunoprecipitation (IP) using monoclonal Ab against c-Myc and loaded in lane 1 of each panel, whereas total cell lysates prior to IP were directly loaded in lane 2 of each panel. Polyacrylamide gel electrophoresis was conducted under native conditions followed by Western blotting using monoclonal Ab against NY-ESO-1 and CRT, and rabbit anti-TLR4 polyclonal Ab (middle panel), and rabbit anti-CRT polyclonal Ab (right panel). Please note the different localization of NY-ESO-1, TLR4, and CRT present in the IP complex and cell lysate. D, co-IP of NY-ESO-1 and mouse TLR4 protein correlated with the polymeric structure of NY-ESO-1. In each panel, lanes 1–4 contained cell lysates of Myc-CaP transduced with retrovirus encoding c-Myc-tagged NY-ESO-1, ESOcs1, ESOcs2, and ESOcs3, respectively. IP was conducted using anti-c-Myc Ab to pull down NY-ESO-1 and its variants, followed by Western blot with a rabbit Ab against TLR4 (left top panel), mAb131 against NY-ESO-1, and its variants (left bottom panel). As the normalized input control, TLR4 present in the total cell lysate prior to the pulldown were shown in the bottom panel. IB, immunoblot; MW, molecular weight.

FIGURE 3. Immunogenicity of NY-ESO-1 and its variants in wild-type and TLR4 knock-out mice. Class-switched IgG Ab were generated from C57BL/10 (A) and the TLR4−/− C57BL/10ScN (B) mice immunized with gene gun delivery of endotoxin-free plasmids. An average OD from three mice per group was plotted based on ELISA detection of specific Ab against the target, i.e. NY-ESO-1, HMGB-1, and β-gal proteins. Both the pre- and post-immunization sera were used at one to five dilutions in ELISA. Serum was regarded positive if OD values increased more than 2-fold against the specific antigenic target after immunization. The entire experiment was repeated once with similar results obtained, whereas key immunization was repeated a third time.
FIGURE 4. Immune adjuvant effects of NY-ESO-1 in vivo. A, IgG Ab against the major mugwort allergen Art v1 were measured 2 weeks after the first gene gun immunization in Balb/c mice with serum dilution at 1–50 as well as 2 weeks after the second immunization with serum diluted at 1–1250. Plasmid encoding the wtArt, wtArt-ESO fusion, and codon-optimized reconstituted Art (recArt) were used for the immunization using three mice/group. The experiment was repeated twice with similar results obtained. B, CA9-specific Ab were induced by immunization with pcDNA-CA9-ESO encoding the CA9-ESO fusion protein but not pcDNA-CA9 encoding CA9 alone. Serum from immunized mice were analyzed against lysates from 293 cells transfected with a plasmid encoding GFP (lane 1), CA9 (lanes 2 and 4), and NY-ESO-1 (lanes 3 and 5) in a Western blot. The full-length CA9 and NY-ESO-1 proteins were indicated in the figure. Lower bands that were shown positive in lane 2 might be CA9 transcript variants. Three Balb/c mice were used for each group; and the results were obtained using pools of serum from three mice/group. C, anti-CA9 Ab from three mice/group was measured by ELISA against recombinant human CA9. A pcDNA-based plasmid encoding the CA9-ESO fusion significantly improved Ab titers against CA9 comparing with saline, pcDNA-CA9, or a mixture of pcDNA-CA9 and pcDNA-ESO. All endotoxin-free plasmid DNA were delivered though intramuscular route to Balb/c mice. The experiment was repeated once with similar results obtained.

NY-ESO-1 as Immune Adjuvant

NY-ESO-1-induced Ab Responses in Mice Are Functions of Its Oligomeric Structure and Host TLR4—NY-ESO-1 and its oligomeric variants provided us with unique examples to study the effect of engaging TLR4 on the resultant adaptive immune responses. To avoid using recombinant proteins in vivo, endotoxin-free plasmid DNA encoding the wild-type NY-ESO-1 and its oligomeric variants, including the natural variant LAGE-1b, were used to immunize C57BL/10 and C57BL/10sC3J (TLR4−/−) mice by a standard gene gun procedure (16). Plasmids encoding β-gal and a membrane-anchored HMGB-1 were used as controls. Two immunizations were conducted at a two-week interval. Two weeks after the second immunization, mice were sacrificed for analysis of serum Ab titers. Antigen-specific, class-switched Ab was efficiently induced by a plasmid encoding NY-ESO-1 and the positive control plasmid pSport-β-gal in wild-type mice (Fig. 3A). Note that ESO(1–74) protein, containing a dominant B cell epitope shared by both NY-ESO-1 and its variants (18), was used for measuring Ab to minimize the potential difference in binding to the wild-type NY-ESO-1 by Ab induced by oligomeric variants. Ab titers induced by ESOcs1 were somewhat reduced, whereas those by ESOcs2 and ESOcs3 were barely detectable. In TLR4 knockout mice (Fig. 3B), NY-ESO-1-specific Ab induced by the plasmid encoding the wild-type protein was significantly decreased; whereas β-gal-specific Ab was essentially the same as in the wild-type mice. This experiment indicated the TLR4-dependent nature of the NY-ESO-1 Ab response, whereas the route (skin) and mechanism (gene gun delivery of plasmid) of immunization was independent of TLR4. Surprisingly, immunization with plasmid DNA encoding HMGB-1, a previously reported protein with potential interactions with TLR4, induced only marginal Ab in TLR4−/− mice (note that HMGB-1 Ab ELISA had a much lower background than NY-ESO-1 and β-gal, Fig. 3B) but not in wild-type mice with intact TLR4. NY-ESO-1 structural variants displayed a correlation between Ab titers and Cys-to-Ser mutations in wild-type mice, but a different pattern in TLR4 knockout mice. For example, ESOcs1 induced relatively high Ab responses in wild-type but not TLR4 knockout mice, assuming the same pattern of immunogenicity as the wild-type NY-ESO-1. In contrast, ESOcs2 induced relatively high titer Ab responses in TLR4 knockout but not wild-type mice, contrasting wild-type NY-ESO-1 but resembling HMGB-1 in its immu-
nogenicity. ESOcs3 remained barely immunogenic in both types of mice. This experiment indicated that IgG Ab responses elicited by NY-ESO-1 were accounted for by its oligomeric structure and the host TLR4 status in vivo.

NY-ESO-1 Serves as Molecular Adjuvant to Augment Immune Responses against Art V1 Allergen and TAA CA9—The fact that polymeric NY-ESO-1 engaged immature DC through cell-surface receptors and was highly immunogenic in mouse and human implied the potential role of NY-ESO-1 as a molecular adjuvant in vivo. The adjuvant effect of NY-ESO-1 on immune responses against allergens was then investigated in mice. A plasmid encoding NY-ESO-1 fused with Art v 1, the major allergen of mugwort pollen (19), was delivered by gene gun to Balb/c mice. Wild-type Art v 1 (wtArt) was known for its poor immunogenicity in mice, for example, DNA vaccines encoding wtArt was nonimmunogenic in Balb/c mice but could be overcome by adapting the sequence of wtArt to mammalian codon usage (reconstituted Art) (20). Fusion with NY-ESO-1 significantly increased the immunogenicity of wtArt and induced faster onset (Fig. 4A) of class-switched IgG after only one immunization. The IgG Ab induced by the fusion protein after one immunization was even more profound than those induced by reconstituted Art, which only reached a similar level only after a second immunization (Fig. 4A).

In another instance, a plasmid DNA encoding fusion of human CA9 (21) with NY-ESO-1, pcDNA-CA9-ESO, was compared with pcDNA-CA9 encoding human CA9 alone after immunization via the intramuscular route. Balb/c mice immunized with a plasmid encoding the fusion protein effectively generated high titer Ab against both the NY-ESO-1 protein and the otherwise less immunogenic CA9 protein (Fig. 4B). In contrast, a plasmid encoding CA9 alone failed to induce class-switched Ab responses under the same conditions. The improvement of CA9 immunogenicity was dependent on the genetic fusion with NY-ESO-1 because a mixture of two plasmids, pcDNA-CA9 and pcDNA-ESO, did not improve the Ab titers under the same experimental conditions (Fig. 4C).

DISCUSSION

NY-ESO-1 is expressed in human cancers of the esophagus, prostate, breast, ovary, lung, stomach, head and neck, bladder, as well as neuroblastoma, hepatoma, myeloma, and melanoma. In normal tissues, NY-ESO-1 expression is restricted to the germ line cells of testis, ovary, and placenta (9). Even though NY-ESO-1 expression is much lower than most TAA in cancer cells (11), it naturally induces a profound helper T cell and class-switched Ab response in cancer patients (12). The fact that spontaneous anti-NY-ESO-1 immune responses occur even in advanced stage cancers despite a severely immunosuppressive tumor environment provides an impetus for us to investigate intrinsic factors contributing to the immunogenicity of NY-ESO-1.

Following our previous report that NY-ESO-1/DC interactions through DC surface CRT might be accounted for the immunogenicity of NY-ESO-1 in human (12), this study provides further details of the NY-ESO-1/DC interaction. Intrinsic factors from NY-ESO-1 and the host, i.e. polymeric structure of NY-ESO-1 and TLR4 were involved in the unique interaction between NY-ESO-1 and the immature DC. Both factors may have directly contributed to the immunogenicity of NY-ESO-1 in mouse and human.

Along the same line, the following processes are presumably responsible for IgG Ab against NY-ESO-1 and its variants in the experiment described in Fig. 3: 1) B cell receptors cross-link and uptake antigens into B cells, which are then matured in the presence of IL-4 secreted by CD4+ T helper cells; 2) uptake of NY-ESO-1 by the CRT-TLR4 receptor complex on DC, which lead to generation of antigen-specific CD4+ T helper cells to provide powerful help to Ab-producing B cells. This study provides evidence that the later process is dependent on strong binding affinity between polymeric NY-ESO-1 and the DC sur-
face CRT-TLR4 complex. On the other hand, we postulate that the former process may slightly favor ESOcs2, which is more soluble and accessible to B cell receptors than the wild-type NY-ESO-1 or ESOcs1. Thus, in wild-type mice, TLR4-dependent antigen uptake and specific helper T cell responses play major roles leading to strong Ab responses against polymeric NY-ESO-1 (Fig. 3A). In contrast, the B cell receptor-mediated process is the dominant factor in TLR4 knock-out animals, leading to relatively strong Ab responses against ESOcs2 (Fig. 3B).

Based on the unique properties of polymeric NY-ESO-1 protein, we exploited its adjuvant effects in two circumstances: generation of prophylactic IgG class Ab against the mugwort pollen allergen Art v 1 and the cell-surface renal cell carcinoma antigen CA9. In both cases, high titer Ab responses were efficiently induced by the fusion genes delivered using a gene gun and via intramuscular injection, respectively. However, strength of the NY-ESO-1 adjuvant effect in comparison with other standard adjuvants has not been defined and will be investigated in future studies.

A hypothesis is proposed to explain the natural immunogenicity and the adjuvant effect of NY-ESO-1 in human: polymeric NY-ESO-1 released from necrotic tumor cells naturally engages immature DC through TLR4 and complement C1q receptor. In the literature, the complement C1q receptor is regarded the same as CRT and forms a receptor complex with TLR4 because CRT does not possess a transmembrane domain and has to interact laterally with a cell-surface receptor (22). We postulate that due to its intrinsic immunogenicity, NY-ESO-1 expression is effectively shut down in normal tissues during evolution to avoid exposure to the innate immune system. In cancer cells, abruptly expressed NY-ESO-1 released from necrotic tumor cells naturally engages immature DC through TLR4 and complement C1q receptor. In the literature, the complement C1q receptor is regarded the same as CRT and forms a receptor complex with TLR4 because CRT does not possess a transmembrane domain and has to interact laterally with a cell-surface receptor (22). We postulate that due to its intrinsic immunogenicity, NY-ESO-1 expression is effectively shut down in normal tissues during evolution to avoid exposure to the innate immune system. In cancer cells, abruptly expressed NY-ESO-1 is released from the cytoplasm of necrotic tumors into the environment and initiates a cascade of immunological events, resulting in spontaneous immunity across many cancer types (Fig. 5). In this regard, we believe NY-ESO-1 assumes dual role as a TAA and its own adjuvant, thus can be categorized as a damage-associated molecular pattern.

In summary, the proposed mechanism of action of the immunogenicity of NY-ESO-1 may find important implications as molecular adjuvants in autoimmune disease, allergy, infectious disease, and cancer immunotherapy.

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