FUSION PROTEIN

FIELD OF THE INVENTION

The present invention relates generally to polypeptides and constructs comprising an antigen derived from one or both of the tumor rejection antigens NY-ESO-1 and LAGE-1.

BACKGROUND TO THE INVENTION

Cancer testis (CT) antigens are a class of tumour-associated antigens with expression normally restricted to germ cells in the testis, ovaries or trophoblast cells. These antigens are not usually expressed in adult somatic tissues. See, Simpson, et al., Nat. Rev. Cancer, 5(8):615-625 (2005); Scanlan, et al., Immunol. Reviews, 188:22-32 (2002); Scanlan, et al., Canc. Immun., 4:1-15 (2004).

The gene regulation of CT antigens is disrupted in cancer patients, leading to the aberrant expression of these antigens in a wide variety of tumours. The first CT antigen to be identified, MAGE-1, was identified in the early 1990s by T-cell epitope cloning (van der Bruggen et al, 1991 Science 13;254(5038):1643-7; van der Bruggen et al, 1999 Science 254:1643-1647; Traversari, et al, 1992 Immunogenetics, 35(3):145-152; and U.S. Patent No. 5,342,774, incorporated by reference). Since then, serological expression cloning technique (SEREX) (Sahin, et al., Proc. Natl. Acad. Sci. USA, 92(25):11810-11813 (1995) and U.S. Patent No. 5,698,396), recombinant antigen expression on yeast surface (RAYS) (Mischo, et al., Canc. Immun., 3:5-16 (2003)) and differential mRNA expression analysis (Gure, et al., Int. J. Canc., 85(5):726-732 (2000)) have led to the identification of approximately 90 CT antigens, and their number is expected to grow in the coming years. The immunogenicity of some CT antigens in cancer patients makes them an ideal target for the development of tumour vaccines.

NY-ESO-1. A cancer testis antigen currently of interest for use in cancer immunotherapy is NY-ESO-1. This antigen was first identified by SEREX in an oesophageal squamous cell carcinoma in the late 90's at the New York Branch of the Ludwig Institute for Cancer Research (Chen, et al., PNAS USA, 94(5):1914-1918 (1997); and U.S. Patent No. 5,804,381, incorporated by reference).

The protein NY-ESO-1 is 180 amino acids in length and can be described as being composed of three regions:

- An N-terminal region about or approximately amino acids 1 to 70,
- A central region about or approximately amino acids 71 to 134, and
- A C-terminal region about or approximately amino acids 135 to 180.

A collagen-like region comprises about or approximately or about amino acids 15 to 73 of the N-terminal region (see Figure 1).

The protein NY-ESO-1 has been found in a wide variety of tumours, including but not limited to ovarian cancer, lung cancer, breast cancer, prostrate, oesophageal cancer, bladder cancer and in melanomas. (Nicholaou T, et al, Immunol Cell Biol. 2006 Jun;84(3):303-17 and Jungbluth, et al. 2001, Int. J. Canc., 92(6):856-860). Spontaneous humoral and cellular immune responses against this antigen have been described in patients with NY-ESO-1-positive tumours, and a number of HLA (Human Leukocyte Antigen) class I- and II-restricted peptides have been identified (Jager, et al., 1998 J. Exp. Med., 187(2):265-270; Yamaguchi, et al., 2004 Clin. Canc. Res., 10(3):890-961; and Davis, et al., 2004 Proc. Natl. Acad. Sci. USA, 101(29):10697-10702). Exemplary of the patent literature are U.S. Patent Nos. 6,140,050; 6,251,603; 6,242,052; 6,274,145; 6,338,947; 6,417,165; 6,525,177; 6,605,711; 6,689,742; 6,723,832; 6,756,044; and 6,800,730, all incorporated by reference.

In a clinical trial, three partially overlapping NY-ESO-1-derived peptides with binding motifs to HLA-A2 (157-167, 157-165 and 155-163) have been used in a vaccine to treat twelve patients with metastatic NY-ESO-1 expressing tumours. This study demonstrated that synthetic NY-ESO-1 peptides can be administered safely and are capable of generating potentially beneficial T cell responses (Jager, et al., 2000 PNAS USA, 97(22):12198-12203).

A number of MHC (major histocompatibility complex) class I and II epitopes in the protein have been identified by different groups see, for example, Figure 1. These epitopes are merely representative of epitopes reported for the protein and the list in Figure 1 is not exhaustive. Furthermore, at least one or more of the epitopes reported and/or listed in Figure 1 have not been confirmed by experimentation. The collagen-like region in the N-terminal contains at least one MHC class I epitope referred to herein as

A31. The central region comprises several MHC class 2 epitopes referred to herein as DR1, DR2, DR4, DR7 and DP4. This region also contains several MHC class I epitopes referred to herein as B35, B51, Cw3 and Cw6. The C-terminal is believed to contain at least two class II epitopes (DR4 and DP4) and one class I epitope (A2).

LAGE-1. A further cancer testis antigen, LAGE-1, has also been identified. Two LAGE-1 transcripts have been described, LAGE-1a and LAGE1b. LAGE-1b is incompletely spliced and codes for a putative protein of approximately 210 amino acids residues, while the LAGE-1a gene product contains 180 amino acid residues (Sun et al. Cancer Immunol Immunother 2006: 55: 644-652).

The N-terminal regions of the LAGE-1 and NY-ESO-1 proteins are highly conserved and are thought to have more than 97% identity. However, LAGE-1 differs from NY-ESO-1 in the central regions which are only 62% identical. The C-terminals of NY-ESO-1 and LAGE-1a are highly conserved (more than 97% identity). However, the C-terminal of LAGE-1b is longer and not conserved and is thought to have less than 50% identity with the same region in LAGE-1a/NY-ESO-1.

General information relating to these proteins is available from the LICR web site (see www.cancerimmunity.org/CTdatabase).

SUMMARY OF THE INVENTION

The present invention provides an immunogenic fusion protein comprising:

(i) NY-ESO-1 or a fragment thereof, linked to

(ii) LAGE-1 or a fragment thereof,

in which at least one of NY-ESO-1 and/or LAGE-1 is truncated or partially truncated, or is a fragment including one or more epitopes of NY-ESO-1 or LAGE-1. The present invention also provides an immunogenic fusion protein comprising:

(i) LAGE-1 or a fragment thereof, linked to

(ii) NY-ESO-1 or a fragment thereof,

in which at least one of NY-ESO-1 and/or LAGE-1 is truncated or partially truncated, or is a fragment including one or more epitopes of NY-ESO-1 or LAGE-1. Thus, polypeptides and fusion proteins comprising truncated or partially truncated NY-ESO-1, or a fragment thereof, including one or more epitopes of NY-ESO-1 are also provided.

Also provided are polypeptides and fusion proteins comprising truncated or partially truncated LAGE-1, or a fragment thereof, including one or more epitopes of LAGE-1. Compositions and methods involving such fusion proteins and polypeptides are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a number MHC (major histocompatibility complex) class I and II epitopes on the NY-ESO-1 protein that have been identified by different groups. These epitopes are merely representative of epitopes reported for the protein thus the list in Figure 1 is not exhaustive. Furthermore, at least one or more of the epitopes reported and/or listed in Figure 1 have not been confirmed by experimentation. The reported amino acid sequence for NY-ESO-1 is found herein in SEQ ID NO:49.

Figure 2 shows construct A, a fusion protein comprising full length NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, the C-terminus of the NY-ESO-1 is fused to the N-terminus of truncated LAGE-1, together with a Histidine affinity tag to provide a fusion protein of 288 amino acids in length. Further details of construct A are given in Table 1 (SEQ ID NO:1; SEQ ID NO:3).

Figure 3 shows construct B, a fusion protein comprising the first third of protein D without its secretion signal (for example amino acids 20 to 127), full length NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, amino acid 127 of protein D is fused to the N-terminus of NY-ESO-1, the C-terminus thereof being fused to the N-terminus of truncated LAGE-1, to provide a fusion protein of 398 amino acids in length. Further details of construct B are given in Table 1 in section 1.6 (SEQ ID NO:2; SEQ ID NO:4).

Figure 4 shows construct C, a fusion protein comprising partially truncated NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, the C-terminus of partially truncated NY-ESO-1 is fused to the N-terminus of truncated LAGE-1, to provide a fusion protein of 242 amino acids in length. Further details of construct C are given in Table 1 (SEQ ID NO:5; SEQ ID NO:7).

Figure 5 shows construct D, a fusion protein comprising the first third of protein D without its secretion signal (for example amino acids 20 to about or approximately 127),

partially truncated NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, amino acid 127 of protein D is fused to the N-terminus of partially truncated NY-ESO-1, the C-terminus thereof being fused to the N-terminus of the truncated LAGE-1, to provide a fusion protein of 352 amino acids in length. Further details of this embodiment are given in Table 1 (SEQ ID NO:6; SEQ ID NO:8).

Figure 6 shows construct E, a fusion protein comprising truncated NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, the C-terminus of truncated NY-ESO-1 is fused to the N-terminus of truncated LAGE-1, to provide a fusion protein of 211 amino acids in length. Further details of construct E are given in Table 1 (SEQ ID NO:9; SEQ ID NO:11).

Figure 7 shows construct F, a fusion protein comprising the first third of protein D without its secretion signal (for example amino acids 20 to about or approximately 127), truncated NY-ESO-1 and truncated LAGE-1, such as LAGE-1a. In this embodiment, amino acid 127 of protein D is fused to the N-terminus of truncated NY-ESO-1, the C-terminus thereof being fused to the N-terminus of truncated LAGE-1, to provide a fusion protein of 321 amino acids in length. Further details of construct F are given in Table 1 (SEQ ID NO:10; SEQ ID NO:12).

Figure 8 shows an alternative embodiment of construct E, namely E', in which the C-terminus of truncated LAGE-1 is fused to the N-terminus of truncated NY-ESO-1, to provide a fusion protein of 212 amino acids in length. Further details of this embodiment, construct E', are given in Table 1 (SEQ ID NO:21; SEQ ID NO:23).

Figure 9 shows construct G, a fusion protein comprising truncated NY-ESO-1, truncated LAGE-1, such as LAGE-1a and the collagen-like region, such as the collagen region from NY-ESO-1. In this embodiment, the C-terminus of the collagen-like region is, for example, fused to the N-terminus of truncated LAGE-1. In turn the C-terminus of the truncated LAGE-1 is fused to the N-terminus of truncated NY-ESO-1, to provide a fusion protein of 289 amino acids in length. Further details of construct G are given in Table 1 (SEQ ID NO:13; SEQ ID NO:15).

Figure 10 shows a schematic of an exemplary recombinant polypeptide comprising NY-ESO-1 with a partially truncated collagen like domain. The epitopes

shown in Figures 10-13 are merely representative of epitopes reported for the protein and have not been confirmed by experimentation.

Figure 11 shows a schematic of an exemplary fusion protein comprising the first third of protein D without its secretion signal (for example amino acids 20 to about or approximately 127) and NY-ESO-1 with a partially truncated collagen-like domain.

Figure 12 shows a schematic of an exemplary recombinant polypeptide comprising NY-ESO-1 with a partially truncated collagen-like domain.

Figure 13 shows a schematic of an exemplary fusion protein comprising the first third of protein D without its secretion signal (for example amino acids 20 to about or approximately 127) and NY-ESO-1 with a truncated collagen-like domain.

Figure 14 is a schematic that shows a number of epitopes identified within the truncated LAGE-1a protein. These epitopes are merely representative of epitopes reported for the protein thus the list is not exhaustive. For the avoidance of doubt, the epitopes reported and/or listed in the figures may or may not have not been confirmed by experimentation (i.e., they may have been predicted, etc.), unless otherwise stated herein. The complete LAGE-1a amino acid sequence is set forth in the sequence listing as SEQ ID NO:58. The complete LAGE-1b amino acid sequence (LAGE-1b not depicted in this Figure) is set forth in the sequence listing as SEQ ID NO:71.

Figure 15 shows a schematic of both NY-ESO-1 and LAGE-1, as well as a number of MHC (major histocompatibility complex) class I and II epitopes. These epitopes are merely representative of epitopes reported for the protein thus the list is not exhaustive; one or more of the epitopes reported and/or listed have not been confirmed by experimentation.

Figure 16 shows a schematic of the NY-ESO-1/LAGE-1 fusion design.

Figure 17 summarizes in schematic fashion fifteen constructs and their production levels. P = protein D; C (grey box) = NY-ESO-1 collagen-like domain; C (white box) = truncated collagen like domain; L = Lage 1 without the collagen like domain; N = NY-ESO-1 without the collagen like domain; black arrow = poly histidine tag; (-) = low production; (+) = some production; (++) = high production; (+++) = best production. The amino acid sequences for eight of the constructs and the nucleotide sequences encoding them are summarized in Table 4 and the sequence listing.

Figure 18 summarizes screening #1, a 76-day trial using CB6F1 mice to assess each of LVL076, LVL079, LVL78, LVL68, LVL020, LVL26, LVL024, LVL30 to determine whether intramuscular immunization with the fusion protein plus adjuvant conferred protection against subcutaneous challenge with transplanted tumors (B16/NYESO1).

Figure 19 summarizes B-16-NY-ESO-1 tumor growth in the control mice used in the 76-day trial.

Figure 20 shows survival of mice immunized with full-length NY-ESO-1, LVL030, LVL068, LVL079, or LVL026.

Figure 21 summarizes the NY-ESO-1-specific immune responses as assessed by ELISA, FACS, and Western Blot and LAGE-1a(without the collagen like domain)-specific immune responses as assessed by ELISA and FACS.

Figure 22 summarizes the experimental design of screening #2, a 105 day trial to determine whether intramuscular immunization with selected fusion proteins plus adjuvant confers protection against B16/NY-ESO-1 challenge and B16/LAGE-1a challenge. B16/NY-ESO-1 challenge is shown.

Figure 23 summarizes screening #2 and shows the B16/LAGE-1a challenge.

Figure 24 shows survival of mice immunized with LVL078, LVL068, full-length NY-ESO-1, LVL024, and LVL076 post-B16/NY-ESO-1 challenge. See Figure 24.

Figure 25 shows survival of mice immunized with LVL076, LAGE-1a without the collagen like region, LVL024, full-length NY-ESO-1, LVL078, or LVL068 post-B16/LAGE-1a challenge.

Figure 26. Columns 1-8, from left to right, show the results of ELISAs carried out to detect possible human collagen-specific immune responses in mice immunized with one of the following: (1) Buffers (control); (2) full-length NY-ESO-1; (3) LAGE-1a without the collagen like domain; (4) LVL068; (5) LVL078; (6) LVL024; (7) LVL076. Positive control (column 8) contains an anti-human collagen 1 monoclonal antibody (mAb anti-human collagen I).

DETAILED DESCRIPTION OF THE INVENTION

Fusion Proteins. The fusion proteins of the invention are useful for the treatment of cancers, and more specifically for the treatment of: melanoma; breast cancer; prostate cancer; bladder cancer including transitional cell carcinoma; lung cancer including non-small cell lung carcinoma (NSCLC); head and neck cancer including oesophageal carcinoma; squamous cell carcinoma; carcinoma of the gastrointestinal tract; liver cancer; brain tumours; leukemia; and various sarcomas.

Based on the expression profiles of LAGE-1 and NY-ESO-1 the fusion protein according to the invention has the potential to be effective in an estimated 37% of breast cancers. The treatment according to the present invention may also be particularly suitable for the treatment of patients not eligible for Her2/neu targeted therapy. The fusion protein of the invention is also predicted to be effective in approximately 35% of prostate cancer patients, 35% of bladder cancer patients, 40% of melanoma patients and 35% of patients with NSCLC (non-small cell lung carcinoma). In one embodiment, the fusion protein of the invention may enable a broader population of patients to be treated because patients having tumours that express both NY-ESO-1 and/or LAGE-1 (including LAGE-1a and LAGE-1b) may be given a fusion protein of the present invention.

The fusion protein according to the invention may also be more immunogenic than its individual component proteins, for the following reasons:

- removal of one or more of the collagen-like domains may reduce potential immunotolerance of the compound caused by its homology with natural endogenous collagen structure, or
- the optional addition of a heterologous fusion partner may further stimulate CD4 T-cell responses. Thus, the fusion proteins are useful for inducing an immunogenic response to a cancer antigen such as NY-ESO-1 or LAGE-1, or both.

The NY-ESO-1 employed in the invention may be full length, partially truncated or truncated NY-ESO-1 or any fragment thereof that includes one or more epitopes capable of raising an immune response to NY-ESO-1. Full length NY-ESO-1 protein in the context of this specification is intended to mean a protein of about or approximately 1 to 180 amino acids and at least 95, 96, 97, 98, 99% or 100% identical, to the naturally

occurring protein (SEQ ID NO:49). As used herein, the term "LAGE-1" refers to one or more LAGE-1 family members such as LAGE-1a and LAGE 1b, as described in the lines below. "Full length LAGE-1a" protein is intended to mean a protein 95, 96, 97, 98, 99% or 100% identical to SEQ ID NO:58. Similarly, "full length LAGE-1b" protein is intended to mean a protein 95, 96, 97, 98, 99% or 100% identical to the naturally occurring protein (SEQ ID NO:71).

In one embodiment, the identity is over the full-length of the sequence. Thus, the invention also extends to said fusion proteins with conservative substitutions. Conservative substitutions are well known and are generally set up as the default scoring matrices in sequence alignment computer programs. These programs include PAM250 (Dayhoft M.O. et al., (1978), "A model of evolutionary changes in proteins", "Atlas of Protein sequence and structure" 5(3) M.O. Dayhoft (ed.), 345-352), National Biomedical Research Foundation, Washington, and Blosum 62 (Steven Henikoft and Jorja G. Henikoft (1992), and "Amino acid substitution matricies from protein blocks"), Proc. Natl. Acad. Sci. USA 89 (Biochemistry): 10915-10919.

In general terms, substitution within the following groups are conservative substitutions, but substitutions between groups are considered non-conserved. The groups are:

i) Aspartate/asparagine/glutamate/glutamine,

ii) Serine/threonine,

iii) Lysine/arginine,

iv) Phenylalanine/tyrosine/tryptophane,

v) Leucine/isoleucine/valine/methionine,

vi) Glycine/alanine.

"Partially truncated" in the context of this specification is intended to mean NY-ESO-1 or LAGE-1 protein (as appropriate) in which the majority of the collagen-like has been region removed but still comprising or consisting of the epitope A31 found in this region.

In one embodiment, partially truncated NY-ESO-1 and/or LAGE-1 comprises or consists of a range of amino acids from amino acid 44, 45, 46, 47, 48, 49, 50, 51 or 52 to amino acid 175, 176, 177, 178, 179 or 180 or any combination of these amino acids,

for example from amino acid 48 to amino acid 180 or from amino acid 46 to 178. In one embodiment partially truncated NY-ESO-1 or LAGE-1 comprises or consists of about or exactly amino acids 48 to 180 (or about or exactly amino acids 48-210 in the case of LAGE-1b). In one embodiment, the term "about" in this context may be taken to mean amino acids up to +/- 10% of the total number of amino acids of the sequence are optionally added or deleted from the sequence. In one embodiment, partially truncated NY-ESO-1 comprises or consists of amino acids 48 to 180 of NY-ESO-1.

In one embodiment, partially truncated LAGE-1b comprises or consists of a range of amino acids from amino acid 44, 45, 46, 47, 48, 49, 50, 51 or 52 to amino acid 205, 206, 207, 208, 209 or 210 or any combination of these amino acids, for example from amino acid 48 to amino acid 210 or from amino acid 46 to 208. In one embodiment partially truncated LAGE-1b comprises or consists of about or exactly amino acids 48 to 210. In one embodiment, the term "about" in this context may be taken to mean amino acids up to +/- 10% of the total number of amino acids of the sequence are optionally added or deleted from the sequence. In one embodiment, partially truncated LAGE-1b comprises or consists of amino acids 48 to 210 of LAGE-1b.

"Truncated" in the context of this specification is intended to mean NY-ESO-1 or LAGE-1 protein (as appropriate) in which the collagen-like region has been removed (including the removal of the A31 epitope). In one embodiment, truncated NY-ESO-1 and/or LAGE 1 comprises or consists of about or exactly amino acids 71-180 (or about or exactly amino acids 71-210 in the case of LAGE-1b).

In one embodiment, truncated NY-ESO-1 or LAGE-1 comprises or consists of a range of amino acids from amino acid 67, 68, 69, 70, 71, 72, 73, 74 or 75 to amino acid 175, 176, 177, 178, 179 or 180 or any combination of these amino acids, for example from amino acid 71 to amino acid 180 or from amino acid 69 to 178. In one embodiment truncated NY-ESO-1 or LAGE-1 comprises or consists of about or exactly amino acids 71 to 180 (or about or exactly amino acids 71-210 in the case of LAGE-1b).

In one embodiment, the term "about" in this context may be taken to mean amino acids up to +/- 10% of the total number of amino acids of the sequence are optionally

added or deleted from the sequence. In one embodiment, truncated NY-ESO-1 or LAGE-1 comprises or consists of amino acids 71 to 180 of NY-ESO-1 or LAGE-1.

In one embodiment, truncated LAGE-1b comprises or consists of a range of amino acids from amino acid 67, 68, 69, 70, 71, 72, 73, 74 or 75 to amino acid 205, 206, 207, 208, 209 or 210 or any combination of these amino acids, for example from amino acid 71 to amino acid 210 or from amino acid 69 to 208. In one embodiment truncated LAGE-1b comprises or consists of about or exactly amino acids 71 to 210. In one embodiment, the term "about" in this context may be taken to mean amino acids up to +/- 10% of the total number of amino acids of the sequence are optionally added or deleted from the sequence. In one embodiment, truncated LAGE-1b comprises or consists of amino acids 71 to 210 of LAGE-1b.

By "other fragments" is intended those which, when incorporated into the fusion protein of the invention, result in a final protein with the desired properties and advantages of the fusion proteins of the invention.

NY-ESO-1. In accordance with the foregoing are provided modified antigens comprising an antigen derived from the tumor rejection antigen NY-ESO-1 wherein the collagen region is partially truncated or completely truncated. In some embodiments, more than the collagen region is removed. In some embodiments, the modified antigen is genetically modified. In some embodiments the modified antigen is recombinant. In some embodiments are provided polypeptides comprising an antigen as described in the preceding sentences. In some embodiments, exemplary polypeptides comprise a heterologous protein, such as protein D from Haemophilus influenzae type B or a fragment thereof. In some embodiments are provided constructs comprising a nucleotide sequence encoding the aforementioned polypeptides.

In some embodiments are provided an immunogenic polypeptide comprising NY-ESO-1 or a fragment thereof, wherein NY-ESO-1 does not include the collagen-like region. In others, NY-ESO-1 is partially truncated or truncated or comprises any fragment thereof that includes one or more epitopes. In some embodiments, such polypeptides have conservative substitutions. In some embodiments, such polypeptides and constructs are useful as a prophylactic for the prevention or substantial amelioration of cancer relapse.

Thus, in some embodiments one or more amino acids are removed from the collagen regions. More specifically, in some embodiments 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73 amino acids are removed from the portion including the collagen region, i.e., roughly amino acids 1-73 of SEQ ID NO:49. The amino acids may be removed from adjacent positions in the collagen region or from positions that are not adjacent. In other words, in some embodiments, an amino acid is removed from any of positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73 or any combination thereof, within that portion of SEQ ID NO:49. Those of skill in the art understand that in some embodiments, portions of the amino acid sequence are preserved such that particular epitopes therein are retained within a resulting polypeptide.

In some embodiments, a fragment of the NY-ESO-1 central region or C-terminal region is utilized. Thus, in some embodiments the polypeptide may comprise one or more fragments of the amino acid sequence set forth in SEQ ID NO:49, i.e., fragments which contain one or more of amino acid positions 74-75, 76-80, 81-85, 86-90, 91-95, 96-100, 101-105, 106-110, 111-115, 116-120, 121-125, 126-130, 131-135, 136-140, 141-145, 146-150, 151-155, 156-160, 161-165, 166-170, 171-175, 176-180, or any combination thereof. Those of skill in the art understand that in some embodiments, the amino acid sequence is preserved such that particular epitopes are retained within a resulting polypeptide.

LAGE-1. In some embodiments are provided a modified antigen comprising an antigen derived from the tumor rejection antigen LAGE-1 wherein the collagen region is partially truncated or completely truncated. In some embodiments, more than the collagen region is removed. In some embodiments, the modified antigen is genetically modified. In some embodiments, the modified antigen is recombinant. In some embodiments are provided polypeptides comprising an antigen as described in the

preceding sentences. In some embodiments the antigen is derived from the tumor rejection antigen LAGE-1a. In some embodiments the antigen is derived from the tumor rejection antigen LAGE-1b. In other embodiments, exemplary fusion proteins comprise a heterologous protein, such as protein D from Haemophilus influenzae type B or a fragment thereof. In some embodiments are provided constructs comprising a nucleotide sequence encoding the aforementioned polypeptides.

In some embodiments are provided an immunogenic polypeptide comprising LAGE-1 or a fragment thereof, wherein LAGE-1 does not include the collagen-like region. In others, LAGE-1 is partially truncated or truncated or comprises any fragment thereof that includes one or more epitopes. In some embodiments, the polypeptide comprises a hybrid of the LAGE-1 polypeptide and the collagen like region of NY-ESO-1. In some embodiments, the polypeptide comprises part, or all, of the NY-ESO-1 collagen region joined to partially truncated or truncated LAGE-1. In some embodiments, such polypeptides have conservative substitutions. In some embodiments, such polypeptides and constructs are useful as a prophylactic for the prevention or substantial amelioration of cancer relapse.

Thus, in some embodiments one or more amino acids are removed from the collagen region, or even from the N-terminal amino acids. More specifically, in some embodiments 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73 amino acids are removed from the collagen region or even the N-terminal amino acids, i.e., roughly amino acids 1-73 of SEQ ID NO:58 (LAGE-1a) or SEQ ID NO:71 (LAGE-1b). The amino acids may be removed from adjacent positions in this region or from positions that are not adjacent. In other words, in some embodiments, one or more amino acids are removed from any of positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73 or any combination thereof, within SEQ ID NO:58 (LAGE-1a) or SEQ ID NO:71 (LAGE-1b). Those of skill in the art understand that in some embodiments, portions of the amino

acid sequence are preserved such that particular epitopes therein are retained within a resulting polypeptide.

In some embodiments, a fragment of the LAGE-1 central region or C-terminal region is utilized. Thus, in some embodiments the polypeptide may comprise one or more fragments of the amino acid sequence set forth in SEQ ID NO:58 (LAGE-1a) or SEQ ID NO:71 (LAGE-1b), i.e., fragments which contain one or more of amino acid positions 74, 75, 76, 77, 78, 79, 80, 81-85, 86-90, 91-95, 96-100, 101-105, 106-110, 111-115, 116-120, 121-125, 126-130, 131-135, 136-140, 141-145, 146-150, 151-155, 156-160, 161-165, 166-170, 171-175, 176-180, or any combination thereof. Those of skill in the art understand that in some embodiments, the amino acid sequence is preserved such that particular epitopes are retained within a resulting polypeptide.

In one aspect the invention provides a fusion protein comprising full length NY-ESO-1.

In one aspect the invention provides a fusion protein comprising partially truncated NY-ESO-1.

In one aspect the invention provides a fusion protein comprising truncated NY-ESO-1.

In one aspect the invention provides a fusion protein comprising full length LAGE-1.

In one aspect the invention provides a fusion protein comprising partially truncated LAGE-1.

In one aspect the invention provides a fusion protein comprising truncated LAGE-1.

In one aspect the LAGE-1 employed in the invention is LAGE-1a.

In one aspect the LAGE-1 employed in the invention is LAGE-1b.

In one aspect of the invention the N-terminal of NY-ESO-1 is fused to the C-terminal of the LAGE-1.

In one aspect of the invention the C-terminal of NY-ESO-1 is fused to the N-terminal of the LAGE-1.

The immunogenicity of the fusion proteins of the invention may be further increased and/or the production properties of the protein further improved by

incorporation of a fragment from a further heterologous antigen, for example protein D, a surface protein of the gram-negative bacterium, Haemophilus influenza B. Further information on immunological fusion partners derived from protein D can be obtained from WO 91/18926.

The proteins for inclusion in a fusion partner of the present invention may be chemically conjugated, or may be expressed as recombinant fusion proteins. In one embodiment, the fusion protein is expressed as a recombinant fusion protein.

The further heterologous fusion partner may assist in providing T helper epitopes (immunological fusion partner), or may assist in expressing the protein at higher yields (expression enhancer). In one embodiment, the further heterologous fusion partner may be both an immunological fusion partner and an expression enhancing partner.

In one embodiment, the protein D or derivative thereof comprises about or exactly the first 1/3 of the protein, for example about or exactly amino acids 1 to 109 of protein D. In this embodiment, amino acids 2-Lys and/or 3-Thr of the native protein D sequence may be substituted with the amino acids 2-Asp and/or 3-Pro. In a further embodiment, the protein D or derivative thereof comprises or consists of about or exactly amino acids 20 to 127 of protein D. In one embodiment, the protein D for use in the present invention does not include the secretion sequence of the protein. Generally, in fusion proteins of the present invention, the protein D derivative is not lipidated.

In one embodiment, the protein D further comprises the amino acids Met, Asp and Pro, for example fused to the N-terminus of the protein D fragment (ie the construct may comprise or consist of "MDP - 20-127 protein D"). It is thought these three additional amino acids may aid the stability of the protein and/or increase the level of the protein expression thereof.

In one aspect the invention provides a fusion protein in which the N-terminal fragment (i.e the first third) of protein D (as described above) is fused to the N-terminus of a fusion protein of the invention or an immunogenic fragment thereof. More specifically, a fusion of protein D and the N-terminus of the fusion protein of the invention may be effected such that the latter replaces the C-terminal-fragment of protein D that has been excised. Thus the N-terminus of protein D becomes the N-terminus of the fusion protein.

Other heterologous fusion partners or fragments thereof may be included in the fusion protein of the invention, instead of or in addition to protein D, for example:

- the non-structural protein from influenzae virus, NS1 (haemagglutinin). Typically, the N-terminal 81 amino acids may be used, although different fragments may be used provided they include T-helper epitopes;
- LytA derived from Streptococcus pneumoniae, which synthesize an N-acetyl-L-alanine amidase LytA coded by the LytA gene (Gene, 43 (1986) page 265-272) such as the repeat portion of the LytA molecule found in the C terminal end, for example starting at residue 178 such as residues 188 - 305. In one embodiment, the heterologous fusion partner is CLytA. In a further embodiment, the heterologous fusion partner is CPC, a fusion protein comprising CLytA-P2-CLytA, as described in WO03/104272. Purification of hybrid proteins containing the C-LytA fragment at its amino terminus has been described in Biotechnology: 10, (1992) page 795-798.

Fusion proteins of the invention may further include an affinity tag, for example, a histidine tail (also known as a his-tag) comprising between 1 to 10, for example 6 or 10 histidine residues. These residues may, for example, be on the terminal portion, such as the N-terminal and/or the C-terminal portion of the protein. The affinity tag may be incorporated to further improve the purification of the protein.

Certain specific fusion proteins of the invention may, for example, be constructed as described in the Figures Each of the embodiments set forth in the Figures represent independent aspects of the invention. Further examples of constructs of fusion proteins according to the present invention are given in Tables 1-4 and in the Sequence Listing.

Nucleic Acids. The present invention also extends to the nucleic acids and polynucleic acids, such as DNA, encoding for fusion proteins of the invention. The processes of the invention may be performed by conventional recombinant techniques such as described in Maniatis et al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989. In particular, a process may comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the fusion protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved, for example by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest. Expression vectors comprising nucleotide sequences encoding fusion proteins of the present invention are novel and also form part of the invention.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules, which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions. Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but are generally E. coli or CHO cells. Suitable vectors include plasmids for example TMCP14 or pET21 or pET26, pcDNA3, bacteriophages, cosmids and recombinant viruses. In one embodiment in which expression is in baculovirus, yeast or CHO host cells, one of the following vectors could be used: pEE14, pPICZA, pPICZB, pPICZC, pDMT-DEST48 and pAcSG2. The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al. cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al. cited above, or "DNA Cloning" Vol. II, D.M.

Glover ed., IRL Press Ltd, 1985. The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen et al., Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

The DNA may be codon optimized by standard techniques to further facilitate expression of the relevant host.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al. and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C. The proteins of the present invention may be expressed in prokaryotes or eukaryotes such as yeast but are often expressed in E. coli. Particular strains of E. coli such as:

- AR58: a cryptic λ lysogen derived from N99 that is gal E::Tn 10,Δ-8(chlD-pgl),Δ-H1(cro-chlA),N^+, and cI857 (ref: Proc.Natl.Acad.Sci.USA vol82,pp.88-92,January 1985 Biochemistry)
- BLR (DE3) Novagen, WI, USA (catalogue number: 69053-4): BLR is a $recA^-$ derivative of BL21, may be employed. Generally a selection marker of, for example kanamycine resistance or ampicillin resistance is incorporated to facilitate identification of the successful incorporation of the recombinant gene/construct into the expression system.

The product is recovered by conventional methods according to the host cell and according to the localisation of the expression product (intracellular or secreted into the culture medium or into the cell periplasm). Thus, where the host cell is bacterial, such as E. coli it may, for example, be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation,

adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

The proteins of the present invention are provided either soluble in a liquid form or in a lyophilised form. The present invention also provides pharmaceutical composition such as a vaccine comprising a fusion protein of the present invention and a pharmaceutically acceptable excipient.

When administered, the therapeutic compositions of the present invention can be administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

The amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors, within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons. It is generally expected that each human dose will comprise 1 to 1000 µg of protein, and preferably 30 - 300 µg.

In one aspect the pharmaceutical compositions used to administer the fusion proteins of the invention will be a vaccine. The vaccine may optionally contain one or more other tumour-associated antigens, polypeptides and/or peptides. For example, members belonging to the MAGE, LAGE and GAGE families.

Combination of NY-ESO-1/LAGE-1 and MAGE. In one embodiment of the present invention there is provided a composition comprising (a) an antigen component comprising a NY-ESO-1 or LAGE-1 antigen or fusion protein as described herein and (b) an antigen component comprising a MAGE antigen or fusion protein. In one embodiment, the composition may further comprise an adjuvant as described herein.

The MAGE antigen for use in the combination may comprise the full length MAGE antigen. Alternatively, the MAGE antigen may comprise an immunogenic portion of MAGE in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids may be deleted from or substituted in the amino acid sequence. In one embodiment of the present invention, 2 amino acids may be deleted from the N-terminus of the MAGE sequence. In one embodiment of the present invention in which the antigen is MAGE-A3 or an immunogenic portion thereof, the sequence of MAGE-A3 may be from amino acid 3 to 314 of MAGE-A3.

In one embodiment of the present invention there is provided a composition comprising an NY-ESO-1/LAGE-1 antigen and/or fusion protein as described herein and a fusion protein comprising a MAGE-A3 antigen. In an alternative embodiment, the fusion protein comprising the MAGE-A3 antigen comprises or consists of the MAGE-A3 antigen and a fusion partner protein comprising about or approximately or about the first 109 amino acids of protein D, in which one or two or more amino acids from protein D are optionally substituted, and in which the signal sequence of protein D is optionally present, in addition to the first 109 amino acids of protein D.

The fusion proteins of the present invention may additionally optionally comprise one or more amino acids as “linkers” between the sequences of the antigen and the fusion partners or fusion partner proteins or between the antigen and a His tail, if present. The amino acids may be unrelated to the sequences of the antigen and/or fusion partner.

Fusion proteins of the present invention, as described herein, may additionally comprise amino acids Met-Asp-Pro at the N-terminal end of the fusion protein sequence. The Met amino acid may be from the original protein D sequence or may be from an unrelated sequence.

In one embodiment, the sequence of a fusion protein comprising MAGE-A3 and protein D for use in combinations of the present invention is shown in SEQ ID NO:98. SEQ ID NO:98, from the N-terminus, comprises the following features:

Amino acids 1-18	signal sequence of Protein D including 1-Met and the substitutions 2-Asp and 3-Pro for the native aa 2-Lys and 3-Thr of protein D
Amino acids 19-127	inclusive of amino acids 20 to 127 of Protein D
Amino acids 128-129	unrelated amino acids Met-Asp at aa 128-129 to create a cloning site
Amino acids 130-441	fragment of MAGE3 (amino acids 3-314 of MAGE3)
Amino acids 442-443	unrelated amino acids Gly-Gly
Amino acids 444-451	7 his tail

The present invention also extends to methods of preparing said vaccines/ compositions and to fusion proteins and vaccines/compositions obtained by or obtainable by the methods described.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds. Powell M.F. & Newman M.J). (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The fusion proteins of the present invention may be adjuvanted in a vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes. Other known adjuvants include CpG containing oligonucleotides. The oligonucleotides are characterised in that the CpG dinucleotide is unmethylated. Such oligonucleotides are well known and are described in, for example WO 96/02555.

In the formulation of the inventions it may be desirable that the adjuvant composition induces an immune response preferentially of the TH1 type. In one embodiment there is provided an adjuvant system including, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-

MPL) together with an aluminium salt. CpG oligonucleotides may also induce a TH1 response and may also be included.

In one embodiment there is provided a composition comprising a fusion protein as described herein and an adjuvant composition comprising the combination of a monophosphoryl lipid A and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. One formulation that may be used comprises QS21, 3D-MPL & tocopherol in, for example, an oil in water emulsion is described in WO 95/17210. Another adjuvant formulation for use in the present invention may comprise QS21, 3D-MPL & CpG or equivalent thereof, for example, in an oil in water emulsion or as a liposomal formulation. Accordingly in one embodiment of the present invention there is provided a vaccine comprising a fusion protein of the invention and an adjuvant, for example as described above. The present invention also extends to methods of preparing vaccines and compositions comprising fusion proteins as described herein.

The present invention also contemplates delivery of nucleic acids, polypeptides or peptides as described herein for vaccination. Delivery of polypeptides and peptides can be accomplished according to standard vaccination protocols which are well known in the art. In another embodiment, the delivery of nucleic acid may be accomplished by ex vivo methods, i.e. by removing a cell from a subject, genetically engineering the cell to include a cancer associated antigen, and reintroducing the engineered cell into the subject. In general, this may involve introduction in vitro of a functional copy of a gene into a cell(s) of a subject, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements, which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO 95/00654. In vivo nucleic acid delivery using vectors such as viruses and targeted liposomes also is contemplated according to the invention.

Abbreviations

CO collagen-like region

W/Ocoll	without collagen-like region (collagen-like domain)
PD1/3	protein D first third

Exemplary embodiments of fusion proteins and the nucleotide sequence encoding same are provided in Tables 1-3.

Table 1. Exemplary embodiments of fusion proteins and the nucleotide sequence encoding same are provided. Each nucleotide sequence is described by subject matter, identified by unique nucleotide sequence identifier (SEQ ID NO:), and set forth in the sequence listing. Each fusion protein is described by subject matter, identified by unique amino acid sequence identifier (SEQ ID NO:), and set forth in the sequence listing.

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
Hybrid Collagen NY-ESO-1/LAGE1a without collagen		
1	**Embodiment A – nucleotide sequence Hybrid Coll NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	Collagen like domain	1-210bp
	NY-ESO-1	1-537bp
	Linker	538-543bp
	LAGE1a	544-846bp
	His-tag	847-864bp
	Stop	865-867bp
2	**Embodiment B – nucleotide sequence 1/3 protein D/Hybrid Coll NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain	334-540bp
	NY-ESO-1	334-867bp
	Linker	868-873bp
	LAGE1a	874-1176bp
	His-tag	1177-1194bp
	Stop	1195-1197bp
3	**Embodiment A – amino acid sequence Hybrid Coll NY-ESO-1/LAGE1a WO coll with His-tag**	
	Collagen like domain	1-70aa
	NY-ESO-1	1-179aa
	Linker	180-181aa

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
	LAGE1a	182-282aa
	His-tag	283-288aa
4	**Embodiment B – amino acid sequence 1/3 protein D/Hybrid Coll NY-ESO-1/LAGE1a WO coll with His-tag**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain	112-180aa
	NY-ESO-1	112-289aa
	Linker	290-291aa
	LAGE1a	292-392aa
	His-tag	393-398aa
	Hybrid Collagen truncated NY-ESO-1/LAGE1a without collagen	
5	**Embodiment C - Hybrid Coll trunc NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	Collagen like domain	1-72bp
	NY-ESO-1	1-399bp
	Linker	400-405bp
	LAGE1a	406-708bp
	His-tag	709-726bp
	Stop	727-729bp
6	**Embodiment D – nucleotide sequence-- 1/3 protein D/Hybrid Coll trunc NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain	334-402bp
	NY-ESO-1	334-729bp
	Linker	730-735bp
	LAGE1a	736-1038bp
	His-tag	1039-1056bp
	Stop	1057-1059bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
7	**Embodiment C - Hybrid Coll trunc NY-ESO-1/LAGE1a WO coll with His-tag**	
	Collagen like domain	1-24aa
	NY-ESO-1	1-133aa
	Linker	134-135aa
	LAGE1a	136-236aa
	His-tag	237-242aa
8	**Embodiment D – amino acid sequence 1/3 protein D/Hybrid Coll trunc NY-ESO-1/LAGE1a WO coll with His-tag**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain	112-134aa
	NY-ESO-1	112-243aa
	Linker	244-245aa
	LAGE1a	246-346aa
	His-tag	347-352aa
Hybrid NY-ESO-1/LAGE1a without collagen like domain and contiguous cystein rich region (8aa)		
9	**Embodiment E – nucleotide sequence Hybrid NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	NY-ESO-1	1-306bp
	Linker	307-312bp
	LAGE1a	313-615bp
	His-tag	616-633bp
	Stop	634-636bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
10	**Embodiment F – nucleotide sequence 1/3 protein D/Hybrid NY-ESO-1/LAGE1a WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	NY-ESO-1	334-636bp
	Linker	637-642bp
	LAGE1a	643-945bp
	His-tag	946-963bp
	Stop	964-966bp
11	**Embodiment E – amino acid sequence Hybrid NY-ESO-1/LAGE1a WO coll with His-tag**	
	NY-ESO-1	1-102aa
	Linker	103-104aa
	LAGE1a	105-205aa
	His-tag	206-211aa
12	**Embodiment F – amino acid sequence 1/3 protein D/Hybrid NY-ESO-1/LAGE1a WO coll with His-tag**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	NY-ESO-1	112-212aa
	Linker	213-214aa
	LAGE1a	215-315aa
	His-tag	316-321aa
	Hybrid Collagen LAGE1a/NY-ESO-1 without collagen	
13	**Embodiment G – nucleotide sequence Hybrid Coll LAGE1a/NY-ESO-1 WO coll (codon optimised)**	
	Collagen like domain of NY-ESO-1	1-210bp
	LAGE1a	211-540bp
	Linker	541-546bp
	NY-ESO-1	547-849bp
	His-tag	850-867bp
	Stop	868-870bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
14	**1/3 protein D/Hybrid Coll LAGE1a/NY-ESO-1 WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain of NY-ESO-1	334-540bp
	LAGE1a	541-870bp
	Linker	871-876bp
	NY-ESO-1	877-1179bp
	His-tag	1180-1197bp
	Stop	1198-1200bp
15	**Embodiment G – amino acid sequence Hybrid Coll LAGE1a/NY-ESO-1 WO coll with His-tag**	
	Collagen like domain of NY-ESO-1	1-70aa
	LAGE1a	71-180aa
	Linker	181-182aa
	NY-ESO-1	183-283aa
	His-tag	284-289aa
16	**1/3 protein D/Hybrid Coll LAGE1a/NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:14)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain of NY-ESO-1	112-180aa
	LAGE1a	181-290aa
	Linker	291-292aa
	NY-ESO-1	293-393aa
	His-tag	394-399aa

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
Hybrid Collagen truncated LAGE1a/NY-ESO-1 without collagen		
17	**Hybrid Coll trunc LAGE1a/NY-ESO-1 WO coll (codon optimised)**	
	Collagen like domain of NY-ESO-1	1-72bp
	LAGE1a	73-402bp
	Linker	403-408bp
	NY-ESO-1	409-711bp
	His-tag	712-729bp
	Stop	730-732bp
18	**1/3 protein D/Hybrid Coll trunc LAGE1a/NY-ESO-1 WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain of NY-ESO-1	334-402bp
	LAGE1a	403-732bp
	Linker	733-738bp
	NY-ESO-1	739-1041bp
	His-tag	1042-1059bp
	Stop	1060-1062bp
19	**Hybrid Coll trunc LAGE1a/NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:17)**	
	Collagen like domain of NY-ESO-1	1-24aa
	LAGE1a	25-134aa
	Linker	135-136aa
	NY-ESO-1	137-237aa
	His-tag	238-243aa

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
20	**1/3 protein D/Hybrid Coll trunc LAGE1a/NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:18)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain of NY-ESO-1	112-134aa
	LAGE1a	135-244aa
	Linker	245-246aa
	NY-ESO-1	247-347aa
	His-tag	348-353aa
Hybrid LAGE1a/NY-ESO-1 without collagen like domain and contiguous cystein rich region (8aa)		
21	**Embodiment E' – nucleotide sequence Hybrid LAGE1a/NY-ESO-1 WO coll (codon optimised)**	
	LAGE1a	1-309bp
	Linker	310-315bp
	NY-ESO-1	316-618bp
	His-tag	619-636bp
	Stop	637-639bp
22	**1/3 protein D/Hybrid LAGE1a/NY-ESO1 WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	LAGE1a	334-639bp
	Linker	640-645bp
	NY-ESO-1	646-948bp
	His-tag	949-966bp
	Stop	967-969bp
23	**Embodiment E' – amino acid sequence Hybrid LAGE1a/NY-ESO-1 WO coll with His-tag**	
	LAGE1a	1-103aa
	Linker	104-105aa
	NY-ESO-1	106-206aa
	His-tag	207-212aa

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
24	**1/3 protein D/Hybrid LAGE1a/NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:22)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	LAGE1a	112-213aa
	Linker	214-215aa
	NY-ESO-1	216-316aa
	His-tag	317-322aa
His N-terminal Hybrid NY-ESO-1/Lage1a without collagen and contiguous cystein rich region (8aa)		
25	**His-Enterokinase site-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	NY-ESO-1	73-375bp
	Linker	376-381bp
	LAGE1a	382-684bp
	Stop	685-687bp
26	**His-Enterokinase site-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:25)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	NY-ESO-1	25-125aa
	Linker	126-127aa
	LAGE1a	128-228aa
27	**His-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-21bp
	NY-ESO-1	22-324bp
	Linker	325-330bp
	LAGE1a	331-633bp
	Stop	634-636bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
28	**His-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:26)**	
	His-tag (6 His)	1-7aa
	NY-ESO-1	8-108aa
	Linker	109-110aa
	LAGE1a	111-211aa
His-N-terminal Hybrid Collagen truncated NY-ESO-1/ LAGE1a without collagen		
29	**His-Enterokinase site-Coll trunc-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	Collagen like domain	73-141bp
	NY-ESO-1	73-468bp
	Linker	469-474bp
	LAGE1a	475-777bp
	Stop	778-780bp
30	**His-Enterokinase site-Coll trunc-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:29)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	Collagen like domain	25-47aa
	NY-ESO-1	25-156aa
	Linker	157-158aa
	LAGE1a	159-259aa
31	**His-Coll trunc-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-21bp
	Collagen like domain	22-90bp
	NY-ESO-1	22-417bp
	Linker	418-423bp
	LAGE1a	424-726bp
	Stop	727-729bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
32	**His-Coll trunc-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:31)**	
	His-tag (6 His)	1-7aa
	Collagen like domain	8-30aa
	NY-ESO-1	31-139aa
	Linker	140-141aa
	LAGE1a	142-242aa
His N-terminal Hybrid collagen NY-ESO-1/LAGE1a without collagen like domain		
33	**His-Enterokinase site-Coll-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	Collagen like domain	73-279bp
	NY-ESO-1	73-606bp
	Linker	607-612bp
	LAGE1a	613-915bp
	Stop	916-918bp
34	**His-Enterokinase site-Coll-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:33)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	Collagen like domain	25-93aa
	NY-ESO-1	25-202aa
	Linker	203-204aa
	LAGE1a	205-305aa
35	**His-Coll-NY-ESO-1/LAGE1a (codon optimised)**	
	His-tag sequence	1-21bp
	Collagen like domain	22-228bp
	NY-ESO-1	22-555bp
	Linker	556-561bp
	LAGE1a	562-864bp
	Stop	865-867bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
36	**His-ColI-NY-ESO-1/LAGE1a (encoded by SEQ ID NO:35)**	
	His-tag (6 His)	1-7aa
	Collagen like domain	8-76aa
	NY-ESO-1	77-185aa
	Linker	186-187aa
	LAGE1a	188-288aa
His-N-terminal Hybrid Lage1a/NY-ESO-1 without collagen and contiguous cystein rich region (8aa)		
37	**His-Enterokinase site-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	LAGE1a	73-378bp
	Linker	379-384bp
	NY-ESO-1	385-687bp
	Stop	688-690bp
38	**His-Enterokinase site-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:37)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	LAGE1a	25-126aa
	Linker	127-128aa
	NY-ESO-1	129-229aa
39	**His-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-21bp
	LAGE1a	22-327bp
	Linker	328-333bp
	NY-ESO-1	334-636bp
	Stop	637-639bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
40	**His-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:39)**	
	His-tag (6 His)	1-7aa
	LAGE1a	8-109aa
	Linker	110-111aa
	NY-ESO-1	112-212aa
His-N-terminal Hybrid Collagen truncated LAGE1a/NY-ESO-1 without collagen		
41	**His-Enterokinase site-Coll trunc-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	Collagen like domain of NY-ESO-1	73-141bp
	LAGE1a	142-471bp
	Linker	472-477bp
	NY-ESO-1	478-780bp
	Stop	781-783bp
42	**His-Enterokinase site-Coll trunc-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:41)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	Collagen like domain of NY-ESO-1	25-47aa
	LAGE1a	48-157aa
	Linker	158-159aa
	NY-ESO-1	160-260aa
43	**His-Coll trunc-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-21bp
	Collagen like domain of NY-ESO-1	22-90bp
	LAGE1a	91-420bp
	Linker	421-426bp
	NY-ESO-1	427-729bp
	Stop	730-732bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
44	**His-Coll trunc-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:43)**	
	His-tag (6 His)	1-7aa
	Collagen like domain of NY-ESO-1	8-30aa
	LAGE1a	31-140aa
	Linker	141-142aa
	NY-ESO-1	143-243aa
His N-terminal Hybrid collagen LAGE1a/NY-ESO-1 without collagen like domain		
45	**His-Enterokinase site-Coll-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-36bp
	Enterokinase site	37-72bp
	Collagen like domain of NY-ESO-1	73-279bp
	LAGE1a	280-609bp
	Linker	610-615bp
	NY-ESO-1	616-918bp
	Stop	919-921bp
46	**His-Enterokinase site-Coll-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:45)**	
	His-tag (10 His)	1-12aa
	Enterokinase site	13-24aa
	Collagen like domain of NY-ESO-1	25-93aa
	LAGE1a	94-203aa
	Linker	204-205aa
	NY-ESO-1	206-306aa
47	**His-Coll-LAGE1a/NY-ESO-1 (codon optimised)**	
	His-tag sequence	1-21bp
	Collagen like domain of NY-ESO-1	22-228bp
	LAGE1a	229-558bp
	Linker	559-564bp
	NY-ESO-1	565-867bp
	Stop	868-870bp

SEQ ID NO:	TABLE 1 CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
48	**His-Coll-LAGE1a/NY-ESO-1 (encoded by SEQ ID NO:47)**	
	His-tag (6 His)	1-7aa
	Collagen like domain of NY-ESO-1	8-76aa
	LAGE1a	77-186aa
	Linker	187-188aa
	NY-ESO-1	189-289aa

Table 2. Additional exemplary embodiments of fusion proteins and the nucleotide sequences encoding same are provided. Each nucleotide sequence is described by subject matter, identified by unique nucleotide sequence identifier (SEQ ID NO:), and set forth in the sequence listing. Each fusion protein is described by subject matter, identified by unique amino acid sequence identifier (SEQ ID NO:), and set forth in the sequence listing.

SEQ ID NO:	TABLE 2. CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
	Partially truncated collagen NY-ESO-1	
50	**Coll trunc NY-ESO-1 (codon optimised)**	
	Collagen like domain	1-72bp
	NY-ESO-1	1-399bp
	His-tag	400-417bp
	Stop	418-420bp
51	**Coll trunc NY-ESO-1 with His-tag (encoded by SEQ ID NO:50)**	
	Collagen like domain	1-24aa
	NY-ESO-1	1-133aa
	His-tag	134-139aa
52	**1/3 protein D/Coll trunc NY-ESO-1 (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain	334-402bp
	NY-ESO-1	334-729bp
	His-tag	730-747bp
	Stop	748-750bp

SEQ ID NO:	TABLE 2. CONSTRUCT DESCRIPTION	SEQUENCE COMPONENTS
53	**1/3 protein D/Coll trunc NY-ESO-1 with His-tag (encoded by SEQ ID NO:52)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain	112-134aa
	NY-ESO-1	112-243aa
	His-tag	244-249aa
	NY-ESO-1 WO coll	
54	**NY-ESO-1 WO coll (codon optimised)**	
	NY-ESO-1	1-306bp
	His-tag	307-324bp
	Stop	325-327bp
55	**NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:54)**	
NY-ESO-1		1-102aa
His-tag		103-108aa
56	**1/3 protein D/NY-ESO-1 WO coll (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	NY-ESO-1	334-636bp
	His-tag	637-654bp
	Stop	655-657bp
57	**1/3 protein D/NY-ESO-1 WO coll with His-tag (encoded by SEQ ID NO:56)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	NY-ESO-1	112-212aa
	His-tag	213-218aa

Table 3. Additional exemplary embodiments of fusion proteins and the nucleotide sequence encoding same are provided. Each nucleotide sequence is described by subject matter, identified by unique nucleotide sequence identifier (SEQ ID NO:), and set forth in the sequence listing. Each fusion protein is described by subject matter, identified by unique amino acid sequence identifier (SEQ ID NO:), and set forth in the sequence listing.

SEQ ID NO:	TABLE 3 DESCRIPTION	SEQUENCE COMPONENTS
Hybrid Collagen LAGE-1a without LAGE-1a collagen-like domain		
59	**Hybrid Coll LAGE-1a WO coll (codon optimised)**	
	Collagen like domain of NY-ESO-1	1-210bp
	LAGE-1a	211-540bp
	His-tag	541-558bp
	Stop	559-561bp
60	**Hybrid Coll LAGE-1a WO coll with His-tag (encoded by SEQ ID NO:59)**	
	Collagen like domain of NY-ESO-1	1-70aa
	LAGE-1a	71-180aa
	His-tag	181-186aa
61	**1/3 protein D/Hybrid Coll LAGE-1a WO coll with His-tag (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain of NY-ESO-1	334-540bp
	LAGE-1a	541-870bp
	His-tag	871-888bp
	Stop	889-891bp
62	**1/3 protein D/Hybrid Coll LAGE-1a WO coll with His-tag (encoded by SEQ ID NO:61)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain of NY-ESO-1	112-180aa
	LAGE-1a	181-290aa
	His-tag	291-296aa

SEQ ID NO:	TABLE 3 DESCRIPTION	SEQUENCE COMPONENTS
Hybrid Collagen truncated LAGE-1a without collagen-like domain		
63	**Hybrid Collagen truncated LAGE-1a without collagen-like domain**	
	Collagen like domain of NY-ESO-1	1-72bp
	LAGE-1a	73-402bp
	His-tag	403-420bp
	Stop	421-423bp
64	**Hybrid Coll trunc LAGE-1a WO coll with His-tag (encoded by SEQ ID NO:63)**	
	Collagen like domain of NY-ESO-1	1-24aa
	LAGE-1a	25-134aa
	His-tag	135-140aa
65	**1/3 protein D/Hybrid Coll trunc LAGE-1a WO coll with His-tag (codon optimised)**	
	MDP initiation sequence	1-9bp
	1/3 protein D	10-333bp
	Collagen like domain of NY-ESO-1	334-402bp
	LAGE-1a	403-732bp
	His-tag	733-750bp
	Stop	751-753bp
66	**1/3 protein D/Hybrid Coll trunc LAGE-1a WO coll with His-tag (encoded by SEQ ID NO:65)**	
	MDP initiation sequence	1-3aa
	1/3 protein D	4-111aa
	Collagen like domain of NY-ESO-1	112-134aa
	LAGE-1a	135-244aa
	His-tag	245-250aa
LAGE-1a without collagen like domain and contiguous cystein rich region (8aa)		
67	**LAGE-1a WO coll (codon optimised)**	
LAGE-1a		1-309bp
His-tag		310-327bp
Stop		328-330bp

SEQ ID NO:	TABLE 3 DESCRIPTION	SEQUENCE COMPONENTS
68	**LAGE-1a WO coll with His-tag (encoded by SEQ ID NO:67)**	
LAGE-1a		1-103aa
His-tag		104-109aa
69	**1/3 protein D/LAGE-1a WO coll (codon optimised)**	
MDP initiation sequence		1-9bp
1/3 protein D		10-333bp
LAGE-1a		334-639bp
His-tag		640-657bp
Stop		6578-660bp
70	**1/3 protein D/LAGE-1a WO coll with His-tag (encoded by SEQ ID NO: 69)**	
MDP initiation sequence		1-3aa
1/3 protein D		4-111aa
LAGE-1a		112-213aa
His-tag		214-219aa

Table 4. The fusion proteins discussed in the Examples and the nucleotide sequences encoding same are provided. Each nucleotide sequence is described by subject matter, identified by unique nucleotide sequence identifier (SEQ ID NO:), and set forth in the sequence listing. Each fusion protein is described by subject matter, identified by unique amino acid sequence identifier (SEQ ID NO:), and set forth in the sequence listing.

TABLE 4		
CONSTRUCT NAME	**NUCLEOTIDE SEQUENCE**	**AMINO ACID SEQUENCE**
LVL020	SEQ ID NO:72	SEQ ID NO:73
LVL024	SEQ ID NO:74	SEQ ID NO:75
LVL026	SEQ ID NO:76	SEQ ID NO:77
LVL030	SEQ ID NO:78	SEQ ID NO:79
LVL068	SEQ ID NO:80	SEQ ID NO:81
LVL076	SEQ ID NO:82	SEQ ID NO:83
LVL078	SEQ ID NO:84	SEQ ID NO:85
LVL079	SEQ ID NO:86	SEQ ID NO:87
LVL106	SEQ ID NO:88	SEQ ID NO:89
LVL151	SEQ ID NO:90	SEQ ID NO:91

TABLE 4		
CONSTRUCT NAME	NUCLEOTIDE SEQUENCE	AMINO ACID SEQUENCE
LVL155	SEQ ID NO:92	SEQ ID NO:93
LVL156	SEQ ID NO:94	SEQ ID NO:95
LVL157	SEQ ID NO:96	SEQ ID NO:97

As will be evident from the sequence listing, many of the constructs of Table 4 have similar designs to one or more embodiment set forth in the preceding tables. For example, LVL068 shares the same design as the embodiment set forth as SEQ ID NO:45, Table 1. LVL076 shares the same design as the embodiment set forth as SEQ ID NO:25, Table 1. LVL078 shares the same design as the embodiment set forth as SEQ ID NO:33, Table 1. LVL079 shares the same design as the embodiment set forth as SEQ ID NO:37, Table 1.

In addition, several of the fusion protein constructs set forth in Table 4, namely LVL155, LVL106, LVL156, LVL157, LVL151, were generated by routine modifications of other fusion protein sequences set forth in Table 4, namely LVL068, LVL030, LVL076, LVL078, LVL024, respectively. Such modifications include the removal of the amino acid residues between protein D and the beginning of the chimers (i.e., the portion derived from either of NY-ESO-1 and LAGE-1) and the removal of the amino acids between the his-tag and the beginning of the chimer. Thus, certain of the fusion proteins of Table 4 correspond closely to other fusion proteins in Table 4. The correspondence between these fusion proteins is set forth in Table 5 and described in greater detail in Example 4.

Table 5. Correspondence between LVL068, LVL030, LVL076, LVL078, LVL024 and the modified LVL155, LVL106, LVL156, LVL157, LVL151.

TABLE 5		
FUSION PROTEIN CONSTRUCT	**CORRESPONDS TO**	**FUSION PROTEIN CONSTRUCT**
LVL068		LVL155
LVL030		LVL106
LVL076		LVL156
LVL078		LVL157
LVL024		LVL151

EXAMPLES

Example 1. NY-LA1 chimeric protein design and production

Several NY-ESO-1/LAGE-1 fusion proteins were designed with and without the collagen-Like domain, and with and without the end terminus of protein D as summarized in Figure 17. The designed constructs were codon optimized for expression in Escherichia coli. The synthetic gene was assembled from oligonucleotides and/or PCR products. The fragment was cloned into pGA4 backbone (AmpR) using KpnI and SacI restriction sites with the addition of NdeI and XhoI sites in the 5' end and the 3' end of the optimized gene respectively.

The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectrometry. The final construct was verified by sequencing. The optimized coding sequence for the different NY/LAGE chimeric constructs was subcloned directly into pET19 (AmpR) multiple cloning site using NdeI and XhoI restriction sites to get the NY/LAGE chimer expression plasmids. For cloning into pET26, PCR primers were designed in order to ad N-terminal Histidine-tail. This amplification resulted in the addition of the 6 Histidines tail in phase with the coding region of the different constructs. This amplified fragment was enzymatically digested with NdeI/XhoI restriction enzymes and the different NY/LAGE chimeric constructs were subsequently cloned into pET26 (KanR) multiple cloning site to get the expression plasmid. The final constructs were verified by sequencing.

Shake-flask production. Growth and induction of bacterial host strain

Culture

Bacteria were grown on 800 ml of Luria-Bertani (LB) broth (BD) + 1% (w/v) glucose (Laboratoire MAT, catalogue number: GR-0101) + antibiotic(Carbenicillin 100 µg/ml for pET19, kanamycin 40 µg/ml for pET26), in 2.5L shaking flask. Cultures were incubated at 37°C for BLR (DE3) cells until an $O.D._{600nm}$ around 0.8.

Induction

At $O.D._{600nm}$ around 0.8, the cultures BLR (DE3) were induced at 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; EMD Chemicals Inc., catalogue number: 5815) and incubated for 16-18 hours at 16°C. 5 to 15 mg of specific protein/800ml have been obtained with construct LVL106, 151, 155 and 157. The protein production for each construct is summarized in Figure 17.

Example 2. Summary of preliminary purification and stability

Extraction and Purification of the Protein

Cells are harvested by centrifugation then disrupted by physical or chemical means and the resulting crude extract retained to isolate the polypeptide of interest.

Purification

The expressed recombinant proteins were solubilized with guanidine hydrochloride solution and loaded on an Immobilized Metal Affinity Chromatography (IMAC) resin. Proteins were then washed on column with 8M and 4M urea solutions before elution by increasing imidazole concentration. Proteins were then desalted in the final 4M urea buffer, pH 7.0 for further use. Purification was evaluated by SDS PAGE and Western Blot, to verify the purity and the identity of the proteins.

Stability test of purified fusion protein

Stability assays were performed at 37°C and proteins were evaluated by SDS-PAGE. Preliminary stability assay did not reveal major issue.

Preliminary solubilization assay

The solubility of the proteins was evaluated as summarized in the following Chart.

BUFFER	CONSTRUCT							
	LVL 076	LVL 079	LVL 78	LVL 68	LVL 020	LVL 26	LVL 024	LVL 30
PBS 1X; 1mM TCEP; 1mM EDTA, pH 7,03	p	S	S	P	P	P	NT	P
20mM Bicine;138mM NaCl; 1mM TCEP; 1mM EDTA, pH 8,68	p	S	P	P	P	P	NT	P
20mM imidazole; 138mM NaCl; 1mM TCEP; 1mM EDTA, pH 5,99	p	S	P	P	P	P	NT	P
10mM Sodium Ac; 5mM NaCl; 1mM TCEP; 1mM EDTA, pH 4.99	S	S	S	S	S	S	NT	S
10mM citrate acid; 5mM NaCl; 1mM TCEP; 1mM EDTA, pH 3.7	NT	NT	S	S	NT	NT	NT	S

Chart 1. Fusion Protein Solubility. Key: P precipate; S no precipitate; NT not tested.

Example 3: IM immunization with fusion proteins

The fusion proteins were evaluated preclinically in a mouse model involving a series of intramuscular immunizations screening experiments, as described below. The mouse model chosen was CB6F1, a first generation resulting from the cross of C57BL6 mice and Balb/c mice. Such mice are commercially available from Charles River Laboratories, Inc., 251 Ballardvale Street, Wilmington, MA 01887-1000. The chosen tumor cell line was B16 (Mouse melanoma cell line), a transplantable murine melanoma commercially available for the study of cancer therapies.

Screening #1

Experimental design. In a 76-day trial, CB6F1 mice were used to assess each of LVL076, LVL079, LVL078, LVL068, LVL020, LVL026, LVL024, LVL030 to determine whether intramuscular immunization with the fusion protein plus adjuvant conferred protection against subcutaneous challenge with transplanted tumor cells (B16/NYESO1). Specifically, mice were immunized intramuscularly with 50µL injections containing 15µg protein and an adjuvant. The adjuvant selected was AS15. AS15 is a liposomal adjuvant formulation comprising QS21, 3D-MPL and CpG.

Trials were carried out with fusion proteins set forth in 1A and 1B, below. Mice were divided into groups of 15 mice/group. Mice were immunized on day 0 and again on day 14 as follows:

Trial 1A

- LVL079
- LVL026
- LVL068
- LVL030

Trial 1B

- LVL076
- LVL020
- LVL078
- LVL024

Controls

- Antigen buffer/AS15 buffer
- Full length NY-ESO-1
- LAGE-1a without the collagen-like domain (CLD)
- MAGE A3

Six mice/group were challenged with subcutaneous transplanted B16/NY-ESO-1 tumors on day 28. Antibody response to NY-ESO-1 full-length, LAGE-1a without collagen like domain, and human collagen was assessed at day 0, 14, 28, and 76 by ELISA (IgG1 and IgG2a). Cell-mediated response was assessed by FACS at day 28 using harvested splenocytes (restimulation--3 pools of 3--with NY-ESO-1 and LAGE-1a peptide pools). The experimental design of screening #1 is summarized in Figure 18.

Results. Of the four controls, only full-length NY-ESO-1 conferred some protection compared with buffer. See Figure 19. Of the groups of mice receiving either full-length NY-ESO-1 or LVL030, two from each group were tumor free at the end of the trial. Of the mice receiving LVL068, four were tumor free at the end of the study. LVL068 and LVL078 conferred longer survival compared with mice that received buffers. See Figure 20. NY-ESO-1-specific immune responses were assessed by ELISA, FACS, and Western blot. LAGE-1a(without the collagen like domain)-specific

immune responses were assessed by ELISA and FACS. See Figure 21. These results are summarized in the following Chart.

Immunogen	B16/NY-ESO-1 Protection	NY-ESO-1 Specific Immunity	LAGE1a Specific Immunity
LVL068	++	++	++
LVL078	+	++	++
LVL076	+	++	+
LVL024	+	++	+
LVL030	+	++	+
LVL020	+	+	+
LVL079	-	+	+
LVL026	-	+	+

Chart 2. Specific Immunity Summary. Key: (-) – lowest response; (+) – medium response; (++) – highest response.

Screening #2

Experimental design. In a 105-day trial, CB6F1 mice were used to assess each of LVL076, LVL078, LVL068, and LVL024 to determine whether intramuscular immunization with the fusion protein plus adjuvant conferred protection against subcutaneous challenge with B16/NYESO1 transplanted tumor cells (after two immunizations) or with B16/LAGE-1a tumor cells (after four immunizations). Specifically, mice were immunized intramuscularly with 50μL injections containing 15μg protein and 25μL of AS15 adjuvant.

Mice were divided into groups of 29 mice/group. Mice were immunized on day 0, 14, 28, and 42 as follows:

Trial

- LVL076
- LVL068
- LVL078
- LVL024

Controls

- Antigen buffer/AS15 buffer
- Full length NY-ESO-1
- LAGE-1a without the collagen-like domain (CLD)
- MAGE A3

Ten mice/group were challenged with subcutaneous transplanted B16/NY-ESO-1 tumor cells on day 28. Nine mice/group were challenged with subcutaneous transplanted B16/LAGE-1A tumor cells on day 56. Sera was taken and antibody response to (i) NY-ESO-1 full-length, (ii) LAGE-1a without collagen like domain, and (ii) human collagen was assessed at day 0, 14, 28, 42 56, 84 and 105 by ELISA (IgG1 and IgG2a). The experimental design of screening #2 is summarized in Figures 22 and 23.

Results

B16-NYESO1 Tumor Challenge.

Of the mice receiving LVL078, two were tumor free for over 50 days post B16-NY-ESO-1 challenge. Of the mice receiving either full-length NY-ESO-1 or LVL024, two from each group were tumor free for over 50 days, three were alive. Of the mice receiving LVL068, three were tumor free and four were alive. Of the mice receiving LVL076, 3 were tumor free and five were alive. See Figure 24.

B16-LAGE1a Tumor Challenge.

All of the mice receiving LVL076 or LAGE-1a without the collagen like region were dead earlier than day 40 post challenge. Of the mice receiving buffer alone, one survived tumor free to the end of the study. Of the mice receiving LVL024, one was tumor free at the end of the study. Of the mice receiving full-length NY-ESO-1, none were tumor free, but one was still alive at the end of the study. Of the mice receiving

LVL078, one was tumor free. Of the mice receiving LVL068, three were tumor free. Of the mice receiving LVL076, three were tumor free at the end of the study. See Figure 25. These results are summarized in the following Chart.

	NY-ESO-1		LAGE-1a	
Immunogen	Protection	Specific Immunity	Protection	Specific Immunity
LVL068	++	++	++	++
LVL078	++	++	++	++
LVL024	±	++	-	+
LVL076	+	+	-	+

Chart 3 Protection versus B16-LAGE1a Tumor Challenge. Key: (-) – lowest; (±) – next lowest response; (+) – medium response; (++) – highest response.

Human collagen-specific Immune Responses

To study whether the collagen like domain of NY-ESO-1 stimulated human collagen-specific immune responses, sera was collected and pooled 14 days post-inoculation from mice immunized with one of the following: (1) Buffers (control); (2) full-length NY-ESO-1; (3) LAGE-1a without the collagen like domain; (4) LVL068; (5) LVL078; (6) LVL024; (7) LVL076. ELISAs were carried out for each of these seven sera pools, as well as for a positive control containing mAb anti-human collagen I. The collagen like domain did not stimulate mouse anti-human collagen I antibody production. See Figure 26. Similar studies (results not shown) were carried out for collagen III and collagen VI; neither mouse anti-human collagen III nor mouse anti-human collagen VI antibody production were detected.

Example 4: Refined constructs

Modifications were carried out using routine cloning techniques on some of the constructs listed in Table 4. Specifically, LVL068, LVL030, LVL076, LVL078, LVL024 were modified to yield LVL155, LVL106, LVL156, LVL157, LVL151. There were two kinds of modifications, first the removal of 5 amino acid residues between protein D and the beginning of the chimers. For example, this modification was carried out with LVL024 (SEQ ID NO:74; SEQ ID NO:75) to yield LVL151 (SEQ ID NO:90; SEQ ID

NO:91). Thus, LVL024 corresponds with LVL 151. The second type of modification was the removal of the amino acids between the his-tag and the beginning of the chimer. This modification was carried out with LVL068 (SEQ ID NO:80; SEQ ID NO:81) to yield LVL155 (SEQ ID NO:92; SEQ ID NO:93). Thus, LVL068 corresponds with LVL 151. Each fusion protein construct that was modified and the fusion protein construct to which it corresponds is set forth in Table 5 of the Description.

As is understood, the modifications described above are not expected to result in functional differences between a fusion protein and its corresponding modified fusion protein. Thus, it is expected that one may utilize each modified fusion protein listed on the right side of the Table 5 interchangeably with its corresponding fusion protein listed on the left hand side of the chart.

Example 5.

Experimental design. In a 105-day trial, CB6F1 mice are used to assess each of LVL068, LVL030, LVL076, LVL078, LVL024 and the modified LVL155, LVL106, LVL156, LVL157, LVL151 to study intramuscular immunization with the fusion protein plus adjuvant against subcutaneous challenge with B16/NYESO1 transplanted tumor cells (after two immunizations) or with B16/LAGE-1a tumor cells (after four immunizations). Specifically, mice are immunized intramuscularly with 50µL injections containing 15µg protein and 25µL of AS15 adjuvant.

Mice are divided into groups of 29 mice/group. Mice are immunized on day 0, 14, 28, and 42 as follows:

Trial

- LVL068
- LVL030
- LVL076
- LVL078
- LVL024
- LVL155
- LVL106
- LVL156

- LVL157
- LVL151

Controls

- Antigen buffer/AS15 buffer
- Full length NY-ESO-1
- LAGE-1a without the collagen domain
- MAGE A3

Ten mice/group are challenged with subcutaneous transplanted B16/NY-ESO-1 tumor cells on day 28. Nine mice/group are challenged with subcutaneous transplanted B16/LAGE-1A tumor cells on day 56. To monitor specific immune response, sera can be taken and antibody response measured to (i) NY-ESO-1 full-length, (ii) LAGE-1a without collagen like domain, and (ii) human collagen at day 0, 14, 28, 42 56, 84 and 105 by ELISA (IgG1 and IgG2a).

The preceding examples are provided by way of illustration, not by way of limitation.

Within the present application, the article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element. The terms “approximately” and “about” as used herein are intended to be optionally deletable or replaceable with the term “exactly”, if required by the applicant, in every instance.

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The above-defined terms are more fully defined by reference to the specification as a whole.

WE CLAIM:

1. A fusion protein comprising:

(a) NY-ESO-1 or a fragment thereof, linked to

(b) LAGE-1 or a fragment thereof,

wherein at least one of NY-ESO-1 and/or LAGE-1 is truncated or partially truncated, or is a fragment including one or more epitopes of NY-ESO-1 or LAGE-1.

2. A fusion protein according to claim 1 in which the NY-ESO-1 is selected from: full length NY-ESO-1, partially truncated NY-ESO-1 or truncated NY-ESO-1 or any fragment thereof that includes one or more epitopes of NY-ESO-1.

3. A fusion protein according to any preceding claim in which the LAGE-1 is selected from: full length LAGE-1, partially truncated LAGE-1 or truncated LAGE-1 or any fragment thereof that includes one or more epitopes of LAGE-1.

4. A fusion protein according to any preceding claim in which the NY-ESO-1 or LAGE-1 is at least 95, 96, 97, 98, 99% or 100% identical to naturally occurring NY-ESO-1 or LAGE-1.

5. A fusion protein according to any preceding claim in which the LAGE-1 is LAGE-1a.

6. A fusion protein according to any preceding claim in which the N-terminus of the NY-ESO-1 is fused to the C-terminus of the LAGE-1.

7. A fusion protein according to any of claims 1 to 5 in which the C-terminus of the NY-ESO-1 is fused to the N-terminus of the LAGE-1.

8. A fusion protein according to any preceding claim in which the fusion protein further comprises a heterologous fusion partner.

9. A fusion protein according to claim 8 in which the heterologous fusion partner is protein D or a derivative or fragment thereof.

10. A fusion protein according to claim 9 in which the protein D derivative comprises about the first 1/3 of protein D, for example amino acids 20 to 127 of protein D.

11. A fusion protein according to claim 9 or 10 in which the protein D derivative is not lipidated.

12. A fusion protein according to any preceding claim further comprising the amino acids Met, Asp and Pro.

13. A fusion protein according to claim 12 in which the amino acids Met, Asp and Pro are fused to the N-terminus of a protein D heterologous fusion partner according to any of claims 9 to 11.

14. A fusion protein according to any preceding claim in which the fusion protein is a recombinant fusion protein.

15. A fusion protein according to any preceding claim in which the fusion protein further comprises an affinity tag.

16. A fusion protein according to claim 15 in which the affinity tag is a histidine tail comprising between 1 to 10 histidine residues.

17. A fusion protein according to any of claims 1 to 5 comprising an amino acid sequence selected from the group consisting of: SEQ ID NO:3, SEQ ID NO:4, SEQ

ID NO:7, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70 SEQ ID NO:73, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:89, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:95, and SEQ ID NO:97.

18. A fusion protein according to any of claims 1 to 5 comprising an amino acid sequence selected from the group consisting of: SEQ ID NO:81, SEQ ID NO:85, SEQ ID NO:93, and SEQ ID NO:97.

19. A nucleic acid molecule encoding the fusion protein of any of claims 1 to 18.

20. A vector comprising the nucleic acid molecule of claim 19.

21. A host cell transformed with a vector of claim 20.

22. An immunogenic composition or vaccine comprising a fusion protein according to any of claims 1 to 18, a nucleic acid molecule as claimed in 19 or a vector as claimed in claim 20.

23. An immunogenic composition as claimed in claim 22, additionally comprising an adjuvant, and/or immunostimulatory cytokine or chemokine.

24. An immunogenic composition or vaccine as claimed in claim 22 or 23 in which the fusion protein is presented in an oil in water or a water in oil emulsion vehicle.

25. An immunogenic composition or vaccine as claimed in claim 23 or 24 comprising one or more of the following adjuvants: 3D-MPL, QS21 or a CpG oligonucleotide.

26. An immunogenic composition or vaccine as claimed in any of claims 22 to 26 additionally comprising one or more other antigens.

27. An immunogenic composition or vaccine as claimed in any of claims 22 to 26 for use in medicine.

28. Use of the fusion protein of claims 1 to 18 or the nucleic acid molecule of claim 19 or the vector of claim 20 or the composition or vaccine of claims 22 to 26 in the preparation of a medicament for treatment of cancer, for example breast melanoma; breast cancer; prostate cancer; bladder cancer including transitional cell carcinoma; lung cancer including non-small cell lung carcinoma (NSCLC); head and neck cancer including oesophageal carcinoma; squamous cell carcinoma; carcinoma of the gastrointestinal tract; liver cancer; brain tumours; leukemia; and various sarcomas.

29. The use of claim 28, for treatment of patients not eligible for Her2/neu targeted therapy.