Published in final edited form as:

Ann N Y Acad Sci. 2009 September ; 1174: 6–17. doi:10.1111/j.1749-6632.2009.04933.x.

The human cancer antigen mesothelin is more efficiently presented to the mouse immune system when targeted to the DEC-205/CD205 receptor on dendritic cells

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Abstract

To develop a tumor vaccine directly targeting tumor antigen to dendritic cells *in situ*, we engineered human mesothelin (MSLN) into an antibody specific for mouse DEC-205, a receptor for antigen presentation. We then characterized both T cell and humoral responses to human MSLN and compared immunizing efficacy of DEC-205-targeted MSLN to nontargeted protein after a single dose immunization. Targeting human MSLN to DEC-205 receptor induced stronger CD4⁺ T cell responses compared to high doses of mesothelin protein. ~0.5% CD4⁺ T cells were primed to produce IFN- γ , TNF- α and IL-2 via intracellular cytokine staining, and the T cells also could proliferate rapidly. The immune response exhibited breadth because the primed CD4⁺ T cells responded to at least three epitopes in the H-2^b background. Targeting MSLN protein to DEC-205 receptor also resulted in cross-presentation to CD8⁺ T cells. Antibody responses against human MSLN were also detected in serum from primed mice by ELISA assays. In summary, targeting of MSLN to DEC-205 improves the induction of CD4⁺ and CD8⁺ T cell immunity accompanied by an antibody response. DEC-205-targeting could be valuable to enhance immunity to MSLN in the setting of cancers where this nonmutated protein is expressed.

Keywords

Dendritic cells; Human mesothelin; DEC-205

Introduction

Mesothelin (MSLN), which is expressed on normal mesothelial cells lining the pleura, peritoneum and pericardium, is overexpressed in several cancers including mesothelioma, ovarian cancer, pancreatic adenocarcinoma, lung adenocarcinoma, uterine serous carcinoma and acute myeloid leukemia.[1-7] The human mesothelin gene encodes a 71-kDa precursor protein that is processed by furin-like proteinases to a 40-kDa membrane-bound protein (MSLN) and a 31 kDa shed fragment called megakaryocyte potentiating factor.[4,8] MSLN knockout mice do not have a detectable phenotype.[9] In humans, it has been suggested that

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Disclosures: Ralph M. Steinman has financial interests in Celldex which is developing anti-DEC-205 antibodies for human use. Li-Zhen He and Tibor Keler (Vice President) are current employees of Celldex.

MSLN allows tumor cell adhesion to mesothelial cells and dissemination of ovarian cancer. [10] A recent study reports that overexpression of MSLN in pancreatic cancer cells induces activation of Stat3, which increases expression of cyclin E and proliferation of the tumor cells.[11] Thus MSLN serves as a tumor differentiation antigen, but its biological functions are under study.

Given its limited expression on normal tissue and overexpression as a nonmutated protein in many human tumor cells, MSLN is being considered as a target antigen for cancer immunotherapy. Jaffee and colleagues immunized pancreatic cancer patients with allogeneic pancreatic tumor cell lines transduced to express granulocyte macrophage colony stimulating factor (GM-CSF). The MSLN in this “G-VAX” vaccine was cross-presented on the HLA class I molecules of the patient, allowing for recognition by potentially protective CD8⁺ cytolytic T lymphocytes (CTL).[12] The identification of additional CTL epitopes from MSLN also supports the potential value of MSLN targeted cancer immunotherapy.[13] These observations (summarized in Table 1) reveal that the human immune system is not tolerant to self MSLN, which can then serve as an antigen to elicit immunity against cancers that overexpress MSLN.

To identify ways to improve the immune response to MSLN, we are carrying out preclinical studies in mice to determine if direct targeting of MSLN to dendritic cells (DCs) *in vivo* improves immunity relative to nontargeted protein. DCs are the most potent antigen presenting cells with the ability to prime the different components of the immune system, both B and T cells. Currently, most DC cancer vaccine trials utilize DCs that are generated *ex vivo*. These approaches allow the investigator to control the quality and antigen loading of the DCs *ex vivo*, but there are limitations to the immune responses that are currently achieved, most likely because the injected DCs do not home in large numbers to the patient's lymphoid tissues, where immunity is initiated. In contrast, the discovery of antigen uptake receptors on DCs, such as DEC-205, has enabled the engineering of monoclonal anti-receptor antibodies to express vaccine proteins, which are then efficiently and specifically targeted to DCs *in situ*, without the need to isolate the DCs. When antigens are incorporated into monoclonal antibodies specific for receptors on DCs, either the DEC-205/CD205 receptor[14-16] or others (reviewed in [17]), large numbers of DCs in lymphoid organs are given an opportunity to capture and process antigens.

Here we pursue DEC-205 as a target for improving the delivery of MSLN to DCs and its subsequent immunogenicity. DEC-205 is a type I transmembrane protein with high levels of expression on DCs localized in the T-cell areas of lymphoid tissues including lymph nodes and spleen from humans.[18,19] Previously, we have shown that a foreign antigen, such as OVA and HIV gag, when targeted to DCs through anti-DEC-205 antibody, can be processed and presented to T cells.[14,16,20] Likewise, human survivin becomes more immunogenic following targeting into mice.[21] DEC-205 targeting increases the efficiency of antigen presentation relative to nontargeted antigen >100 fold.[22] A study in the B16 melanoma model shows that targeting of melanoma antigen to DEC-205 together with a DC maturation stimulus is able to reject small established tumors in a majority of mice.[23]

We hypothesized that human MSLN would be efficiently processed and presented by DCs targeted with anti-DEC-205 mAb fused to human MSLN. When we evaluated this approach in C57BL/6 mice, we found that specific targeting of human MSLN to DCs allowed for more efficient induction of CD4⁺/CD8⁺ T cell responses, relative to nontargeted MSLN protein, as well as humoral responses.

Materials and Methods

Animals

C57BL/6 mice and IFN γ R knockout mice (B6 background) were purchased from Jackson Labs. DEC-205^{-/-} mice were generated and provided by Dr. M. Nussenzweig, and are available from Jackson Labs. Mice were maintained under specific pathogen free conditions and used at 6-8 wk of age according to the guidelines of our Institutional Animal Care and Use Committee.

Fusion human mesothelin mAbs and recombinant proteins

DNA for human mesothelin (MSLN) aa 296-578 was cloned in frame into the COOH terminus of anti-DEC-205 heavy chain as previous described.[24] Fusion mAb was expressed by transient transfection (calcium-phosphate) in 293T cells in serum-free DMEM medium supplemented with Nutridoma SP (Roche). The mAb was purified on protein G columns (GE Healthcare) and characterized by SDS-PAGE and Western blot using anti-mouse IgG-HRP (SouthernBiotech) or anti-MSLN antibody (Covance). FLAG-tagged MSLN was prepared by cloning MSLN (aa 296-578) into the plasmid pFLAG-CMV3 expression vector (Sigma-Aldrich) using *Hind III* and *Not I* restriction enzyme sites. Similarly, supernatant was collected from transient transfected 293T cells and FLAG-MSLN was purified using anti-FLAG (M1) column (Sigma-Aldrich) following product manual. The protein was analyzed by SDS-PAGE gel and Western blot using anti-MSLN antibody (Covance) or anti-FLAG antibody (Sigma-Aldrich).

Antigen targeting and maturation of DCs *in vivo*

Anti-DEC-205-MSLN fusion mAb or FLAG-MSLN fusion protein were injected i.p. with or without DC maturation stimulus, which was 50 μ g poly IC (InVivoGen) together with 25 μ g 1C10 agonistic anti-CD40 mAb (produced by Northeast Biodefense Center).[25]

Human and mouse MSLN peptides

Overlapping (staggered by 4 amino acids) human and mouse MSLN 15-mer peptides were synthesized by H. Zebroski in the Proteomics Resource Center of The Rockefeller University. The use of peptides overcomes in large part the need for antigen processing by antigen presenting cells during the immune assays. The 64- and 71-member human and mouse MSLN peptide libraries were divided into 6 pools as a source of antigens in assays for MSLN-specific immunity. The respective human MSLN peptide pools span from 296-355 aa (pool 1), 346-405 aa (pool 2), 396-455 (pool 3), 446-505 (pool 4), 496-505 (pool 5), 546-622 aa (pool 6) of the human MSLN protein, and the respective mouse MSLN peptide pools span from 293-358 aa (pool 1), 348-413 aa (pool 2), 403-458 aa (pool 3), 448-510 aa (pool 4), 500-557 aa (pool 5), 547-584 aa (pool 6) of the mouse MSLN protein.

Assays for MSLN-specific immune T cells

To identify MSLN-responsive T cells, bulk splenocytes were stimulated for 6 h with pools of MSLN peptides (2 μ g/ml), or as controls, HIV gag p17 peptide library (2 μ g/ml) or medium alone. The cultures included 2 μ g/ml anti-CD28 (clone 37.51), to provide costimulation of the T cells. 5 μ g/ml brefeldin A (Sigma-Aldrich) was added for the last 4 h to accumulate intracellular cytokines. Cells were washed, incubated for 15 min at 4°C with 2.4G2 mAb to block Fc γ R and stained with Live/Dead Fixable Aqua vitality dye (Invitrogen), FITC- or Alexa 700-conjugated anti-CD4 (RM4-5), PerCP-Cy5.5-conjugated anti-CD8 (53-6.7) and Pacific Blue-conjugated anti-CD3 (17A2) (eBioscience) for 20 min at 4°C. Cells were permeabilized (Cytotfix/Cytoperm Plus; BD Biosciences) and stained with APC-conjugated anti-IFN- γ , PE-conjugated anti-IL-2 and PECy7-conjugated anti-TNF- α .

mAbs for 20 min at 4°C (BD Biosciences), resuspended in stabilizing fixative (BD Bioscience) and 10^5 live-CD3⁺ events were acquired using a BD LSR II flow cytometer. Data were analyzed with FlowJo software (Tree Star, Inc.). CFSE dilution assay was used to assess the proliferative capacity of primed T cells.[26] Bulk splenocytes (10^7 cells/ml) were labeled with 2.5 μ M CFSE (Invitrogen) at 37°C for 10 min. CFSE-labeled T cells were restimulated with MSLN peptide pools (2 μ g/ml), individual peptides, or medium alone in the presence of anti-CD28 (2 μ g/ml) for 4 days in 5 ml round-bottom tubes (Falcon). CFSE dilution was analyzed by flow cytometry, often in combination with intracellular cytokine staining of cells restimulated for the last 6 h of culture.

ELISA for anti-MSLN antibodies

To detect MSLN-specific antibody, we coated high-binding ELISA plates (Nunc) with 10 μ g/ml of MSLN protein overnight at 4°C. Plates were washed three times with PBS/0.1% Tween-20 and blocked with PBS/0.1% Tween-20/5% BSA for 1 h at 37°C. Serial dilutions of serum were added to the plates and incubated for another 1 h at 37°C. Secondary goat anti-mouse Fc-specific antibodies conjugated with horseradish peroxidase (Southern Biotech) were added and visualized with tetramethylbenzidine (TMB) (eBioscience) at room temperature for 15-30 min. The values of OD₄₅₀ are presented in this study.

Results

Expression of functional anti-DEC-205-MSLN fusion antibody

To deliver human MSLN to DCs in lymphoid tissues, MSLN was cloned in frame into the heavy chain of anti-mouse DEC-205 mAb as previously described.[24] To improve the secretion of fusion mAb, we removed the GPI anchoring sequences of MSLN. The fusion mAb was successfully expressed and secreted, with the heavy chain of the chimeric mAb detected at 87 kDa by SDS-PAGE (the MSLN insertion is ~ 40 kDa) instead of 50 kDa for mouse Ig heavy chain (Figure 1A). To test whether fusion mAb retained its ability to bind to mouse DEC-205 receptor, we did binding assays using a stable Chinese hamster ovary (CHO) cell transfectant that expressed mouse DEC-205 receptor on the surface. As shown in Figure 1B, anti-DEC-205-MSLN mAb bound to CHO-mDEC-205 cells but not control CHO-Neo cells. We also expressed a soluble FLAG-tagged MSLN construct in 293T cell transfectants and purified the secreted antigen using an anti-FLAG affinity column (Figure 1A). We could then compare the immunogenicity of these two forms of MSLN in mice.

Targeting MSLN to DEC-205 increases MSLN-specific CD4 T cell responses

To overcome the tolerance induced when antigen is presented by immature DEC-205-DCs in the steady state, we vaccinated mice with MSLN along with adjuvants.[15,27,28] We used the combination of poly IC and an agonistic anti-CD40 mAb (1C10) as previously described.[21] To detect responding T cells, we used a library of 15-mer “mimotope” peptides staggered every 4 amino acids along the MSLN sequence. The combination of anti-DEC-205-MSLN and adjuvants, but neither alone, induced the formation of CD4⁺ T cells that recognized MSLN but not a nonspecific antigen HIV gag (Figure 2A). A fraction of the IFN- γ producing cells also produced IL-2 (Figure 2A, right).

To compare the efficiency of protein vaccination, graded doses of anti-DEC-205-MSLN mAb or soluble nontargeted MSLN protein in combination with poly IC and anti-CD40 were injected into wild type C57BL/6 mice. The frequency of IFN- γ , IL-2 and TNF- α secreting MSLN-specific CD4⁺ T cells were measured two weeks after vaccination. Anti-DEC-205-MSLN vaccination was at least 100 times more efficient at eliciting IFN- γ -producing CD4⁺ T cell immunity than soluble nontargeted MSLN protein (Figure 2B, top). The same vaccination experiment was also performed in DEC-205 knockout mice to show

the requirement of DEC-205 for efficient immunization (Figure 2B bottom). Interestingly, human MSLN-specific CD4⁺ T cells were also detected in liver (data not shown). These data show that targeting MSLN to DEC-205 greatly increases the efficiency of induction of CD4⁺ T cell immunity to a protein vaccine.

Anti-DEC205-MSLN fusion mAb induces strong and broad CD4 T cell responses

To assess the breadth of the T cell response, we divided the MSLN peptide library into six pools each containing 10-14 peptides. Two weeks after a single dose vaccination, we used these peptide pools to detect MSLN-specific T cell responses in splenocytes. The CD4⁺ T cells responded to peptide pools 1, 2, 4 and 5 (Figure 3A, B). We then identified CD4⁺ T cell epitopes using individual peptides from the reactive peptide pools. The reactive mimotope peptides are summarized in Table 2. Five mimetopes corresponding to at least three antigenic epitopes were identified in C57BL/6 mice.

To determine the proliferative capacity of the primed CD4⁺ T cells, splenocytes were labeled with CFSE to follow their cell division during 4 days of culture in the presence of MSLN peptides. We detected proliferating CD4⁺ T cells, some of which also produced IFN- γ specifically to rechallenge with MSLN peptide pools 1, 2, 4 and 5 (Figure 3C, D). Therefore, a single vaccination with anti-DEC-205-MSLN in combination with poly IC and anti-CD40 induces strong and broad CD4⁺ T cell responses as measured by cytokine production and proliferation upon antigen rechallenge.

Cross-presentation of MSLN protein by anti-DEC-205 fusion mAb

Previously we have shown that targeting HIV gag p24 protein to human DEC-205 receptor mediated cross-presentation of p24 on MHC class I in various HLA haplotypes.[22] To study the ability of anti-DEC-205 to present MSLN to CD8⁺ T cells, we restimulated the primed splenocytes with MSLN peptides and analyzed IFN- γ production from CD8⁺ T cells. While we did not detect responses by CD8⁺ T cells in a 6 h stimulation assay, the CD8⁺ T cells proliferated significantly after stimulation with MSLN peptides, particularly in pool 2 (aa346-405), and the proliferating CD8⁺ T cells also produced IFN- γ (Figure 4) and TNF- α (data not shown). Together, these results demonstrate that one dose of anti-DEC-205-MSLN fusion mAb lead to cross-presentation of MSLN on MHC class I, eliciting expansion of CD8⁺ T cells capable of producing IFN- γ .

Targeting MSLN to DEC-205 induces serum anti-MSLN antibodies

To study humoral immunity induced by DC-targeted MSLN protein, we tested serum anti-MSLN antibody responses to graded doses of anti-DEC-205-MSLN mAb. The titers of anti-MSLN antibody increased with increasing doses of priming protein (Figure 5). Thus MSLN targeted via DEC-205 can result in enhanced B and T cell immunity.

Discussion

Each element of the immune system has the potential to resist cancer and an effective cancer vaccine may need to induce a coordinated response including CD4, CD8 T cells, and antibodies.[29] Here we found that a single low dose of a protein vaccine combined with two DC maturation stimuli, poly IC together with agonistic anti-CD40 mAb, induced strong human MSLN-specific CD4⁺, CD8⁺ T cell and antibody responses. The efficacy of the MSLN vaccine was greatly increased by targeting the antigen within a mAb to DEC-205, relative to untargeted MSLN protein. Generally, the targeting of antigen to DEC-205 on steady-state immature DCs does not activate immune responses, and instead leads to tolerance.[15,27,28] Therefore, the addition of DC maturation stimuli is important to induce tumor immunity. Similar to previous studies, we found that a combination of agonist anti-

CD40 mAb and TLR3 receptor ligand poly IC with antibody targeted protein is able to induce strong immune response without further boost. However, primary T cell responses were not detected when using poly IC as the only adjuvant. In contrast poly IC in a prime-boost regimen did lead to CD4⁺ T cell responses as previously described,[30] although weaker compared to the combination of poly IC and anti-CD40 (data not shown).

Previously, several CD8⁺ CTL epitopes of human MSLN have been identified in different MHC haplotypes (Table 1).[31-33] Our study shows that human MSLN targeted to DEC-205 receptor was able to be processed and presented on both MHC class I and II products of H-2^b mice. Multiple peptides were recognized by CD4⁺ T cells from vaccinated mice, and at least one peptide by CD8⁺ T cells. We detected these responses by intracellular cytokine staining, an assay that detects more robust immune responses. However, while the responses were Th1 in type and included T cells that could proliferate in response to MSLN peptide, we were unable to break tolerance and observe T cell responses to mouse MSLN. Thus, mice seem to differ from human pancreatic cancer patients in whom responses to self or human MSLN have been documented.[12]

Accumulating evidence reveals an important role of CD4⁺ T helper cells in tumor immunity. CD4⁺ T cells provide helps to CD8⁺ T cells at various phases of immune responses, both priming and maintenance of functional memory.[34] In addition, CD4⁺ T cells can mediate tumor eradication independently of CTL.[35-38] Therefore, successful cancer vaccines will likely require activation of tumor-specific CD4⁺ T cells. Here we show that a strong CD4⁺ T cell response was induced with a single vaccination. Importantly, the response was broad, with recognition of at least 3 epitopes in the H-2^b background. We also saw a relatively high functional avidity of these CD4⁺ T cells, i.e., as low as 30 ng/ml of the 15-mer mimotope peptides were needed to activate primed T cells in immune assays. Consistent with our previous studies, targeting of human MSLN to DEC-205 receptor was much more efficient in inducing CD4⁺ T cell responses compared to nontargeted soluble MSLN protein.[16,21] Previously we reported that targeting of a xenogeneic form of a self protein (human survivin) to DEC-205 receptor in Balb/c mice was able to break tolerance and induced detectable although lower affinity immune response against the murine form of survivin.[21] In this case, human and mouse survivin share 85% homology in amino acid sequence. However, human and murine MSLN share only about 55% homology in amino acid sequence. The anchor residue sequences of our identified CD4 epitopes show some differences between human and murine sequences (Table 2), so that a lack of antigen presentation may explain the lack of the corresponding anti-mouse MSLN peptide response. Another possibility is that strong self-tolerance develops against mouse MSLN, and our current vaccine conditions are not sufficient to break this tolerance.

DEC-205 is highly conserved in mammals. Actually human DEC-205 and murine DEC-205 share ~90% amino acid homology. In preliminary vaccination studies (data not shown) using human DEC-205 Tg mice, human DEC-205 shows similar properties as a receptor for human DEC-205-MSLN targeting. A recent study using cultured human PBMC shows that targeting a viral Ag EBNA1 to human DEC-205 stimulates protective T cell responses, making it a potentially useful target for future human vaccine trials.[39]

In summary, targeting of a tumor antigen, human MSLN, to DEC-205 receptor on DCs *in vivo* is efficient for inducing broad immunity in mice. The contribution of T cell and humoral immunity elicited by the DEC-205-targeting strategy is currently being tested in different mouse tumor models. In addition, the vaccine strategy may need to be combined with other strategies such as immune checkpoint blockade.[40] Since human MSLN is expressed in a non-mutated form in many cancers and is known to be recognized by the human immune system (see Introduction), our study suggests that targeting human MSLN to

DCs via DEC-205 receptor be evaluated for immunogenicity in patients with cancer. Possibly a strong immune response to one protein will ignite spreading of the immune response through the processing of killed tumor cells, or possibly the MSLN antigen will need to be supplemented with additional vaccine proteins to achieve resistance to tumors.

Acknowledgments

This work was supported by National Institute of Health Grant AI 13013.

We thank Henry Zebroski for synthesizing mesothelin and HIV gag p17 peptide libraries, Christine Trumpfheller, Maria Paula Longhi, Juliana Idoyaga and Olga Mizenina for technique support and manuscript reading, and Judy Adams for help with graphics.

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Figure 1. Successful genetic engineering of human mesothelin (MSLN) into the heavy chain of the anti-mDEC-205 mAb. A. 4-15% SDS page gel under reducing conditions: FLAG-MSLN, empty-anti-mDEC-205 (purified from NLDC145 hybridoma) anti-mDEC-205-MSLN. B. Binding assay to CHO-mDEC-205 and control CHO-Neo cells, using graded concentrations (0.01-1 $\mu\text{g/ml}$) of anti-mDEC-205-MSLN. The FACS signal is stained for anti-mouse IgG.

Figure 2.

DEC-205-targeting improves the efficacy of MSLN immunization. A. C57BL/6 mice were vaccinated with 5 μ g anti-DEC-205-MSLN with or without maturation stimulus (50 μ g poly IC and 25 μ g anti-CD40 mAb). Two weeks later, we assessed the percentage of IFN- γ and IL-2 producing CD4 $^{+}$ T cells in CD3 $^{+}$ splenic T cells using a pool of overlapping 15-mer peptides from human MSLN. One of two similar experiments is shown. B. Wild type C57BL/6 mice or DEC-205 KO mice were vaccinated i.p. with graded doses of anti-DEC-205-MSLN (30 μ g, 3 μ g, 0.3 μ g) or purified FLAG-MSLN protein (30 μ g, 3 μ g, 0.3 μ g) together with poly IC and anti-CD40 mAb. Two weeks later, splenocytes were restimulated with the MSLN peptide library, and IFN- γ -secreting CD4 $^{+}$ T cells were assessed by intracellular cytokine staining. One of two similar experiments is shown.

Figure 3.

Broad CD4⁺ T cell responses to a single dose of anti-DEC-205-MSLN fusion mAb vaccine. A. C57BL/6 mice were vaccinated i.p. with anti-DEC-205-MSLN (5 μg) plus poly IC (50 μg) and anti-CD40 (25 μg) in 200 μl PBS. Two weeks later, splenocytes were restimulated with six human MSLN peptide pools (2 μg/ml) in the presence of anti-CD28 mAb (2 μg/ml) and IFN-γ secreting CD4⁺ T cells were assessed by intracellular cytokine staining. One of three similar experiments is shown. B. As in A, but to monitor immunity, splenocytes were labeled with CFSE and restimulated *in vitro* with six MSLN peptide pools (2 μg/ml) and anti-CD28. At day 3.5, T cells were restimulated for 6 h with same MSLN peptide pools (2 μg/ml) and intracellular IFN-γ production in CFSE-low or proliferating T cells was assessed. One of three similar experiments is shown. Averages and SEM for three similar experiments are shown in C and D.

Figure 4. Vaccination with anti-DEC-205-MSLN plus poly IC and anti-CD40 mAb induces CD8⁺ T cells that proliferate upon MSLN encounter. Bulk splenocytes were labeled with CFSE and restimulated *in vitro* with MSLN peptide pools and anti-CD28. At day 3.5, T cells were restimulated for 6 h with MSLN peptide pools and intracellular IFN-γ was measured in CD3⁺ CD8⁺ gated populations. One of three similar experiments is shown.

Figure 5.

Antibody response following a single vaccination. Two weeks after immunization with graded doses of anti-DEC-205-MSLN in the presence of poly IC and anti-CD40, serum anti-MSLN IgG antibody titers were measured by ELISA. One of three similar experiments is shown.

Table 1
Previously defined mesothelin epitopes recognized by CD8⁺ T cells

Previously defined human and mouse MSLN epitopes recognized by CD8⁺ T cells. MPF: megakaryocyte potentiating factor.

	MHC	Position	Sequence	Reference
Human	HLA-A2	540-549	KLLGPHEVGL	[43]
		530-538	VLPLTVAEV	[31] [13] [44]
		20-28 (MPF ^a)	SLLFLLFSL	[31] [13] [44]
	HLA-A24	435-443	FYPGYLCSL	[31]
	HLA-A3	83-91 (MPF)	ELAVALAQK	[31]
		225-233 (MPF)	ALQGGGPPY	[31]
Mouse	H-2D ^b	406-414	GQKMNAQAI	[32]
	H-2K ^b /D ^b	351-358	LSIFKHKL	[45]
		612-619	LIWIPALL	[45]

^a MPF: Megakaryocyte-potentiating factor

Table 2
Comparison of human and mouse mesothelin epitopes

Identification of MSLN mimotope peptides from 15-mer peptide pools that stimulate IFN- γ secretion by CD4⁺ T cells from C57BL/6 mice after anti-DEC-205-MSLN vaccination. C57BL/6 mice were immunized i.p. with anti-DEC-205-MSLN in combination with 50 μ g poly IC and 25 μ g anti-CD40. Two weeks later, bulk splenocytes were restimulated with single peptides from reactive MSLN peptide pools (2 μ g/ml). Percentages of responding CD4⁺ T cells are shown. Potential I-A^b restricted MSLN epitopes were predicted by <http://tools.immuneepitope.org> using a consensus method.[41] Shadowed amino acids are anchor residues (P1, P4, P6 and P9).[42] Mouse MSLN sequences share some of the anchor residues with human.

Position	Sequence Alignment Top: human Bottom: mouse	% of hMSLN responding CD4 ⁺ T cells
296-310	EVEKTACPSGKKARE DAEQKACPPGKEPYK	0.40
341-355	AIPFTYEQLDVLKHK EIPFTYEQLSIFKHK	0.86
346-360	YEQLDVLKHKLDELY YEQLSIFKHKLDKTY	0.79
446-460	PEELSSVPSSIWAV PQDLHSPSSVMWLW	0.85
451-465	SVPPSSIWAVRPQDL SVPSSVMWLWGPQDL	0.69