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α_2-macroglobulin co-administered *in vivo* promotes antigen delivery and presentation

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ABSTRACT

Administered *in vivo*, covalent receptor-recognized α_2-macroglobulin (α_2M^*)-antigen complexes enhance humoral and cell-mediated immunity. We hypothesized that *in vivo* α_2M^*-encapsulation could be promoted in the setting of vaccines that co-deliver α_2M^* with unbound antigen, thereby eliminating the need to prepare complexes *in vitro*. Mice immunized intradermally with co-delivered α_2M^* and OVA demonstrated antigen-specific immune responses, including anti-tumor responses, similar to those elicited by conjugated α_2M^*-OVA complexes. Enhanced immunity appears to result from *in vivo* α_2M^*-encapsulation of antigen. This finding represents a significant advancement in the development of α_2M^* as an antigen delivery vehicle capable of enhancing the presentation of subunit vaccines.

Keywords: α_2-Macroglobulin-Antigen Complexes; Antigen Presentation/Processing; Vaccination; Cytokines; Spleen

1. INTRODUCTION

Previous studies have demonstrated that antigen encapsulation by α_2-macroglobulin (α_2M)[1] enhances antigen-specific immune responses, both *in vitro* and *in vivo* [1-7]. For these studies, α_2M-encapsulated antigen complexes were typically prepared by *in vitro* incubation of amine-activated α_2M, designated α_2M^*, with an 8 to 100-fold-molar excess of antigen in the presence of heat [3,4,7]. These α_2M^*-antigen complexes were then purified by size-exclusion chromatography to remove unbound antigen. While producing α_2M^*-antigen complexes is not extremely difficult on a small scale, it would be more challenging to produce the complexes and perform the necessary quality control on an industrial scale.

While the maximal incorporation of antigen into α_2M^* occurs following 24 h of incubation at 37˚C, some incorporation of antigen occurs at much earlier time periods [8,9]. It is thought that α_2M^*-encapsulation occurs naturally *in vivo* as a mechanism of targeting antigen for uptake, processing, and presentation by professional antigen presenting cells [1]. It, however, is unknown whether *in vivo* α_2M^*-encapsulation could be promoted in the setting of a vaccine that co-delivers α_2M^* with unbound antigen. A high local concentration of antigen may be necessary to drive incorporation into α_2M^*. Because most routes of injection result in rapid dissipation of antigen, conditions that result in a depot effect, for example intradermal injection and alum absorption, should promote α_2M^*-encapsulation *in vivo*. In this study, we investigate the ability of α_2M^*, co-administered with unbound antigen, to enhance antigen-specific immune responses through *in vivo* encapsulation, resulting in enhanced antigen delivery.

2. MATERIALS AND METHODS

2.1. Purification and Activation of Murine and Human α_2M

Purification and amine-activation of murine α_2M was performed as previously described [7,8,10,11]. Human α_2M was purified from fresh, frozen human plasma, obtained from the American Red Cross (Durham, NC, USA), according to a previously published protocol [12].

Native human α_2M was converted to the "amine-activated" form (α_2M^*) by incubation in 200 mM ammonium bicarbonate at room temperature for 1 h. Buffer exchange into PBS was achieved using a 5 mL disposable de-salting column. Native human α_2M was converted to the "trypsin-activated" form (α_2M-T) by incubation with a 5-fold molar excess of trypsin (Worthington Biochemical, Lakewood, NH) for 1 h at room temperature. The Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL) was then added to inhibit proteolytic activity. Excess trypsin and protease inhibitors were removed by consecutive passes over a 100 kDa centrifugal concentrator (Pall Life Sciences, Ann Arbor, MI). Purified protein contained less than 10 pg of endotoxin per mg of protein, as determined by a commercial assay kit (Limulus Amebocyte Lysate Kinetic-QCL by Cambrex, Walk-

ersville, MD).

2.2. Encapsulation of OVA into Murine α_2M^*

Complexes of amine-activated α_2M^* and Alexa Fluor® 647-conjugated ovalbumin (OVA) (Molecular Probes, Eugene, OR) were prepared as previously described [7]. Molar ratio of incorporation was approximately 3:1 OVA: α_2M^*, as determined by fluorescence quantification.

2.3. Time-Dependent Study of OVA Encapsulation into Human α_2M

A 3-fold molar excess of OVA was incubated at 37˚C with either α_2M^* or α_2M-T for 0.5 to 24 h. Samples were then immediately analyzed by native PAGE.

2.4. Cells and Cell Culture

The MO5 cell line, an OVA-transfected subclone of B16 melanoma, was a kind gift from Dr. Kenneth Rock (University of Massachusetts Medical School, Worcester, MA). MO5 cells were cultured in complete media supplemented with 2 mg/mL G418. Murine splenocytes were harvested and cultured as previously described [7].

2.5. Mice

Female C57/BL6 mice were obtained from Charles River Laboratories (Raleigh, NC). All mice were housed in the Duke University Animal Facility, an AAALAC approved facility. All experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol.

2.6. Intradermal Immunization and Tumor Challenge

Prior to tumor challenge, C57/BL6 mice were immunized by intradermal injection into the right ear pinna with 10 µL antigen or PBS, with or without the addition of α_2M^* or CpG 1826, 5'-TCCATGACGTTCCTGACGT-T-3' (Midland Certified Reagent Co., Midland, TX, USA). The treatment groups (n = 5; each receiving 1.35 µg OVA/injection) included the following: OVA alone; OVA administered with 10 µg CpG 1826; α_2M^*-OVA; α_2M^*-OVA administered with 10 µg CpG 1826; α_2M^* co-administered with unbound OVA; and α_2M^* co-administered with unbound OVA with 10 µg CpG 1826. The quantity of α_2M^* administered with unbound OVA was equivalent to the amount of α_2M^* present in the α_2M^*-OVA preparations (6 µg).

Preparations of α_2M^* and OVA were kept in separate containers on ice until immediately prior to injection in order to minimize the possibility of *in vitro* α_2M^*-encapsulation. Mice were subsequently boosted at days 35 and 63, consistent with our previously published immunization protocol [7]. Control groups (n = 5) received intradermal injections of PBS, CpG 1826, or α_2M^* alone. Serum anti-OVA IgG was monitored every 2 weeks by ELISA, as previously described [7]. Mice were injected s.c. in the left flank with 10^4 MO5 tumor cells in Matrigel™ basement membrane matrix (BD Biosciences PharMingen) at week 14. Staining of mouse PBLs with iTAg™ MHC Tetramer H2-K^b SIINFEKL-PE (Beckman Coulter, Fullerton, CA) and Caltag™ FITC-conjugated Rat anti-Mouse CD8a antibody (Invitrogen Corp., Carlsbad, CA) was performed 2 weeks following tumor implantation, as previously described [7], in order to quantify the proportion of CD8+ T cells specific for the H2-K^b-restricted CTL epitope of OVA, the SIINFEKL peptide ($OVA_{257-264}$). Tumor diameters were measured using digital calipers, and tumor volume was calculated using the equation $V = 0.4\ ab^2$, where a and b are the longest and shortest diameters, respectively. Mice were euthanized when tumor volume reached 2 cm^3.

For the detection of fluorescently-labeled OVA encapsulated by α_2M^* *in vivo*, three mice were injected intradermally in the right ear pinnae with 3:1 OVA:α_2M^* (30 µg:10 µg) in PBS. For comparison, one mouse was injected with only OVA (30 µg) and another was injected with only α_2M^* (10 µg). After 1.5 h, mice were euthanized, and ear pinnae were flushed with 3 × 20 µL PBS. The collected fluid samples were analyzed by native PAGE, and incorporation of antigen was measured directly by fluorescence imaging using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.7. [^{3}H]-Thymidine Proliferation Assay

Splenocytes harvested from mice that had been previously immunized with OVA and CpG 1826 (as above) were pulsed for 6 h with 2.5 µM OVA, either free or co-delivered with amine-activated α_2M^* or trypsin-activated α_2M-T (7.5 µM), in serum-free media. As controls, cells were also pulsed with Con A (5 µg/mL) and α_2M^* containing no antigen. Cells were then washed, resuspended in complete media, loaded at 2.5×10^5 cells per well onto a 96-well flat-bottom plate and cultured for 3 d at 37˚C in a humidified 5% CO_2 incubator. Cultured cells were treated with 1 µCi/well [methyl-^{3}H]thymidine (PerkinElmer, Waltham, MA) 18 h prior to harvesting. Incorporation of [^{3}H]thymidine was measured using a Tri-Carb 2100 TR liquid scintillation counter (PerkinElmer, Boston, MA).

2.8. Statistical Analysis

For *in vitro* studies, the Student's *t*-test was performed to determine *P* values and ascertain statistical signifi-

cance between two treatments. For *in vivo* studies (antibody titers, tetramer staining, and tumor growth), ANOVA was performed, followed by multiple comparison procedures (Tukey) to determine differences between groups. Significance between Kaplan-Meier survival curves was determined by log-rank Mantel-Cox analysis. The level of significance used was 0.05.

3. RESULTS

3.1. Antigen Encapsulation by α_2M^* Occurs Rapidly and Can Be Detected Following as Little as 30 min of Incubation

Consistent with a previous report [8], maximal incorporation of antigen by α_2M^* occurs after 24 h of incubation at 37˚C (**Figure 1**). However, some association of antigen with α_2M^* can be observed at 30 min. This association likely represents covalent antigen incorporation by α_2M^* because it is not observed with proteolytically-activated α_2M-T. Although proteolytic thiol ester cleavage in α_2M is irreversible, thiol ester cleavage by primary amines can be reversed with heat, allowing the incorporation of new antigens by α_2M [11]. A small association between OVA and α_2M-T was observed following 24 h of incubation.

3.2. Co-Administration of α_2M^* with Unbound Antigen Enhances Humoral and Cell-Mediated Immunity to a Similar Degree as Conjugated α_2M^*-Antigen Complexes

To determine if co-delivery of antigen with unbound α_2M^* could enhance immune responses *in vivo*, groups of naïve C57/BL6 mice (n = 5) were immunized intradermally with OVA and unbound α_2M^*, with or without the addition of an immunostimulatory adjuvant, CpG 1826. This study was performed concurrently with a previously reported experiment investigating immune responses to prepared α_2M^*-OVA complexes [7]. Separate unbound α_2M^* and OVA preparations were kept on ice and combined immediately prior to injection in order to minimize the possibility of α_2M^*-encapsulation occurring *in vitro*. Following two booster injections (days 35 and 63), mice were challenged with a subcutaneously implanted OVA-expressing B16 melanoma flank tumor.

For both conjugated α_2M^*-OVA and unconjugated α_2M^* + OVA groups, the development of anti-OVA IgG antibody was first observed 8 weeks following initial injection (result not shown). End-point titers (antibody titers at the time of tumor implantation; week 14) are shown in **Figure 2(a)**. OVA administered either with unbound α_2M^* or the well-characterized adjuvant CpG 1826 produced similar antibody titers in immunized mice.

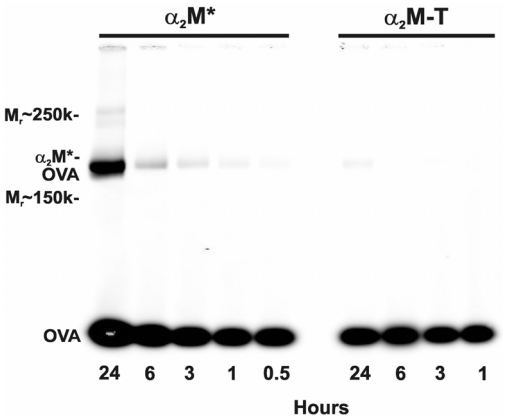

Figure 1. Time-dependence of α_2M^* incorporation of antigen. A 3-fold molar excess of OVA was incubated at 37˚C with either amine-activated α_2M^* or proteolytically-activated α_2M-T for 0.5 to 24 h. Native PAGE demonstrates co-migration of fluorescently-labeled OVA (45 kDa) with α_2M^* dimers (360 kDa) after 30 min of incubation; maximal comigration can be observed after 24 h of incubation. For incubation periods less than 24 h, no association between OVA and α_2M-T is observed. Previous studies have shown no incorporation of OVA into native α_2M [7].

Although the mean antibody titers produced by conjugated α_2M^*-OVA were greater than those elicited with OVA and unbound α_2M^*, the difference between these groups was not found to be statistically significant.

Tetramer staining of PBLs was performed 2 weeks following tumor implantation in order to observe expansion of the antigen-specific CD8+ T cell population in these mice (**Figure 2(b)**). Mice treated with OVA and unbound α_2M^* with the addition of CpG, however, did elicit expansion of the OVA-specific CD8+ T cell population, which was significant compared to the control groups. Although the OVA with unbound α_2M^* without CpG group appeared to elicit some degree of expansion of the OVA-specific CD8+ T cell population, the tetramer staining population for this group was not significantly greater than that of the control groups (PBS, CpG, or α_2M^* alone). Conjugated α_2M^*-OVA treatment groups appeared to elicit greater expansion of antigen-specific CTLs than the OVA with unbound α_2M^* groups, the differences between these treatment groups were not found to be statistically significant. The greatest expansion of OVA-specific CD8+ T Cell population was observed in the α_2M conjugated-OVA with the addition of CPG.

3.3. Co-Administration of α_2M^* with Unbound Antigen Enhances Anti-Tumor Immune Responses

To investigate the anti-tumor response of co-administered α_2M^* vaccinated mice were challenged with OVA

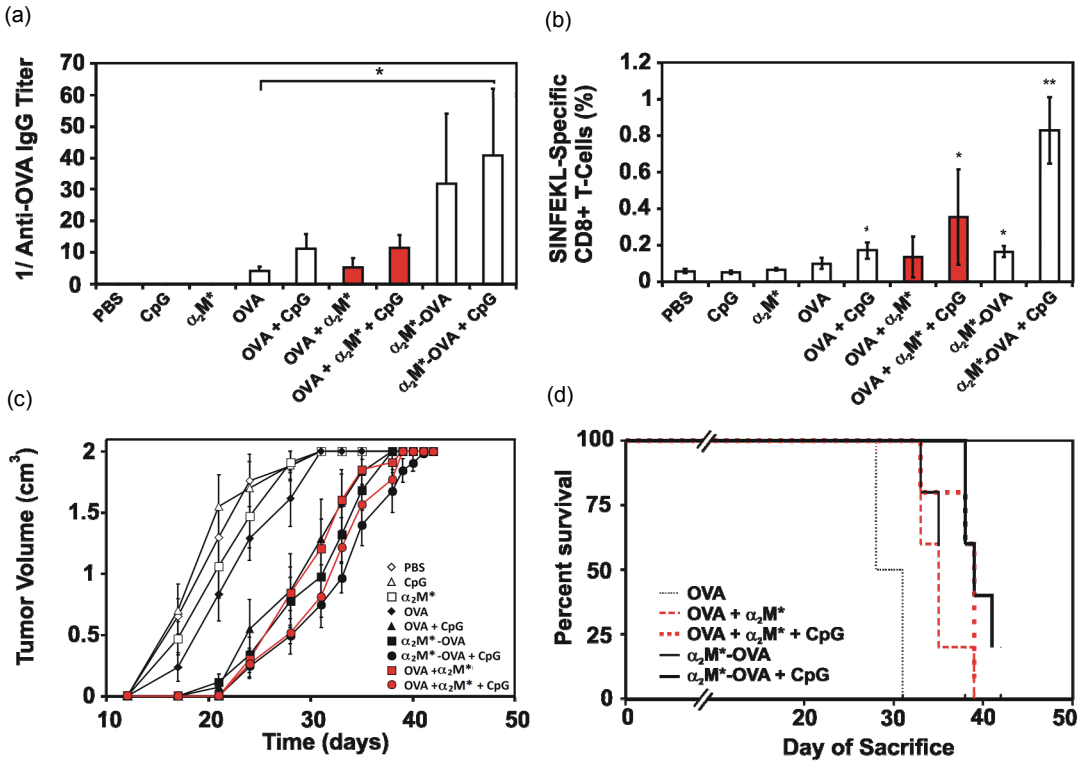

Figure 2. Co-delivery of α_2M^* with antigen enhances tumor protection *in vivo*. End-point titers (a), tetramer staining of PBLs (b), and tumor growth (c) are shown for each mouse treatment group. For clarity, groups treated with α_2M^* co-administered with unbound OVA are shown in red. Values indicate mean ± SEM. *P* values ($^*P < 0.05$; $^{**}P < 0.005$) indicate differences between the respective treatment group and each of the three control groups (PBS, CpG, α_2M^*). The bracket in Panel A indicates a comparison between the indicated groups ($^*P < 0.05$). (d) Kaplan-Meier plot depicts survival of treatment groups.

expressing BIG melanoma flank tumors. Anti-tumor responses elicited by the co-delivery of α_2M^* with unbound OVA were found to be similar to those observed with α_2M^*-OVA. Observable growth of OVA-expressing tumors over time is shown in **Figure 2(c)**. Mice immunized with OVA and unbound α_2M^*, with or without CpG, demonstrated delayed tumor growth compared to each of the control groups, tumor growth for these groups was not significantly different from the α_2M^*-OVA treatment groups. Survival of mice treated with co-delivered α_2M^* and unbound OVA, either with or without CpG, was significantly prolonged ($P < 0.005$) compared to OVA treatment alone (**Figure 2(d)**). However, survival of these mice did not differ significantly from that of mice treated with conjugated α_2M^*-OVA.

3.4. Encapsulation of Antigen by α_2M^* Occurs *in Vivo* in the Setting of Intradermal Injection

We hypothesized that enhanced *in vivo* immune responses with α_2M^* and OVA co-delivery were the result of *in vivo* encapsulation of antigen. The conditions of antigen delivery, including the depot effect caused by intradermal injection and the 37˚C environment of the mouse, are similar to the conditions used to successfully incorporate antigen into α_2M^* *in vitro* [8]. To determine if such *in vivo* encapsulation of antigen could occur in this setting, we similarly intradermally injected the ear pinnae of mice with a 3:1 molar ratio of OVA:α_2M^*. For comparison, mice were injected with either OVA or α_2M^* alone. After 1.5 h, the mice were euthanized, and the ear pinnae were flushed with 3 × 20 µL PBS. The fluid that was recovered was analyzed by native PAGE (**Figure 3**). The detection of fluorescently labeled OVA co-migrating with α_2M^* dimers in these mice confirmed the occurrence of *in vivo* encapsulation of OVA into α_2M^*.

3.5. Enhanced Immune Responses with α_2M^* Co-Administration Result from Encapsulation of Antigen, Rather than Ligation of α_2M^* Receptors

The detection of *in vivo* α_2M^* encapsulation suggests a mechanism for the enhanced *in vivo* immune responses discussed above, it was also possible that ligation of the α_2M^* receptor, low-density lipoprotein receptor-related protein 1 (LRP-1)/CD91, in the absence of antigen en-

capsulation, may also contribute to this enhanced immunity. To investigate this possibility, splenocytes harvested from OVA-immunized mice were treated for 6 h with OVA and either unconjugated amine-activated α_2M^* or proteolytically-activated α_2M-T. After 3 days, cell proliferation was measured by [^{3}H]thymidine incorporation (**Figure 4**). Cell proliferation was increased approximately two-fold following co-delivery of α_2M^* with antigen. However, co-delivery of α_2M-T, which is receptor-recognized but incapable of incorporating new antigen, did not enhance proliferation. Therefore, we concluded that this enhanced response is secondary to α_2M^*-encapsulation and not ligation of the α_2M^* receptor, (LRP-1)/CD91.

4. DISCUSSION

It has been suggested that new generation vaccines will largely consist of purified recombinant proteins [13]. However, formulations of purified protein are frequently poor at eliciting humoral and cell-mediated immunity. Therefore, the development of adjuvants and antigen delivery vehicles that are efficacious, as well as cost-effective and practical, is of extreme importance.

The highly conserved proteinase inhibitor α_2M has received attention in recent years for its ability to entrap diverse macromolecules and target them for rapid receptor-mediated uptake by professional antigen presenting cells. Antigen delivery by α_2M^* elicits 100 to 1000-fold enhanced antibody titers against protein and peptide based vaccines and vaccine candidates, such as hepatitis B surface antigen [3] and the HIV envelope gp120 C4-V3 peptide [4]. Complexes of α_2M^* and trypanosomal proteinases have been shown to activate CD4+ T cells more efficiently than antigen alone [14] and to stimulate the production of antibodies that effectively inhibit activity of the enzyme [15]. Furthermore, our laboratory has recently demonstrated that α_2M^*-encapsulation enhances antigen-specific CTL responses and protection against antigen-presenting tumors [7]. Although these studies have established that α_2M^*-encapsulation can be achieved with relative ease on a small scale, assuming adequate resources and training in biochemical techniques, the large scale production of α_2M^*-antigen complexes may present new challenges. Therefore, achieving enhanced immunologic responses with co-administered α_2M^*, avoiding the steps of *in vitro* incorporation and isolation of complexes, represents a significant advance for this antigen delivery vehicle.

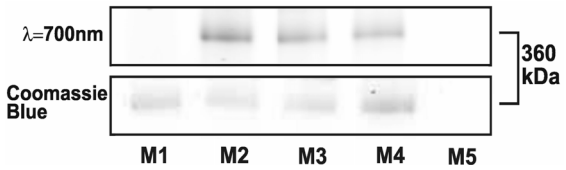

Figure 3. Incorporation of antigen by α_2M^* occurs *in vivo*. Five mice received 10 μL intradermal injections in the left ear pinnae. One mouse (M1) was injected with only α_2M^*, three mice (M2, M3, M4) were injected with a 3:1 molar ratio of OVA:α_2M^*, and one mouse (M5) was injected with only OVA. After 1.5 h, mouse ears were flushed with 3 × 20 μL PBS, and collected fluid was analyzed by native PAGE (top: infrared fluorescence scan at λ = 700 nm, bottom: Coomassie stain).

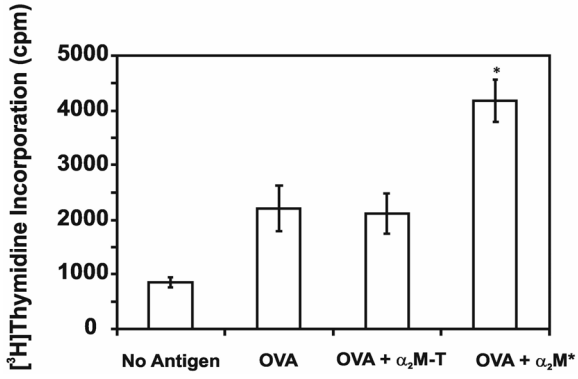

Figure 4. Co-delivery of α_2M^* with antigen enhances cell proliferation *in vitro*. Splenocytes harvested from OVA-immunized mice were treated for 6 h with OVA, either alone or in combination with α_2M^* or α_2M-T. After 3 days of culture, cell proliferation was measured by [^{3}H]-thymidine incorporation. As a control, incorporation by cells treated with no antigen is also shown. Each concentration was assayed in triplicate; values are mean ± SD. Results are representative of three experiments. $^*P < 0.05$ compared to OVA or OVA + α_2M-T.

5. CONCLUSION

Our findings demonstrate that co-delivery of α_2M^* with unbound antigen can enhance humoral and cell-mediated immunity, resulting in improved anti-tumor responses, to similar degree as α_2M^*-antigen complexes prepared *in vitro*. These enhanced immune responses with α_2M^* co-delivery appear to result from *in vivo* encapsulation of antigen, rather than a direct effect of ligating LRP by α_2M^* not carrying bound antigen α_2M^* receptors. The capacity of α_2M^* to promote antigen delivery *in vivo* results from the rapidity with which it encapsulates local macromolecules. Antigens encapsulated by α_2M^* are targeted for rapid receptor-mediated uptake by professional antigen presenting cells, resulting in efficient antigen processing and presentation. We conclude that administration of α_2M^* in the context of a high localized concentration of antigen, such as that which can be achieved with a depot, facilitates antigen delivery and presentation. These findings represent a significant advancement in the use of α_2M^* as an antigen delivery vehicle.

6. ACKNOWLEDGEMENTS

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ABBREVIATIONS

α_2M: α_2-macroglobulin;
α_2M^*: amine-activated α_2M;
α_2M-T: trypsin-activated α_2M;
α_2M^*-OVA: α_2M^*-encapsulated ovalbumin;
CpG 182: 5'-TCCATGACGTTCCTGACG-TT-3';
LRP-1: low-density lipoprotein receptor-related protein 1;
OVA: ovalbumin;
$OVA_{257-264}$: H2-K^b-restricted CTL epitope of OVA (SIINFEKL peptide).

