Receptor-Mediated Antigen Delivery by $\alpha_2$-Macroglobulin:
Effect on Cytotoxic T Lymphocyte Immunity and Implications for Vaccine Development

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pathology in the Graduate School
of Duke University

2009
ABSTRACT

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Abstract

The receptor-recognized form of α2-macroglobulin (α2M*) targets antigens (Ag) to professional Ag-presenting cells (APCs) for rapid internalization, processing, and presentation. When employed as an Ag delivery vehicle, α2M* amplifies major histocompatibility complex (MHC) class II presentation as demonstrated by increased antibody (Ab) titers. Recent evidence, however, suggests that α2M*-encapsulation may also enhance Ag-specific cytotoxic T lymphocyte (CTL) immunity. In these studies, we demonstrate that α2M*-delivered Ag (ovalbumin, OVA) enhances the production of specific in vitro and in vivo CTL responses.

Murine splenocytes expressing a transgenic T cell receptor (TCR) specific for CTL peptide OVA257-264 (SIINFEKL) demonstrated up to 25-fold greater IFN-γ and IL-2 secretion when treated in vitro with α2M*-OVA compared to soluble OVA. The frequency of IFN-γ-producing cells was increased ~15-fold as measured by ELISPOT. Expansion of the OVA-specific CD8+ T cells, as assayed by tetramer binding and [3H]thymidine incorporation, and cell-mediated cytotoxicity, as determined by a flow cytometric assay, were also significantly enhanced by α2M*-OVA. Furthermore, CTL responses were observed at Ag doses tenfold lower than those required with OVA alone.

We also observed enhanced humoral and CTL responses by naïve mice following intradermal immunization with α2M*-OVA. These α2M*-OVA-immunized mice displayed increased protection against a subcutaneously implanted OVA-expressing tumor, as demonstrated by delayed tumor growth and prolonged animal survival. The anti-tumor response observed with α2M*-mediated Ag delivery was comparable to that
of an accepted vaccine adjuvant (CpG 1826) and appeared superior to a cell-based vaccine technique.

To further understand the mechanism underlying this enhanced CTL immunity, subsets of professional APCs capable of cross-presenting α₂M*-encapsulated Ag were investigated. Although both dendritic cells (DCs) and macrophages appear to stimulate some degree of cross-priming in response to α₂M*-encapsulated Ag, CD8⁺CD4⁻ and CD8⁻CD4⁺ DCs appear to do so with the greatest efficiency. The implications of this finding to the ongoing debate regarding the relative contributions of APC subsets to Ag cross-presentation and the determinants of which cells cross-present with high efficiency are discussed.

These observations demonstrate that α₂M*-mediated Ag delivery promotes cross-presentation resulting in enhanced Ag-specific CTL immunity. Considered in the context of previous work, these results support α₂M* as an effective Ag delivery system that may be particularly useful for vaccines based on weakly immunogenic subunits or requiring dose sparing.
Dedication

This dissertation is dedicated to:

My mother, who always cares for me

My father, who is always proud of me

My big sisters, Cynthia and Tricia, for always being my role models

My mentor, Sal, for always being my cheerleader

And my husband, Rob, for being my companion in joy
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List of Abbreviations

$\alpha_2$M, $\alpha_2$-macroglobulin

$\alpha_2$M*, receptor-recognized/activated $\alpha_2$M

$\alpha_2$M*-OVA, $\alpha_2$M*-encapsulated ovalbumin

Ab, antibody

ADCC, Ab-dependent cell-mediated cytotoxicity

Ag, antigen

alum, aluminum salt

ANOVA, analysis of variance

APC, Ag-presenting cell

BSA, bovine serum albumin

C, complement C region, constant region of Ig

CD, cluster of differentiation

CFA, complete Freund’s adjuvant

CFSE, 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester

Con A, concanavalin A

CpG, cytosine guanine dinucleotide

CpG 1826, 5’ – TCCATGACGTTCCGCGGT – 3’

cpm, counts per minute

CSF, colony-stimulating factor

CTL, cytotoxic T lymphocyte

Da, Dalton

DC, dendritic cells

DMEM, Dulbecco’s modified Eagle’s medium
DN, double negative
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay
ELISPOT, enzyme-linked immunospot
E:T ratio, effector to target ratio
Fab, Ag-binding fragment
FBS, fetal bovine serum
FDA, United States Food and Drug Administration
FITC, fluorescein isothiocyanate
g, gram
GM-CSF, granulocyte-macrophage CSF
Grp78, glucose regulatory protein 78
h, hour
HBSS, Hanks’ balanced salt solution
HEL, hen egg lysozyme
HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
HIV, human immunodeficiency virus
HRP, horseradish peroxidase
IFN, interferon
Ig, immunoglobulin
IL, interleukin
i.p., intraperitoneal
ISCOM, immunostimulating complex
i.v., intravenous
kDa, kiloDalton
LCMV, lymphocytic choriomeningitis virus
LRP-1, low-density lipoprotein receptor-related protein (or CD91)
MΦ, macrophage
mAb, monoclonal Ab
2-ME, 2-mercaptoethanol
mg, milligram
MHC, major histocompatibility complex
min, minute
ml, milliliter
µg, microgram
µl, microliter
m.w., molecular weight
MPL, monophosphoryl lipid A
n, number in study or group
NK cell, natural killer cell
ODN, oligodeoxynucleotides
OVA, ovalbumin
OVA\textsubscript{257-264}, H2-K\textsuperscript{b}-restricted CTL epitope of OVA (SIINFEKL peptide)
p, probability
PAGE, polyacrylamide gel electrophoresis
PAMP, pathogen-associated molecular pattern
PBL, peripheral blood leukocyte
PBMC, peripheral blood mononuclear cell
PBS, phosphate-buffered saline
PE, phycoerythrin
pI, isoelectric point
RBC, red blood cell
Rs, Stokes radius
s.c., subcutaneous
SD, standard deviation
SDS, sodium dodecyl sulfate
SE, standard error
TAP, transporter associated with Ag processing
TCR, T cell receptor for Ag
Tc, cytotoxic T cell
Tc1, type 1 CD8+ CTL
Th, T helper cell
TLR, toll-like receptor
TG, thioglycollate
TNF, tumor necrosis factor
VPL, virus-like particle
wk, week
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“If I have seen further it is by standing on the shoulders of giants.”
Isaac Newton, letter to Robert Hooke, 1676

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1. Introduction

1.1 Vaccine Adjuvants and Antigen Delivery Systems

First introduced by Edward Jenner over two hundred years ago, vaccines continue to be among the most cost-effective and efficacious therapies for the treatment and prevention of a wide range of infectious diseases. While a few previously devastating diseases, such as smallpox and polio, have been nearly eradicated by vaccination, scientists have been thus far unsuccessful at developing effective vaccines against many of today’s most deadly and burdensome global infections including human immunodeficiency virus (HIV) (1), tuberculosis (2), and hepatitis C (3). Furthermore, despite the advent of vaccines against hepatocellular carcinoma-associated hepatitis B and cervical cancer-associated human papilloma virus, the long-standing hope to develop efficacious vaccines for the treatment and prevention of human cancers remains largely unrealized (4).

Efforts to improve the safety of administered vaccines have raised particular challenges with regard to eliciting protective immune responses. Early vaccines were primarily based on either live attenuated or killed microorganisms; as a result, all of the signals required to induce an effective immune response were generally present in the different microbial components delivered (5). However, concerns regarding the safety of live attenuated vaccines (e.g. virulence reversion) and our increasing knowledge of the molecular pathobiology of human disease has caused vaccine development to turn increasingly toward the delivery of subunit vaccines, which contain purified antigens (Ag) rather than whole microorganisms.

Although safer and often relatively simple to manufacture, subunit vaccines are extremely poor at eliciting CD8\(^+\) T-cell immunity. As a result, the utility of subunit
vaccines for the prevention and treatment of certain viral infections, cancers, and other diseases for which immunity is mediated by cytotoxic T lymphocytes (CTLs) is extremely limited (6). Therefore, the addition of adjuvants or other agents intended to enhance or otherwise modify the immunogenicity of subunit vaccines is critical. Although aluminum salts (alum) are still the only vaccine adjuvants approved for use in humans by the United States Food and Drug Administration (FDA) (7), researchers continue to work on developing adjuvants targeted at specifically stimulating desired immunological results (i.e. protective humoral or cytotoxic responses). Vaccine adjuvants that have been investigated include emulsions (8-14), microbial derivatives (15-21), polysaccharides (22, 23), CpG motifs (24), cytokines (25-28), and specific Toll-like receptor (TLR) agonists (29, 30). Additionally, Ag delivery systems such as liposomes (31, 32), nano-beads (33-35), immunostimulating complexes (ISCOMs) (8, 13, 36), and virus-like particles (VLPs) (37) have been studied as alternative methods of inducing desired host immune responses (29). Examples of various types of vaccine adjuvants and Ag delivery systems are summarized in Table 1.

It is now known that the failure of subunit vaccines to stimulate CTL immunity can be explained by the fact that these exogenously delivered Ags are not presented on the MHC class I molecules of professional Ag presenting cells (APCs) (6) (see section 1.4). Therefore, vaccine formulations that introduce these Ags in way that promotes their cross-presentation will induce CTL immunity (6). Current methods proposed for enhancing the cross-presentation of subunit vaccines include generating particulate Ag formulations. Such preparations are taken up by professional APCs and presented on MHC class I and II molecules, resulting in stimulation of both CTL and CD4 T-cell responses (33, 38, 39). Furthermore, vaccine compositions of dendritic cells (DCs) that
have been treated \textit{ex vivo} to cross-present Ag have been regarded with considerable interest in recent years (40-42).

Table 1: Types of vaccine adjuvants and Ag delivery systems with examples

<table>
<thead>
<tr>
<th>Minerals</th>
<th>Aluminum salts\textsuperscript{1,2}</th>
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<td>Calcium salts</td>
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</table>

| Emulsions         | Complete Freund’s Adjuvant (CFA)      |
|                   | Incomplete Freund’s Adjuvant (IFA)    |
|                   | MF59\textsuperscript{2}               |
|                   | Syntex adjuvant formulation (SAF)     |

<table>
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<tr>
<th>Particulate delivery vehicles</th>
<th>Biodegradable microspheres</th>
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<tr>
<td></td>
<td>Immunostimulating complexes (ISCOMs)</td>
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<td>Liposomes</td>
</tr>
<tr>
<td></td>
<td>Saponins (QS-21)</td>
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<tr>
<td></td>
<td>Virosomes\textsuperscript{2}</td>
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<tr>
<td></td>
<td>Virus-like particles (VLPs)</td>
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<tr>
<th>Microbial products</th>
<th>Cholera toxin</th>
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<tr>
<td></td>
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<tr>
<td></td>
<td>\textit{Escherichia coli} heat-labile toxin</td>
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<td></td>
<td>Monophosphoryl lipid A</td>
</tr>
<tr>
<td></td>
<td>Muramyl dipeptide</td>
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<td>Pertussis toxin</td>
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<tr>
<th>Cytokines and cells</th>
<th>Dendritic cells</th>
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<tr>
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<td>GM-CSF</td>
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<td></td>
<td>IL-12</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Licensed for human use in the United States.
\textsuperscript{2}Licensed for human use in several European countries.

Table adapted from (43-45).
1.2 $\alpha_2M^*$ and Receptor-Mediated Ag Delivery

Although originally characterized as a protease inhibitor, the serum protein $\alpha_2$-macroglobulin ($\alpha_2\text{M}$) has been recognized in recent years as a potent Ag delivery vehicle. This 718-kilodalton (kDa) tetrameric glycoprotein is an abundant and highly conserved member of the thiolester superfamily, which includes complement components C3 and C4 (46). $\alpha_2\text{M}$ is present in high, typically micromolar, concentrations in the plasma of humans, mice, and other animal species (47). Like the complement components, $\alpha_2\text{M}$ is proteolytically cleaved to achieve its functionally activated form, termed $\alpha_2\text{M}^*$. This proteolytic cleavage of the “bait region” within each $\alpha_2\text{M}$ subunit triggers a major conformational change that permits the entrapment and subsequent inhibition of proteases from all four mechanistic classes (48, 49).

During this activation, the thiolesters within each $\alpha_2\text{M}^*$ subunit are subject to nucleophilic attack, allowing for incorporation of a variety of non-proteolytic peptides, proteins, and growth factors as well as CpG oligodeoxynucleotides (ODN) (47, 50, 51). In addition, activation of $\alpha_2\text{M}$ exposes receptor recognition sites on each of its four subunits which permit the binding of $\alpha_2\text{M}^*$ to the scavenger receptor low-density lipoprotein receptor-related protein (LRP-1), also known as CD91. Cells known to express LRP-1/CD91 include monocytes/macrophages (52, 53), DCs (54, 55), B cells (56), and fibroblasts (57, 58). Professional APCs and other LRP-1/CD91+ cells rapidly internalize $\alpha_2\text{M}^*$ via receptor-mediated endocytosis, resulting in the removal of entrapped proteases from the extracellular space and the introduction to the cell of any other $\alpha_2\text{M}^*$-bound molecules (59-61). A second cell surface receptor for $\alpha_2\text{M}^*$, identified as the glucose regulated protein Grp78, appears to associate with LRP-1/CD91 and facilitate $\alpha_2\text{M}^*$-induced cell signaling (59, 60, 62).
The observation that $\alpha_2$M* can entrap and bind diverse macromolecules during its transient proteinase-activated state before undergoing rapid internalization by macrophages and other cells led to the hypothesis that $\alpha_2$M* may mediate receptor-enhanced Ag uptake by professional APCs. Consistent with this hypothesis, it was found that hen egg lysozyme (HEL) when complexed to $\alpha_2$M* demonstrates enhanced macrophage uptake, processing, and presentation to T cells \textit{in vitro} (63). Furthermore, rabbits injected with $\alpha_2$M*-HEL demonstrated up to 500-fold greater IgG titers compared to those treated with HEL alone, a response comparable to that observed with HEL in complete Freund’s adjuvant (CFA) (61).

Based on these findings, as well as the known properties of $\alpha_2$M*, a unique role for $\alpha_2$M* in the processing and presentation of exogenous Ags was proposed (Figure 1) (61). Areas of inflammation feature not only enriched levels of proteases but also high concentrations of native $\alpha_2$M, due to increased plasma extravasation and local synthesis by macrophages and fibroblasts (64-66). Activated by proteases in the presence of antigenic proteins, $\alpha_2$M* would carry a proteinaceous sampling of the extracellular environment. Following the receptor-mediated endocytosis of $\alpha_2$M* by macrophages or other professional APCs, bound proteins would be processed and presented, presumably on MHC class II, for surveillance by CD4$^+$ T cells (61).

As interest in utilizing $\alpha_2$M* as an Ag delivery vehicle grew, our laboratory developed a novel nonproteolytic method of encapsulating Ags within $\alpha_2$M* (67). When incubated with ammonia, $\alpha_2$M is cleaved to form $\alpha_2$M* without proteolysis. Heating this $\alpha_2$M*-NH$_3$ (at 50°C for 5 h or 37°C for 24 h) results in nucleophile loss and thiolester regeneration, restoring $\alpha_2$M’s ability to inhibit proteases and allowing it to bind new available proteins (Figure 2). This method was first used to successfully incorporate HEL
and bovine insulin by heating $\alpha_2M^\ast$-NH$_3$ in the presence of these proteins (67). This approach avoids the possibility of Ag degradation by proteinases and removes the competition between proteinase and Ag for available thiolesters, allowing for higher efficiency of Ag incorporation. Diverse proteins have been successfully incorporated into $\alpha_2M^\ast$ using these methods (Table 2). Furthermore, $\alpha_2M^\ast$-Ag complexes created in this manner have been shown to bind macrophages with an affinity identical to that of proteolytically-activated $\alpha_2M^\ast$ (68). As a result, this nonproteolytic suggested even more potential for targeted Ag delivery via $\alpha_2M^\ast$. 
Figure 1: α₂M*-delivered Ag processing by professional APCs.

1, Diffusion of proteins in and out of the native α₂M “trap,” (א). 2, Proteolytic cleavage of the α₂M “bait” region, results in capture of the proteinase, (ב), and potential Ag, (ג). 3, Binding of receptor-recognized α₂M, (ד), to the LRP-1/CD91 scavenger receptor, (ה), on a macrophage or other professional APC results in rapid internalization and intracellular signaling. The receptor is recycled to the cell surface after releasing its ligand. 4, Partial degradation of the antigenic proteins in endosomal compartments, bold circle. 5, Intersection with MHC class II molecules, (ו), and presentation of the MHC-associated Ag fragment at the cell surface. 6, Specific stimulation of T-helper (Th) cells in conjunction with costimulatory signals (i.e., CD28/B7), leading eventually to (ז) antibody (Ab) synthesis by plasma B cells. (Figure adapted from (61)).
Native $\alpha_2$M is converted to its receptor-recognized conformation, $\alpha_2$M*, by reaction with either a proteinase or small nucleophiles, such as NH$_3$ or primary amines. When heated in the presence of excess ligand, $\alpha_2$M* produced with NH$_3$ undergoes transient conversion to $\alpha_2$M, the ligand is covalently incorporated into the $\alpha_2$M cage-like structures, and $\alpha_2$M reverts to the receptor-recognized $\alpha_2$M*. This can be viewed chemically as a mechanism of nucleophilic exchange.
Table 2: Examples of the diverse proteins that have been incorporated into α₂M* via nonproteolytic methods

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rs (Å)</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>Incorporation¹ (Mol/Mol α₂M*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin</td>
<td>35.5</td>
<td>66.4</td>
<td>5.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>20.3</td>
<td>28.7</td>
<td>6.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Transferrin</td>
<td>28.0</td>
<td>75.2</td>
<td>6.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>22.4</td>
<td>25.7</td>
<td>8.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Hen Egg Lysozyme</td>
<td>16.1</td>
<td>14.3</td>
<td>9.3</td>
<td>3.9</td>
</tr>
<tr>
<td>RNase (Bovine)</td>
<td>19.2</td>
<td>13.7</td>
<td>8.6</td>
<td>5.1</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>--</td>
<td>14.2</td>
<td>4.8</td>
<td>8.0</td>
</tr>
<tr>
<td>β-lactalbumin</td>
<td>--</td>
<td>18.3</td>
<td>4.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>--</td>
<td>41.6</td>
<td>7.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Hepatitis B Surface Ag</td>
<td>--</td>
<td>25.4</td>
<td>7.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Epidermal Growth Factor</td>
<td>--</td>
<td>6.2</td>
<td>4.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Platelet Derived Growth Factor</td>
<td>--</td>
<td>14.3</td>
<td>9.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>--</td>
<td>47.1</td>
<td>6.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table adapted from (69).
¹Incorporation values refer to human α₂M*; similar results found with rabbit and mouse α₂M*.
1.3 $\alpha_2M^*$ and the Adaptive Immune Response

Several studies have demonstrated that Ag delivery via $\alpha_2M^*$ can enhance specific humoral immune responses. In addition to the findings with $\alpha_2M^*$-HEL discussed above, $\alpha_2M^*$-encapsulation has been shown to produce 100 to 1000-fold enhancement of antibody (Ab) titers to established, but poorly immunogenic, vaccine Ags, such as hepatitis B surface Ag (HBsAg) (70), and to peptide-based vaccine candidates, such as the HIV envelope gp120 C4-V3 peptide (71). The observation that $\alpha_2M^*$-encapsulation causes increased humoral immune responses to Ag is expected based on its rapid and efficient process of delivering Ag to endosomal compartments, where they can be processed and presented on MHC Class II (61). However, until recently, little was known about the impact of $\alpha_2M^*$-encapsulation on CTL responses.

Preliminary studies in our laboratory have indicated that $\alpha_2M^*$-delivery of Ags may in fact influence CTL responses. A study conducted in collaboration with Dr. Herman Staats found that mice injected with the $\alpha_2M^*$-encapsulated HIV C4-V3 peptide, $\alpha_2M^*$-C4-V3, demonstrate a 10 to 20-fold enhancement of CTL activity (Liao et al., unpublished data). Additionally, studies conducted in collaboration with Dr. Hilliard Seigler, Duke Department of Surgery, found that peripheral blood mononuclear cells (PBMCs) collected from melanoma patients and exposed to $\alpha_2M^*$-encapsulated tyrosinase peptide in vitro exhibited enhanced cell-mediated immune responses as demonstrated by increased interferon-γ (IFN-γ) secretion (unpublished data). These findings suggest that, in addition to stimulating enhanced humoral immunity, $\alpha_2M^*$ may also play a role in enhancing CTL responses. Given its mechanism of receptor-mediated uptake into endosomes, this discovery would suggest that exogenously delivered $\alpha_2M^*$-bound Ags undergo cross-presentation onto MHC class I.
Studies from another research group have emerged which concur with the hypothesis that $\alpha_2$M*-delivered Ags can be cross-presented and stimulate enhanced CTL responses. Binder et al. reported that a peptide sequence bound to $\alpha_2$M* primes CTL responses in immunized mice and that such immunization decreases tumor growth and increases survival in mice injected with tumor cells displaying this peptide sequence (72, 73). While these findings provide significant evidence for the ability of $\alpha_2$M* to enhance CTL responses, the commercially-produced $\alpha_2$M used in these studies was most likely pre-activated and unable to efficiently encapsulate Ags, resulting in the low level of peptide binding reported by the authors. The peptide used in these studies is suspected to have bound non-specifically to the exterior of $\alpha_2$M*, rather than be successfully incorporated within the $\alpha_2$M* cage-like structure. As a result, these studies may not adequately represent the biological potential of utilizing $\alpha_2$M* as an Ag delivery vehicle (74-76). Therefore, while these reports further support the role of $\alpha_2$M* in enhancing CTL responses, there is still a need for further study to explore and elucidate this process.
1.4 Cross-Presentation: Mechanism and Significance

For years, it was generally believed that MHC class I molecules presented peptides that were derived exclusively from endogenous proteins, synthesized within cells and degraded in the cytosol. This MHC class I presentation by professional APCs can then stimulate resting T cells to clonally expand and express effector functions, including cytolytic activity and cytokine secretion. However, this “direct presentation” failed to explain how CTL responses are elicited against cancer cells or viruses that do not infect professional APCs or how tolerance is acquired to self-Ag not expressed by professional APCs. In 1976, this apparent discrepancy was resolved when M.J. Bevan demonstrated that CTL responses could be elicited against exogenous Ags (77, 78). Bevan found that animals immunized with fully allogenic cells generated CTL responses specific for minor Ags from the graft presented on MHC class I molecules of the host. This process of exogenous Ag presentation on MHC class I was termed cross-presentation and the subsequent activation of CD8+ cytotoxic T cells termed cross-priming.

Although cross-presentation was originally thought to be an obscure phenomenon limited to transplant immunity, it is now recognized as a major mechanism of immune surveillance. Without cross-presentation, the immune system would be unable to mount CTL responses against viral infections or mutations that occur exclusively in parenchymal cells rather than in bone marrow-derived professional APCs (6). Increasingly, cross-presentation is also being recognized as important in eliciting CTL responses against viruses that can infect both parenchymal and professional APC cells. Since some viruses impair the functional capability of infected professional APCs, cross-presenting rather than virally infected cells seem to be responsible for stimulating
immunity (79, 80). Cross-presentation has also come to be appreciated for its contribution to the development of tolerance to self-Ag, a process sometimes termed cross-tolerance (81). DCs, as well as macrophages to some degree, appear to be the principal cells capable of cross-presenting Ags in vivo (see section 1.5).

Phagocytosis of particulate Ag and pinocytosis of soluble Ag are typically considered the chief mechanisms by which professional APCs acquire proteins for cross-presentation. Phagocytosis has been shown to be crucial for the presentation of particulate or bead-bound ovalbumin (OVA) by macrophages (82, 83) and DCs (84), respectively. Recently, however, several specific cell-surface receptors have been identified that appear to favor cross-presentation (81). Fc receptors, for example, permit the cross-presentation of immune complexes on both CD8+ and CD8- DCs (85). The mannose receptor, furthermore, appears to be required for the cross-presentation of soluble glycoproteins (86). Ags targeting the C-type lectin CD205, an endocytic receptor abundant on DCs and in lymphoid tissues, efficiently elicit CD8+ T cell-mediated immunity (87). Dectin-1, another member of the C-type lectin-like receptor family, appears to be involved in the in vitro uptake and cross-presentation of cellular Ags by human monocyte-derived DCs (88). Also, Ags complexed to heat shock proteins are taken up by specific receptors on professional APCs, a process which appears to enhance cross-presentation (89).

Multiple pathways seem to contribute to the processing and cross-presentation of exogenous Ags. The phagosome-to-cytosol pathway (Figure 3), in which Ags are hydrolyzed by proteasomes in the cytosol and transported by the transporter associated with Ag processing (TAP) to MHC class I molecules in the endoplasmic reticulum (ER), appears to be the principal pathway for most Ags in vivo (6). Certain Ags, including
proteins associated with E. coli and some viruses, are cross-presented at least in part by an alternate mechanism termed the vacuolar pathway (Figure 4). In this pathway, Ag is degraded by endosomal proteases, particularly cathepsin S; the resulting peptides are bound by MHC class I molecules probably in the endocytic compartment itself (6). Yet another possible alternate pathway has been proposed for the cross-presentation of soluble proteins. This pathway was based on the observation that some exogenous soluble proteins can be internalized in DCs and transported into the ER (90) where they are processed by the ER-associated degradation pathway (ERAD) (91). Of note, although cross-presented Ag can be acquired in several different forms including RNA or DNA, peptides, or peptide-heat shock protein (HSP) complexes, recent evidence indicates that cellular proteins are the major source of cross-presented Ags in vivo (92-94).
Figure 3: Phagosome-to-cytosol pathway of cross-presentation

Exogenous Ag is internalized into phagosomes or micropinosomes and then transferred into the cytosol. Ag is then hydrolyzed by proteosomes in the cytosol; the resultant oligopeptides are transported by TAP and loaded onto MHC class I molecules in the endoplasmic reticulum (ER). The mechanism by which Ag is translocated from phagosomes to the cytosol is not understood. ER-resident proteins (e.g. TAP, MHC I, Sec61) have been found in a subset of phagosomes, often presumed to be the result of ER-phagosome fusion. These “ergosomes” are thought to be fully equipped to export Ag to the cytosol for cross-presentation. Figure adapted from (6, 81, 95).
Figure 4: Vacuolar pathway of cross-presentation

In this pathway, internalized Ags are degraded in phagosomes by cathepsin S and other endosomal proteases. Resulting peptides may be loaded onto MHC class I molecules that have trafficked into the vesicle from the plasma membrane or from the ER. Figure adapted from (6, 81).
1.5 Professional APCs Involved in Cross-Presentation

The precise identity of the principal cross-presenting cells has been the subject of much study and debate. Even before any clear mechanisms had been proposed to describe cross-presentation, it was believed that this process was likely unique to a small subset of cells. Although all nucleated cells express MHC class I, it seemed unlikely that most cell types would cross-present exogenous Ag as this might result in the inappropriate targeting of healthy cells for destruction by the immune system (6). In the 1990s, K.L. Rock’s research group tested the hypothesis that cross-presentation was performed by a unique subset of APCs. They found that cross-presenting cells were indeed localized within lymphoid organs and when explanted ex vivo could generate MHC class I-presented peptides from Ags in the extracellular medium (96-99). Cells that demonstrated this ability to cross-present were further found to be co-purified with DCs and macrophages (98, 99).

Subsequent studies have demonstrated that DCs (100, 101), macrophages (82, 102), B cells (103) and even neutrophils (104) are capable of presenting MHC class I-restricted exogenous Ags in vitro (105). In vivo, however, DCs and macrophages are generally considered to be the only significant cross-presenting cells. Despite evidence that B cells can cross-present in vitro, the observation that cross-presentation is not impaired in B cell-deficient mice suggests that they play only a minor or redundant role in vivo (6).

Several groups have identified the primary cross-presenting cell, at least in viral models, as a bone marrow-derived (106, 107) or a CD11c+ DC (108, 109). Consistent with the earlier findings by Rock et al. described above (96-99), Jung et al. (108) demonstrated the critical role of DCs in cross-presenting Ags in vivo. In this study, they generated transgenic mice expressing the diphtheria toxin receptor under the control of the CD11c
promoter. When CD11c<sup>+</sup> DCs were ablated by treatment with diphtheria toxin, these transgenic mice failed to generate a CD8<sup>+</sup> T-cell response to cell-associated Ag and intracellular pathogens. It was, therefore, concluded that cross-presentation by DCs <em>in vivo</em> is critical for eliciting CD8<sup>+</sup> T-cell responses to certain Ags. Of note, however, no specific subsets of DCs were examined in this model. While the evidence for DC involvement was compelling, the co-involvement of macrophages could not be disproven given that marginal zone and sinusoidal macrophages from the spleen and lymph nodes of these mice were also depleted, as demonstrated in a subsequent study (110).

Some <em>in vivo</em> data also exists to support the role of macrophages in cross-presentation. Direct <em>in vivo</em> studies using intravital microscopy to visualize APC-T cell interactions in lymph nodes are often cited as evidence for this role. In this case, mice were infected with green fluorescent protein (GFP)-expressing vaccinia virus. The responding CD8<sup>+</sup> T cells were found to cluster around GFP-negative cross-presenting APCs that were both CD11c<sup>+</sup> (DCs) and CD11c<sup>-</sup> (presumably macrophages) (111).

Most evidence, however, suggests that DCs are most effective at eliciting protective cytotoxic immunity. As professional APCs, DCs are reported to be 100-1000 times more potent than macrophages <em>in vitro</em>. Although both peptide-pulsed macrophages and DCs have been found to stimulate CD8<sup>+</sup> T cells when injected <em>in vivo</em>, it takes 10-fold more Ag to prime naïve T cells with macrophages compared to DCs (112). Notably, however, macrophage numbers increase rapidly at sites of infection and inflammation; this scenario in which macrophages are much more abundant than DCs suggests that they may indeed play a significant role in T-cell priming (112).
Although the Ag presentation machinery of all professional APCs is not considered significantly different, each cell type appears to have acquired adaptations that fit with its specific role (81). B cells, for example, in accordance with their role of recruiting T cell help for humoral responses, present Ag proportional to the quantity of Ag detected; thus, the duration of the humoral response is generally proportional to the pathogen burden (113). Macrophages, acting as a first line of defense against invading pathogens, express high levels of many lysosomal proteases (114) and maintain an acidic endosomal environment (115) which support a high capacity for rapid degradation of internalized material. DCs, however, have developed specializations that favor effective Ag presentation, such as efficient Ag uptake, regulation of Ag degradation, and enhanced transport of Ag from endosomes to the cytosol (105, 116).

DCs represent a heterogenous cell type that can be broadly categorized as conventional and plasmacytoid DCs. Conventional DCs can be further subdivided into ‘lymphoid’ and ‘myeloid’ DCs (105, 117, 118). The relative contribution of these subsets to cross-priming CD8+ T cells is not yet fully understood. The CD8+ DC, however, has been identified in several studies as the primary APC for stimulating naïve CD8+ T cell responses in mice that were either virally infected or immunized with cell-associated Ag (85, 119, 120). The form of Ag, however, seems to influence the cell subset involved in cross-priming. Cell-associated OVA, for instance, is cross-presented by CD8+ DCs while immune complexes of OVA are cross-presented by both CD8+ and CD8- DCs (85).

If α2M*-encapsulated Ags can indeed be cross-presented, discovering which cell types are involved in this cross-presentation would serve to further elucidate the processing of this unique Ag delivery vehicle. Although early studies of α2M*-Ag delivery emphasized receptor-mediated uptake by macrophages, more recent work
suggests that DCs are critical to eliciting immune responses to $\alpha_2\text{M}^*$-encapsulated Ags. Studies in human PBMCs by J.P. Hart demonstrated that, although both CD14$^+$ monocytes and lin$^-$ CD11c$^+$ DCs are capable of rapidly internalizing $\alpha_2\text{M}^*$, only Ag presentation by DCs resulted in significant T cell stimulation (55). The finding that CD11c$^+$ DCs play a significant role in stimulating adaptive immune responses to $\alpha_2\text{M}^*$-delivered Ag is not surprising given their well-accepted efficiency as professional APCs. Notably, however, these studies investigated blood monocytes, generally considered to be poor APCs, rather than fully differentiated tissue macrophages. Given the studies that establish DCs as critical to cross-priming, the bone marrow-derived DC seems a likely suspect in the cross-presentation of $\alpha_2\text{M}^*$-Ag complexes. However, the relative contribution of tissue macrophages in cross-presenting $\alpha_2\text{M}^*$-encapsulated Ags also merits investigation.
1.6 Research Objectives

The goal of this work is to investigate the ability of $\alpha_2 M^*$-mediated Ag delivery to promote cross-presentation and enhance Ag-specific CTL immunity. The approaches employed to establish the effect of $\alpha_2 M^*$-encapsulation on CTL responses and to identify subsets of professional APCs capable of cross-presenting $\alpha_2 M^*$-encapsulated Ag are described within the following experimental objectives:

**Objective 1.** Examine *in vitro* CTL responses to Ag delivered via $\alpha_2 M^*$. Demonstrate increased production of type 1 CD8$^+$ CTL (Tc1) cytokines with $\alpha_2 M^*$-encapsulation of Ag. Demonstrate enhanced expansion of Ag-specific CD8$^+$ T cells with $\alpha_2 M^*$-delivered Ag. Demonstrate enhanced cell-mediated cytotoxicity with $\alpha_2 M^*$-encapsulated Ag.

**Objective 2.** Examine *in vivo* CTL responses in naïve mice immunized with $\alpha_2 M^*$-encapsulated Ag. Characterize stimulation and expansion of Ag-specific CD8$^+$ T cells in mice that receive $\alpha_2 M^*$-encapsulated Ag versus soluble Ag. Demonstrate protective immunity of mice immunized with $\alpha_2 M^*$-encapsulated Ag to Ag-presenting tumors.

**Objective 3.** Identify cells capable of cross-presenting $\alpha_2 M^*$-encapsulated Ag. Characterize the ability of DC subsets and macrophages to cross-present $\alpha_2 M^*$-encapsulated Ags and stimulate cross-priming (i.e. activate CD8$^+$ cytotoxic T cells).
2. Experimental Procedures

2.1 Methods for analysis of in vitro CTL responses to $\alpha_2M^*$-encapsulated Ag

2.1.1 Mice

Female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC). Female MHC class I-restricted OVA-specific TCR transgenic mice (C57BL/6-Tg(TcraTcrtb)1100Mjb/J, commonly called OT-1 mice) were purchased from Jackson Laboratories (Bar Harbor, ME). 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.

2.1.2 Purification and activation of murine $\alpha_2M$

Purification of $\alpha_2M$ was performed using endotoxin-free plasma, columns, and buffers and followed a protocol modified from that previously described (67, 68, 121). Mouse plasma was purchased from Harlan (Indianapolis, IN). $\alpha_2$-Macroglobulin was separated from mouse plasma by consecutive precipitation at 4% and 16% polyethylene glycol (Fluka, Switzerland) followed by DEAE Sephadex Fractionation (Sigma-Alrich, St. Louis, MO) and further purified over a Sephacryl S-300 sizing column (Amersham Biosciences, Uppsala, Sweden). Native $\alpha_2$M was converted to the “activated” form ($\alpha_2M^*$) by incubation in 200mM ammonium bicarbonate at 37°C overnight. Activated protein was then dialyzed for 4h into PBS and stored at 4°C. Purified protein contained less than 10pg of endotoxin per mg of protein, as determined by a commercial assay kit (Limulus Amebocyte Lysate Kinetic-QCL by Cambrex, Walkersville, MD).
2.1.3 Encapsulation of OVA into $\alpha_2\text{M}^*$

Amine-activated $\alpha_2\text{M}^*$ was incubated with a 40-fold molar excess of Alexa Fluor® 647-conjugated OVA (Invitrogen - Molecular Probes, Eugene, OR) for 18h at 37°C. The resultant $\alpha_2\text{M}^*$-encapsulated OVA was purified by Sephacryl S-300 size exclusion chromatography (Amersham Biosciences, Uppsala, Sweden) to remove unbound OVA. Purified $\alpha_2\text{M}^*$-OVA contained less than 23pg of endotoxin per mg of protein, as determined by the above mentioned commercial assay kit. Successful incorporation and purification was confirmed by native and SDS PAGE of $\alpha_2\text{M}^*$-OVA complex. Molar ratio of incorporation was determined by fluorescence quantification.

2.1.4 IFN-γ and IL-2 ELISAs

Spleens were harvested from OT-1 mice and single cell suspensions were prepared by repeated sub-capsular injection of complete culture media (high glucose phenol-red free DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 10mM HEPES, 0.1mM non-essential amino acids, and 50µM 2-ME). Following hypotonic lysis of RBCs, splenocytes were loaded into 96-well plates at 2.5x10^5 cells per well. In our initial studies examining the dose-dependence of Tc1 cytokine secretion, various concentrations of OVA or $\alpha_2\text{M}^*$-OVA were added to each well, and cells were incubated for 24 or 48h at 37°C in a humidified 5% CO₂ incubator. Positive controls included Con A (Sigma–Aldrich, St. Louis, MO) at 5µg/ml and SIINFEKL peptide (AnaSpec, San Jose, CA) at 0.5µM. Negative controls included PBS, amine-activated $\alpha_2\text{M}^*$ containing no Ag, and Alexa Fluor® 647-conjugated bovine serum albumin or BSA647 (Molecular Probes, Eugene, OR). Cell supernatants were then harvested and stored at −20°C until IFN-γ and IL-2 could be quantified using DUO-ELISA kits (R&D Systems, Minneapolis, MN). Assays comparing the cytokine response
to fluorescently labeled OVA versus unlabeled OVA found the biologic effect to be comparable (data not shown). Therefore, throughout this document, OVA and α₂M*-OVA refer to the fluorescent species.

In subsequent studies of Ag uptake, splenocytes were prepared as above, but cells were treated with 0.25µM OVA or α₂M*-OVA for 30min to 8h, washed, and then incubated for an additional 48h. Supernatants were then harvested and IFN-γ secretion was measured by ELISA as above.

### 2.1.5 IFN-γ ELISPOT

The number of cells stimulated to produce IFN-γ was examined using the BD Mouse IFN-γ ELISPOT Kit (BD Biosciences Pharmingen, San Diego, CA). Briefly, OT-1 splenocytes were added at 5x10⁵ cells per well to the 96-well ELISPOT plate, which was pre-coated with an anti-IFN-γ capture Ab. The splenocytes were incubated with Ag (0.0125-0.05µM α₂M*-OVA or free OVA) for 24 or 48h at 37°C in a humidified 5% CO₂ incubator. Positive controls included Con A at 5µg/ml and SIINFEKL peptide at 0.5µM. Negative controls included PBS, 0.02µM amine-activated α₂M* containing no Ag, and 0.05µM BSA647. After several washes to remove cells, the plate was incubated with a biotinylated anti-IFN-γ detection Ab for 2h at room temperature and then washed again. The ELISPOT plate was then incubated for 1h with Streptavidin-HRP, washed, and color development was achieved using the 3-amino-9-ethylcarbazole substrate reagent kit. Following the development of spots, the reaction was stopped with water, the plate was air-dried, and spots were counted using a CTL-ImmunoSpot® plate reader (CTL Analyzers, Cleveland, OH).
2.1.6 Rate of Ag uptake

Thioglycollate (TG)-elicited murine peritoneal macrophages were harvested from C57BL/6 mice by peritoneal lavage and plated at 2.5x10^5 cells per well in complete media in a 96-well plate. Cells were incubated at 37°C for 2h and washed to remove non-adherent cells. Cells were treated with 0.05µM OVA or α2M*-OVA and incubated at 37°C for 5min to 2h. Cells were then washed four times with ice-cold PBS to remove unbound Ag. Uptake of Ag was measured directly by fluorescence imaging using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska).

2.1.7 Tetramer binding

Single cell suspensions of C57BL/6 and OT-1 splenocytes were prepared as above and combined at a 20:1 (C57BL/6:OT-1) ratio. These diluted OT-1 cell suspensions were treated with OVA or α2M*-OVA (0.001 to 0.05µM) for 4h, washed, and then incubated at 37°C for a total of 4 days. Cells were stained with iTAg™ MHC Tetramer H2Kb SIINFEKL-PE (Beckman Coulter, Fullerton, CA) and Caltag™ FITC-conjugated Rat anti-Mouse CD8a Ab (Invitrogen Corp., Carlsbad, CA) at room temperature for 30min and then fixed with 0.5% paraformaldehyde and stored light-protected at 4°C until flow cytometric analysis (Guava EasyCyte Plus system, Guava Technologies, Hayward, CA).

2.1.8 [³H]Thymidine proliferation assay

OT-1 splenocytes were pulsed for 4h with 0.001 to 0.05µM OVA, either free or encapsulated within α2M*. As controls, cells were pulsed with Con A (5µg/ml) or α2M* containing no Ag (0.02µM, equivalent to the highest concentration of α2M*-OVA). Cells were then washed, loaded at 5x10^5 cells per well onto a 96-well flat-bottom plate, and cultured 4 days at 37°C in a humidified 5% CO2 incubator. Cultured cells were treated with 1µCi/well [methyl-³H]Thymidine (PerkinElmer, Waltham, MA) 24h prior to
harvesting. [³H]thymidine incorporation was measured using a Tri-Carb 2100 TR liquid scintillation counter (PerkinElmer, Boston, MA).

### 2.1.9 Ag-specific cell-mediated cytotoxicity

Specific lysis of OVA-presenting cells was assayed using the Guava Cell Toxicity kit (Guava Technologies, Hayward, CA). OT-1 Splenocytes were pulsed for 4h with 0.01µM OVA or α₂M*-OVA. Following treatment with Ag, splenocytes were washed and cultured at 5x10⁵ cells per well in a flat-bottom 96-well plate for 48h at 37°C in a humidified 5% CO₂ incubator. OVA-expressing MO5 tumor cells were used as targets for this assay. The MO5 cell line, an OVA-transfected subclone of B16 melanoma, was a kind gift from Dr. K. Rock (University of Massachusetts Medical School, Worcester, MA). Target cells were stained with CFSE and added to the OT-1 effector cells to achieve effector:target ratios between 1:1 and 20:1. Cells were centrifuged at 50 x G for 2min and then incubated at 37°C for 4h. Cells were then incubated with 7-amino-actinomycin (7-AAD) for 10min to stain dead cells. The percentage of killed target cells was determined using the Guava EasyCyte Plus System. To determine the precise proportion of killed MO5 targets, the background percentage of dead MO5 cells in suspension (when not exposed to effector cells) was subtracted from the total percentage of dead MO5 cells.

### 2.1.10 Statistical analysis

For the in vitro studies, the Student’s t test was performed to determine p values and ascertain statistical significance between OVA and α₂M*-OVA treatments. The level of significance used was 0.05.
2.2 Methods for analysis of in vivo CTL responses to α2M*-encapsulated Ag

2.2.1 Adoptive Transfer and Immunization

Donor CD8+ T cells were purified from OT-1 splenocytes (harvested as in 2.1.4) by depletion of peripheral erythrocytes and other leukocytes using the BD Imag™ Mouse CD8 T Lymphocyte Enrichment Set (BD Biosciences Pharmingen, San Diego, CA). Briefly, cells were incubated with the following biotin-conjugated mAbs: anti-mouse CD4, clone Gk1.5; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse CD49b, clone HMα2; anti-mouse TER-119, clone TER-119. Labeled cells were then removed using streptavidin-conjugated magnetic particles. Purity of CD8+ T cells was determined to be > 95% by flow cytometry.

Each host C57BL/6 mouse (obtained and housed as in 2.1.1) received 3.5x10^6 CD8+ OT-1 cells in 1ml HBSS by i.p. injection. Intraperitoneal injection, rather than i.v., was used to deliver donor cells in this study based upon the observation that i.p.-delivered adoptive transfer cells localize more efficiently in the spleen (122). For three days following adoptive transfer, mice received daily intradermal injections of 0.35µg OVA, either free or encapsulated within α2M*, in PBS; the three injections occurred at 24, 48, and 72h following adoptive transfer. Control mice received an equivalent (10µl) volume of PBS. Murine α2M* was purified and α2M*-OVA was prepared as in sections 2.1.2. and 2.1.3. To distinguish between the effects of α2M*-mediated Ag delivery and any direct immunostimulatory effect of α2M*, control mice received α2M* containing no Ag (2.2µg, equivalent to the concentration of α2M*-OVA). Mice were sacrificed 7 days following the first injection of Ag, spleens were harvested, and OVA-specific CD8+ T cells were quantified by tetramer staining as above.
2.2.2 Intradermal Immunization of Naïve Mice

C57BL/6 mice were immunized by intradermal injection into the right ear pinna with 10µl of Ag or PBS, with or without the addition of α2M* or CpG 1826, 5′-TCCATGACGTTCCGACGTT-3′ (Midland Certified Reagent Company, Midland, TX). The treatment groups (n=5; each receiving 1.35µg OVA per injection) included the following: OVA alone; OVA administered with 10µg CpG 1826; α2M*-OVA; and α2M*-OVA administered with 10µg CpG 1826. Mice were subsequently boosted at days 35 and 63. Control groups (n=5) received intradermal injections of PBS, CpG 1826 alone, or α2M* containing no Ag (6µg, equivalent to the amount of α2M* present in the α2M*-OVA preparations).

In a separate experiment, C57BL/6 mice (n=5 per group) were immunized intradermally with 10⁶ bone marrow-derived DCs loaded ex vivo with either SIINFEKL or an irrelevant control peptide, lymphocytic choriomeningitis virus (LCMV) NP205 (see 2.2.3). These mice received only one injection.

2.2.3 Culture and Ag loading of bone marrow-derived DCs

At day 0, both tibia and femur bones were collected from a C57BL/6 mouse and flushed with media (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 2mM L-glutamine, and 50µM 2-ME) to harvest bone marrow. Following RBC lysis, bone marrow cells were plated at 1x10⁶ cells per ml in media supplemented with GM-CSF and IL-4 (40ng/ml each; Sigma-Aldrich, Saint Louis, MO). At day 3, 75% of the media and non-adherent cells were removed and replaced with fresh media. At day 6, non-adherent and adherent cells were collected (3mM EDTA in PBS was used to free adherent cells) and re-plated at 1x10⁶ cells per ml in media supplemented with GM-CSF an IL-4 (as above). At day 10, mature DCs were ready for stimulation and Ag loading.
Peptide, either SIINFEKL or the irrelevant control peptide, LCMV NP205 (YTVKYPNL) was added to the DC culture plates at 100ng/ml in combination with 5ng/ml CpG 1826 to promote activation. After 24h, mature Ag-loaded DCs were collected for animal vaccination. Phenotype of cultured DCs was verified by flow cytometry using the following Abs: PE-Cy7-conjugated hamster anti-mouse CD11c, PE-conjugated rat anti-mouse CD40, PE-conjugated hamster anti-mouse CD80, and PE-conjugated rat anti-mouse CD86 (BD Biosciences, San Jose, CA).

2.2.4 Determination of serum Ab titer

Serum anti-OVA IgG in immunized mice was monitored every 2 wks by ELISA. Approximately 200µl of peripheral blood was collected from each mouse by puncturing the submandibular vein with a 4mm animal lancet (Medipoint, Mineola, NY). Serum was isolated by centrifugation in serum separator tubes and stored at -20°C until analysis. Ninety-six well plates were coated with 10µg/ml OVA in sodium carbonate buffer at 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween20 (PBS-T) and blocked with SuperBlock Blocking Buffer in PBS (Pierce, Rockford, IL) for 1h at room temperature. Serial dilutions of mouse sera were then loaded into the 96-well plates, which were incubated overnight at 4°C. Plates were again washed with PBS-T, loaded with a 1:10,000 dilution of biotinylated rabbit anti-mouse IgG (H+L) (Pierce, Rockford, IL), and incubated overnight at 4°C. Plates were then washed, loaded with Ultra-sensitive ABC peroxidase staining complex (Pierce, Rockford, IL) for 30min at room temperature, washed again, and incubated with 1-Step Ultra TMB-ELISA substrate solution (Pierce, Rockford, IL) for 20min. Colorimetric change was stopped using 1M H₂SO₄ and absorbance was measured at 450nm.
2.2.5 Tetramer staining of mouse PBLs

Tetramer staining of mouse PBLs was performed after collecting ~200µl of whole blood from each immunized mouse by puncturing the submandibular vein with a 4mm animal lancet (Medipoint, Mineola, NY). Staining with tetramer and anti-CD8a Ab was performed as in 2.1.7. RBCs were lysed according to manufacturer’s directions using the iTag™ MHC Tetramer Lyse Reagent supplemented with iTag™ MHC Tetramer Fixative Reagent (Beckman Coulter, Fullerton, CA). Cells were then washed, fixed with 0.5% paraformaldehyde and stored light-protected at 4°C until flow cytometric analysis.

2.2.6 Tumor Challenge

Mice were injected subcutaneously in the left flank with 10⁴ MO5 tumor cells in Matrigel™ Basement Membrane Matrix (BD Biosciences Pharmingen, San Diego, CA) at wk 14 (DC-immunized mice were injected with tumor at wk 4). Tetramer staining of mouse PBLs (as described above) was performed 2 wks following tumor implantation. Tumor diameters were measured using digital calipers and tumor volume was calculated using the equation \( V = 0.4ab^2 \), where \( a \) and \( b \) are the longest and shortest diameters, respectively. Mice were sacrificed when tumor volume reached approximately 2cm³.

2.2.7 Statistical analysis

For the majority of in vivo studies (Ab 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.
2.3 Methods for analysis of cross-presentation of $\alpha_2M^\ast$-encapsulated Ag by professional APC subsets

2.3.1 Isolation of DC subsets and macrophages

Splenocytes from 30 C57BL/6 mice were harvested as in 2.1.4, pooled, and cultured overnight at 37°C. Macrophages were isolated by positive selection using anti-mouse CD11b magnetic particles (BD Biosciences Pharmingen, San Diego, CA). Dendritic cells were isolated by negative selection (depletion of other leukocytes) using the BD Imag™ Mouse Dendritic Cell Enrichment Set (BD Biosciences Pharmingen, San Diego, CA). Briefly, cells were incubated with the following biotin-conjugated mAbs: anti-mouse CD2 (LFA-2), clone RM2-5; anti-mouse CD3e (CD3 e chain), clone 145-2C11; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse CD49b (Integrin $\alpha_2$ chain), clone HMo2; anti-mouse CD147 (Basigin), clone RL73; anti-mouse Ly-6G and Ly-6C (Gr-1), clone RB6-8C5; anti-mouse TER-119/erythroid cells, clone TER-119. Labeled cells were then removed using streptavidin-conjugated magnetic particles. This population of CD11c$^\ast$ DCs was further separated into CD8$^\ast$, CD4$^\ast$, and double negative (DN; CD8$^\ast$, CD4$^\ast$) subsets by successive incubation with anti-mouse CD8 and anti-mouse CD4 magnetic particles (BD Biosciences Pharmingen, San Diego, CA). Phenotypes were confirmed by flow cytometry using the following Abs: PE-Cy7-conjugated hamster anti-mouse CD11c (BD Biosciences, San Jose, CA), PE-conjugated rat anti-mouse CD11b (BD Biosciences, San Jose, CA), PE-conjugated rat anti-mouse CD4 (BD Biosciences, San Jose, CA), and Caltag™ FITC-conjugated Rat anti-Mouse CD8a Ab (Invitrogen Corp., Carlsbad, CA).
2.3.2 Cross-presentation assays

Purified DC subsets and macrophages were pulsed for 2 h with 0.1µM OVA or α2M*-OVA. As a control, some APCs were not exposed to Ag. Effector OT-1 CD8+ T cells were purified by depletion of erythrocytes and other leukocytes as in 2.2.1. Effector OT-1 CD8+ T cells were then loaded into 96-well plates at 1x10^5 cells per well and incubated at a 1:10 ratio (APC:T cell) with DCs (CD8+, CD4+, DN) or macrophages (CD11b+). Cell supernatants were collected after 48h for detection of IFN-γ by ELISA (as in 2.1.4). After 3 days of culture, T cell proliferation was determined by [3H]thymidine incorporation. Cells were treated with 1 µCi/well [methyl-3H]thymidine at day 2, 18h before being harvested on day 3 as in 2.1.8.

2.3.3 Statistical analysis

As in 2.1.10, the Student’s t test was performed to determine p values and ascertain statistical significance between OVA and α2M*-OVA treatments. As in 2.2.7, ANOVA was performed followed by multiple comparison procedures (Tukey) to determine p values and ascertain statistical significance between APC-pulsed treatment groups. The level of significance used was 0.05.
3. Effect of $\alpha_2\text{M}^*$-mediated Ag delivery on \textit{in vitro} CTL responses

3.1 Introduction

Concerns regarding the safety of live attenuated and whole microorganism vaccines, as well as expanding knowledge of molecular pathobiology, have stimulated increased interest in the delivery of subunit vaccines. Although generally considered safe and relatively simple to manufacture, subunit vaccines are typically poor at eliciting CD8$^+$ T cell immunity and, therefore, have limited utility in the prevention and treatment of viral infections, cancers, and other diseases for which immunity is mediated by CTLs. The failure of subunit vaccines to stimulate CTL immunity depends on the lack of presentation of these exogenously delivered Ags on the MHC class I molecules of professional APCs. Therefore, vaccine formulations that introduce these Ags in a manner that promotes their cross-presentation should improve CTL immunity (6). Although numerous vaccine adjuvants and Ag delivery vehicles have been studied, few are able to specifically enhance cross-presentation and most are plagued by significant toxicity or manufacturing issues that often preclude human use (123, 124).

Although originally characterized as a proteinase inhibitor, $\alpha_2$-M has come to be appreciated as a potent Ag delivery vehicle. Upon activation with proteinases or small nucleophiles, such a NH$_3$ or primary amines, $\alpha_2\text{M}^*$ undergoes a major conformational change, allowing it to entrap and covalently bind diverse macromolecules which are then targeted for rapid internalization via LRP-1/CD91 (63). The ability of $\alpha_2\text{M}^*$-mediated Ag delivery to enhance specific humoral immune responses up to 1000-fold has been well documented (70, 71). Given the rapid and efficient process by which $\alpha_2\text{M}^*$ is known to deliver Ag to endosomal compartments, the observation that $\alpha_2\text{M}^*$-
mediated Ag delivery leads to increased MHC class II presentation and enhanced humoral immunity is not unexpected (61). While it has been reported that gp96 (125) and heat shock fusion proteins (56) can enhance cross-presentation in a CD91-dependent process, until recently, little has been known about the impact of α2M*-encapsulation on MHC class I presentation or the stimulation of CTL responses.

In this study, we examine the effect of receptor-mediated Ag (ovalbumin, OVA) delivery by α2M* on in vitro Ag-specific CTL responses. We find that delivery of OVA by α2M* increases the secretion of type 1 CD8+ CTL (Tc1) cytokines, expands OVA-specific CD8+ T cells, and enhances Ag-specific cell-mediated cytotoxicity. Consistent with previous reports, this enhanced biologic response appears to correlate with enhanced Ag uptake. These results suggest that α2M*-encapsulation enhances cross-presentation of exogenous Ag at least in vitro and provide support for the continued investigation of α2M* as an Ag delivery system with potential applications for enhancing the efficacy of subunit vaccines.
3.2 Results

3.2.1 Encapsulation of OVA into α₂M

Native and SDS PAGE of α₂M*-OVA complexes demonstrated successful incorporation of OVA into α₂M* as well as effective removal of free OVA (Figure 5). α₂-Macroglobulin dissociates into disulfide linked dimers under non-reducing conditions and can be observed migrating primarily as a single band. Under SDS non-reducing conditions, the direct infrared fluorescence scan demonstrates the Alexa Fluor® 647 label co-migrating with the α₂M* dimers (m.w. 360 kDa) rather than in the location of free OVA (m.w. 45 kDa). Ovalbumin remains covalently associated with α₂M* under SDS reducing conditions. Fluorescence quantification of complexes revealed a molar ratio of incorporation of ~2.5-3.5:1 (OVA:α₂M*). Consistent with α₂M* incorporation ratios observed for other unlabeled as well as radiolabeled proteins [18-20, 22], this finding demonstrates that α₂M*-encapsulation of the full-length OVA protein was achieved with high efficiency.
As shown here, 4-15% SDS-PAGE under non-reducing conditions demonstrates isolated \( \alpha_2 \text{M}^* \)-OVA complexes after column purification and concentration, OVA, \( \alpha_2 \text{M}^* \), and molecular weight markers. Ovalbumin is present at twice the concentration as that in \( \alpha_2 \text{M}^* \)-OVA, and \( \alpha_2 \text{M}^* \) concentrations in both lanes are equivalent. \( \alpha_2 \)-Macroglobulin* (m.w. 718 kDa) dissociates into disulfide linked dimers (m.w. 360 kDa) under non-reducing conditions and is observed migrating primarily as a single band. In the infrared fluorescence scan, free OVA (m.w. 45 kDa) can be identified as a major band in lane 3. In lane 2, however, OVA is shown to migrate in association with \( \alpha_2 \text{M}^* \). \( \alpha_2 \)-Macroglobulin* but not OVA is visible on Coomassie staining due to the small mass of OVA loaded on the gel. Infrared fluorescence scan at \( \lambda = 700 \) nm (left) and Coomassie stain (right) are shown.
3.2.2 $\alpha_2\text{M}^*$-encapsulation of OVA enhances dose-dependent IFN-$\gamma$ and IL-2 secretion

To better understand the impact of $\alpha_2\text{M}^*$-encapsulation on CTL immunity, we investigated the secretion of Tc1 cytokines (IFN-$\gamma$ and IL-2) in response to $\alpha_2\text{M}^*$-OVA. IFN-$\gamma$ is produced by activated Tc1 CD8$^+$ T cells as well as Th1-differentiated CD4$^+$ T cells, progenitor Th0 cells, DCs, and natural killer (NK) cells (126, 127). IFN-$\gamma$ performs numerous functions that enhance adaptive immunity, including the upregulation of Ag processing and presentation by professional APCs (128), and is critical for the propagation of CD8$^+$ CTLs. Therefore, measurements of IFN-$\gamma$ secretion and IFN-$\gamma$-secreting cells are often used as indicators of CTL activity (129). IL-2, another Th1/Tc1 cytokine, is produced primarily by activated CD4$^+$ and CD8$^+$ T cells, functioning to support the clonal expansion and differentiation of CTLs (130).

Splenocytes from OT-1 mice were incubated with either OVA or $\alpha_2\text{M}^*$-OVA for 24 or 48h. Various concentrations of Ag were studied and IFN-$\gamma$ secretion was measured by ELISA. At both 24 and 48h, incubation with OVA and $\alpha_2\text{M}^*$-OVA demonstrated dose-dependent increases in IFN-$\gamma$. However, at either time, IFN-$\gamma$ responses rose above baseline levels at lower concentrations of $\alpha_2\text{M}^*$-OVA compared to OVA alone. At 48h, a twofold higher concentration of OVA, 0.5$\mu$M, was required to elicit approximately the same response as that stimulated by 0.25$\mu$M $\alpha_2\text{M}^*$-OVA, suggesting more efficient immune stimulation with $\alpha_2\text{M}^*$-encapsulation than with Ag alone. In addition, the IFN-$\gamma$ response at 48h ranged from three to 13-fold greater with $\alpha_2\text{M}^*$-OVA compared to OVA alone (Figure 6A). Although detectable enhancement of IFN-$\gamma$ secretion was small at the lowest tested concentration, 0.0625$\mu$M OVA, the enhancement seen with $\alpha_2\text{M}^*$-mediated delivery of OVA achieved statistical significance at all other tested concentrations.
Figure 6: Splenocyte cytokine responses to OVA and α2M*-OVA challenge

Splenocytes from OT-1 mice which express a transgenic MHC class I-restricted TCR that recognizes the SIINFEKL (OVA\textsubscript{257-264}) peptide were incubated for 48h with varying concentrations of OVA or α\textsubscript{2}M*-OVA. Secretion of A) IFN-γ and B) IL-2 were measured by ELISA. In C and D, splenocyte cytokine responses to BSA647, α\textsubscript{2}M*, and SIINFEKL are shown. For reference, cytokine responses to OVA, either alone or incorporated into α\textsubscript{2}M*, are shown for the highest concentration tested in A and B, 0.5µM. Secretion of IFN-γ and IL-2 are shown in C and D, respectively. Each concentration was assayed in triplicate; values are mean ± SD. Results representative of three experiments. Where SD bars are not evident, it is because the SD was too small to be seen on the scale of the graph. Data for 24h incubation are not shown. *, p<0.05; **, p<0.005; ***, p<0.0001, between α\textsubscript{2}M*-OVA and OVA responses, as calculated by the Student’s t test.
OVA and α₂M*-OVA also produced dose-dependent increases in splenocyte IL-2 production at 24 and 48h. Similar to the IFN-γ studies, an increase in IL-2 secretion was seen with lower concentrations of α₂M*-OVA compared to OVA alone. At 48h, a > fourfold concentration of OVA was required to elicit a similar degree of IL-2 secretion as that seen with α₂M*-OVA (Figure 6B). Increases in IL-2 production at 48h ranged from nearly threefold up to fivefold with α₂M*-OVA compared to free OVA. This enhancement of IL-2 secretion with α₂M*-delivery of OVA was found to be statistically significant at all tested OVA concentrations. These data suggest significant amplification of the CTL response, particularly at low Ag concentrations, with α₂M*-encapsulation of OVA. Of note, similar cytokine data was obtained with MHC class II-restricted OVA-specific TCR transgenic mice (termed DO11.10), which express CD4+ TCRs specific for OVA323-339 (data not shown).

To ensure that any observed differences in cytokine secretion were the result of α₂M* receptor-mediated Ag delivery and not the immunogenicity of the fluorescent label or the α₂M* molecule itself, splenocytes were also incubated with α₂M* containing no Ag and BSA647. The concentration of BSA647 used was equivalent to the highest concentration of OVA tested, 0.5μM. The concentration tested of α₂M* containing no Ag was equivalent to the amount of α₂M* present, 0.2μM, at the highest concentration of α₂M*-encapsulated OVA, 0.5μM. As a positive control, splenocytes were treated with the H2-Kb-restricted CTL epitope of OVA, the SIINFEKL peptide (0.5μM). Unlike native OVA, the SIINFEKL peptide can bind directly to surface MHC class I molecules without internalization or further processing by APCs. As expected, these cells exhibited the greatest degree of Tc1 cytokine secretion. Treatment with either BSA647 or α₂M* alone, however, resulted in no detectable increase in IFN-γ or IL-2 secretion (Figure 6C,D).
These findings demonstrate that the enhanced Tc1 cytokine secretion observed with α2M*-OVA is not related to the immunogenicity of α2M* or the fluorescent species.

3.2.3 α2M*-encapsulation of Ag enhances the number of IFN-γ-producing Tc1 cells

In addition to demonstrating enhanced secretion of Tc1 cytokines with α2M*-encapsulation, we also quantified the reactive cells responsible for secretion. By ELISPOT assays we observed that the frequency of activated cells producing IFN-γ increased ~15-fold with α2M*-OVA compared to OVA alone (Figure 7). At 48h, significantly increased numbers of IFN-γ-producing T cells were observed at low concentrations of α2M*-OVA, 0.0125µM; twofold higher concentrations of OVA, 0.025µM, were required to elicit similar counts of IFN-γ-producing cells. The increased frequency of IFN-γ-producing cells with α2M*-encapsulation was statistically significant at all tested concentrations of Ag. Similar results were also observed at 24h (data not shown). As seen with the ELISA studies, treatment with BSA647 or α2M* containing no Ag failed to stimulate a detectable IFN-γ response. In summary, the number of IFN-γ-producing cells was observed to be dose-dependent and the magnitude of enhancement of IFN-γ-producing cells observed with α2M*-OVA compared to OVA was similar to that seen in the ELISA studies. Therefore, we conclude that the increase in Tc1 cytokine secretion observed with α2M*-encapsulation is primarily the result of an increased number of stimulated cytokine-producing T cells.
Splenocytes harvested from OT-1 mice were incubated for 48h with varying concentrations of OVA or α₂M*-OVA. Representative wells are shown in A). Quantified spot number, i.e. cells stimulated to produce IFN-γ, is represented as a function of Ag concentration in B) where cells treated with OVA are represented by (■) and cells treated with α₂M*-OVA are represented by (□). All conditions were assayed in triplicate and are represented as mean ± SD. Results representative of three experiments. *, p<0.005; **, p<0.0001, between α₂M*-OVA and OVA responses, as calculated by the Student’s t test.
3.2.4 Rapid uptake of α₂M*-encapsulated Ag enhances the CTL response

Previous studies have demonstrated that the high capacity LRP-1/CD91 receptor allows rapid uptake of α₂M*-encapsulated Ags even at low Ag concentrations (55, 63, 131). Consistent with earlier published studies, we observed significantly greater uptake of α₂M*-OVA per unit time compared to free OVA (Figure 8A). We, therefore, hypothesized that the in vitro enhancement of CTL responses to α₂M*-encapsulation would be even more pronounced following brief Ag exposure. OT-1 splenocytes were again incubated with OVA (0.25 µM), either free or encapsulated within α₂M*; cells were then washed and placed in fresh media after 30min to 8h and cultured for an additional 48h. As expected, IFN-γ secretion was increased (up to 25-fold) in the cells treated with α₂M*-OVA (Figure 8B). This enhancement was statistically significant for all time-points. Consistent with previous findings, this result supports the hypothesis that the rapid receptor-mediated uptake of α₂M*-encapsulated Ags results in enhanced adaptive immune responses in conditions of low Ag concentration or brief Ag exposure, as would often be expected physiologically. Based on these findings, all subsequent studies were performed with 4h pulses of Ag.
Figure 8: Rapid uptake of α₂M*-encapsulated Ag enhances the biological response

A) Murine TG-elicited peritoneal macrophages were treated with either OVA or α₂M*-OVA for durations of 5min to 2h. Cells were then washed and the quantity of OVA taken up by cells was measured directly by fluorescent imaging. B) OT-1 splenocytes were pulsed for 30min to 8h with either OVA or α₂M*-OVA. After 48h of subsequent incubation at 37°C, IFN-γ secretion was measured by ELISA. Each time-point was assayed in quadruplicate in A and triplicate in B; values are mean ± SD. Results representative of three experiments. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, between α₂M*-OVA and OVA responses, as calculated by the Student’s t test.
3.2.5 Expansion of Ag-specific CD8$^+$ T cells with $\alpha_2$M*-encapsulation

While the cytokine ELISA and ELISPOT data suggest an enhanced CTL response with $\alpha_2$M*-encapsulated Ag, direct evidence of Ag-specific CD8$^+$ T cell expansion was also obtained by an MHC class I tetramer assay (Figure 9A,B). Since OT-1 CD8$^+$ T cells are already specific for H-2K$^b$-SIINFEKL, we diluted OT-1 splenocytes 1:20 with SIINFEKL-naive C57BL/6 splenocytes in order to increase our ability to detect significant expansion of Ag-specific T cells. These cells were then pulsed for 4h with various concentrations of OVA or $\alpha_2$M*-OVA, washed, and cultured for an additional 4 days. Splenocytes pulsed with $\alpha_2$M*-OVA demonstrated up to 8-fold enhancement of tetramer binding compared to cells pulsed with the equivalent amount of free OVA. Furthermore, when averaged over three separate experiments, this increased tetramer binding with $\alpha_2$M*-OVA compared to OVA was found to be statistically significant for all tested Ag exposures. Thus, in addition to enhancing secretion of Tc1 cytokines, $\alpha_2$M*-encapsulation also results in a greater expansion of Ag-specific CD8$^+$ T cells available to mount a CTL response.

To further demonstrate that $\alpha_2$M*-delivery enhances T cell proliferation, we also performed a tritiated thymidine incorporation assay (Figure 9C). Consistent with the above tetramer studies, cells treated with $\alpha_2$M*-OVA demonstrated significantly enhanced proliferation compared to OVA-treated cells. More than tenfold greater concentrations of free OVA were required to elicit the same degree of proliferation as that seen with $\alpha_2$M*-OVA. At any given OVA concentration investigated, a four to sixfold enhancement of proliferation was observed with $\alpha_2$M*-OVA compared to OVA alone. This increase in cell proliferation with $\alpha_2$M*-delivery of Ag was found to be statistically significant at all OVA concentrations tested. This finding further supports
Figure 9: Expansion of Ag-specific CD8\(^+\) T cells with OVA and \(\alpha_2\)M\(^*\)-OVA

Suspensions containing a 20:1 ratio of C57BL/6:OT-1 splenocytes were incubated for 4h with OVA, either free or encapsulated within \(\alpha_2\)M*. Cells were then washed to remove remaining Ag and incubated for another 4 days at 37°C. A) Comparison of Ag-specific CD8\(^+\) T cell expansion with OVA and \(\alpha_2\)M*-OVA for a representative experiment. B) The concentration dependence of Ag-specific T cell expansion as determined by pooling the results of three experiments. Cells treated with OVA represented by ■ and cells treated with \(\alpha_2\)M*-OVA represented by □. C) Cell proliferation stimulated by OVA and \(\alpha_2\)M*-OVA. OT-1 splenocytes were incubated for 4h with various concentrations of either OVA (■) or \(\alpha_2\)M*-OVA (□). After 4 days of culture, cell proliferation was measured by [\(^3\)H]thymidine incorporation. Each condition was assayed in triplicate; values are mean ± SD. Results representative of three experiments. *, \(p<0.01\); **, \(p<0.001\); ***, \(p<0.0001\), between \(\alpha_2\)M*-OVA and OVA responses, as calculated by the Student’s t test.
our previous observation that $\alpha_2$M*-delivery of Ag results in enhanced Ag-specific T cell proliferation.

3.2.6 Enhanced Ag-specific cell-mediated cytotoxicity with $\alpha_2$M*-encapsulation

While the previous data clearly indicate increased expansion of Ag-specific CD8$^+$ T cells with $\alpha_2$M*-OVA, they do not directly demonstrate improved cytolytic activity. To evaluate the functionality of these expanded CTLs, we performed a flow cytometric cytotoxicity assay (Figure 10) and found that OT-1 splenocytes pulsed with $\alpha_2$M*-OVA demonstrate significantly enhanced cytotoxicity against OVA-expressing MO5 tumor cells compared to OVA-pulsed cells. For each of the conditions examined, treatment with $\alpha_2$M*-OVA stimulated approximately twofold greater lysis by effector cells compared to treatment with OVA alone. Furthermore, lysis observed with OVA treatment did not differ significantly from treatment with PBS. No significant cell lysis was observed under any tested condition against B16 melanoma cells, which do not present OVA. These findings verify the suggestion of previous results that $\alpha_2$M*-mediated Ag delivery increases Ag-specific cell lysis.
Figure 10: Enhancement of Ag-specific cytotoxicity with α₂M*-OVA

OT-1 splenocytes, pulsed with either 0.01μM OVA (□) or α₂M*-OVA (△), were incubated for 4h with MO5 cells. Splenocytes pulsed with PBS only are represented by ◊. Ag-specific cell lysis was measured as the percentage of dead target MO5 cells. Each condition was assayed in triplicate; values are mean ± SD. Results representative of three experiments. *, p<0.01; **, p<0.001, between α₂M*-OVA and OVA responses, as calculated by the Student’s t test.
3.3 Discussion

Given the necessity of potent CTL responses for the prevention and treatment of numerous human diseases, including viral infections and cancers, the development of practical and effective adjuvants or Ag delivery systems that promote CTL immunity is imperative. $\alpha_2$-Macroglobulin incorporates a wide variety of non-proteolytic Ags when co-incubated with such Ags during proteolytic activation (67). Ags incorporated in this manner demonstrate enhanced MHC class II presentation to Ag-specific T cells in vitro and increased Ab production in rabbits in vivo (61, 63). A non-proteolytic method of Ag incorporation developed and employed in subsequent studies has shown increased Ab titers to HBsAg and HIV envelope gp120 C4-V3 peptide with $\alpha_2$M*-Ag complexes relative to Ag alone (70, 71). These studies, however, were limited to measurements of humoral immune responses.

Here we demonstrate that $\alpha_2$M*-Ag complexes also amplify CTL responses. When OVA is incorporated into $\alpha_2$M*, Tc1 cytokine (IFN-\(\gamma\) and IL-2) production is enhanced up to 25-fold compared to OVA alone. Detectable CTL responses are observed with Ag doses of $\alpha_2$M*-OVA two- to tenfold less than those needed with OVA alone. In addition to demonstrating a quantifiable enhancement of Tc1 cytokine production, we also show an ~15-fold increase in the number of IFN-\(\gamma\)-secreting Tc1 cells with $\alpha_2$M*-OVA and that these cells, as demonstrated by tetramer assay and \(^3\)H\]thymidine incorporation, represent an expansion of the Ag-specific CD8\(^+\) T cell population. Furthermore, this Ag-specific CD8\(^+\) T cell population was effectively primed and demonstrated cytolytic activity against an Ag-presenting tumor cell line in vitro.

The observation that $\alpha_2$M*-mediated Ag delivery is capable of enhancing specific CTL responses provides new support for the use of $\alpha_2$M* as an Ag delivery vehicle.
Although originally thought to promote only MHC class II presentation (61), it now appears evident that $\alpha_2M^*$-mediated Ag delivery also promotes the cross-presentation of exogenous Ag onto MHC class I. This finding, evidenced by the enhanced Ag-specific CTL responses described here, suggests that $\alpha_2M^*$, given its ability to incorporate diverse Ag, may be quite useful for the delivery of subunit vaccines. Furthermore, these results indicate that $\alpha_2M^*$ may serve as a helpful resource for the study of cross-presentation and cross-priming.

The findings presented here are consistent with previous reports of enhanced CTL immunity with $\alpha_2M^*$-Ag complexes (72, 73). These reports demonstrated that $\alpha_2M^*$-bound peptides stimulate IFN-$\gamma$ secretion by cells that express the $\alpha_2M^*$ receptor, CD91, and that mice immunized with these $\alpha_2M^*$-bound peptides exhibit relative protection against Ag-presenting tumors. In contrast to these reports, here we demonstrate that a full-length protein, rather than a peptide, may be incorporated and delivered by $\alpha_2M^*$, processed by APCs, and cross-presented to stimulate CTL immunity. We have also further characterized the impact of $\alpha_2M^*$-mediated Ag delivery on CTL immunity by examining the activation and expansion of Ag-specific CD8$^+$ CTLs by ELISPOT and both $in vitro$ and $in vivo$ tetramer assays. Furthermore, we believe that the high efficiency by which $\alpha_2M^*$ incorporates the whole OVA protein in this study (2.5-3.5:1 OVA:$\alpha_2M^*$), compared to the relatively low incorporation of peptide presented in the above mentioned studies (~0.05:1 peptide: $\alpha_2M^*$), represents an improved model for examining the biological potential of utilizing $\alpha_2M^*$ as an Ag delivery vehicle.

In conclusion, we demonstrate in this study that receptor-mediated delivery of Ag by $\alpha_2M^*$ promotes cross-presentation and stimulation of Ag-specific CTL responses. When considered in the context of previous publications, these results suggest that $\alpha_2M^*$
is an effective Ag delivery system that enhances both humoral and cell-mediated immunity. Other characteristics of α₂M*, including its favorable safety profile, cost-effectiveness, and flexibility to efficiently incorporate both small ligands and large proteins, further support its potential utility for vaccines based on weakly immunogenic subunits.
4. Effect of $\alpha_2\text{M}^*$-mediated Ag delivery on \textit{in vivo} CTL responses

4.1 Introduction

Experts predict that new generation vaccines will be increasingly comprised of purified recombinant proteins (132). However, formulations of purified protein are frequently poorly immunogenic. Specifically, exogenous protein Ag does not readily enter the MHC class I presentation pathway and, therefore, cannot efficiently elicit a CTL response (133). Vaccine formulations must elicit effective CTL immunity in order to confer preventative and therapeutic effect against cancers and many chronic infectious diseases. Therefore, the addition of novel adjuvants and Ag delivery systems that promote the cross-presentation of these exogenous Ags is critical (6). An ideal vaccine adjuvant should enhance specific immune responses while minimizing toxicity (29).

Despite their structural and mechanistic diversity, adjuvants are sometimes broadly categorized by whether they have direct immunostimulatory effects on APCs or whether they function as delivery systems to promote antigen uptake into APCs (134). Those adjuvants known to have direct immunostimulatory effects are often derived from pathogens (e.g. MPL, CpGs) and are referred to as displaying pathogen-associated molecular patterns (PAMPs). Antigen delivery systems, however, are designed to specifically target antigens for uptake by professional APCs (e.g. mimicking the dimensions and surface properties of pathogens) (29).

In addition to traditional molecular adjuvants, cell-based vaccine technologies have also received significant attention in recent years. Several groups have employed vaccines consisting of Ag-pulsed DCs for the immunotherapy of cancer (135, 136). In this
approach, patient progenitor cells are collected and cultured to produce DCs that are then loaded with tumor Ag in an effort to enhance the anti-tumor response.

The activated form of $\alpha_2$M* is unique as an Ag delivery vehicle that targets Ag for rapid uptake by a specific cell-surface receptor. Although the results from Chapter 3 indicate the $\alpha_2$M*-delivered Ag are efficiently cross-presented in vitro, it is necessary to investigate the effect of $\alpha_2$M*-mediated Ag delivery in vivo in order to further assess the potential of this delivery system in enhancing the efficacy of subunit vaccines. Furthermore, any impact that $\alpha_2$M*-encapsulation may have on in vivo CTL responses should be compared to that of other investigative adjuvants and technologies so that its practical utility can be understood in the context of other vaccine formulations.

In this study, we examine the effect of receptor-mediated Ag (OVA) delivery by $\alpha_2$M* on in vivo Ag-specific CTL responses. We find that mice immunized with $\alpha_2$M*-encapsulated Ag exhibit enhanced humoral immunity, as demonstrated by elevated Ab titers, and increased proliferation of Ag-specific CD8+ T cells, as demonstrated by tetramer staining. Furthermore, these mice also display enhanced protection against an Ag-expressing subcutaneous tumor. Finally, the enhanced response observed with $\alpha_2$M*-OVA-immunization was comparable to that observed with a well-studied vaccine adjuvant (CpG 1826) and superior to an immunization protocol with ex vivo-prepared peptide-pulsed DCs. These results further support $\alpha_2$M* as an effective Ag delivery system, capable of enhancing both humoral and CTL immune responses.
4.2 Results

4.2.1 Ag-specific CD8\(^+\) T cell expansion following adoptive transfer

In an initial effort to probe the effect of \(\alpha_2\)M*-encapsulation on \textit{in vivo} CTL responses, the activation and expansion of adoptively transferred OVA-specific CD8\(^+\) T cells following \(\alpha_2\)M*-OVA-immunization was studied. Purified naïve CD8\(^+\) OT-1 T cells were delivered i.p. to C57BL/6 mice on day 0. For three days following adoptive transfer, mice received daily intradermal injections of PBS, \(\alpha_2\)M* containing no Ag, OVA, or \(\alpha_2\)M*-OVA (n=3 for each group). On day 7, mice were sacrificed and splenocytes were harvested and stained with H-2K\(^b\)/SIINFEKL tetramer (Figure 11). Mice immunized with OVA demonstrated an approximately 50 percent greater proportion of SIINFEKL-specific CD8\(^+\) T cells compared to the PBS control mice. Delivery of \(\alpha_2\)M* in the absence of Ag did not result in significant expansion of Ag-specific CTLs. Mice immunized with \(\alpha_2\)M*-OVA, however, demonstrated a threefold expansion of SIINFEKL-specific CD8\(^+\) T cells compared to the PBS control mice. This CTL expansion with \(\alpha_2\)M*-OVA was significantly greater than that with OVA alone \((p<0.05)\). Consistent with the \textit{in vitro} assays discussed in Chapter 2, this result indicates that \(\alpha_2\)M*-mediated Ag delivery is capable of enhancing specific CTL responses in biological systems.
Figure 11: Expansion of adoptively transferred SIINFEKL-specific CD8\(^+\) T cells

Following i.p. adoptive transfer of isolated OT-1 CD8\(^+\) T cells, C57BL/6 mice received three daily injections of PBS, empty \(\alpha_2M^*\), OVA, or \(\alpha_2M^*\)-OVA. Expansion of SIINFEKL-specific CD8\(^+\) T cells was quantified by H-2K\(^b\)/SIINFEKL tetramer binding. Values are mean ± SD (n=3 per group). The CTL expansion with \(\alpha_2M^*\)-OVA was found to be significantly greater than that with OVA alone (p<0.05).
4.2.2 Immunization with $\alpha_2$M*-encapsulated Ag enhances humoral and cell-mediated immunity

While the previous in vitro data as well as the adoptive transfer study suggest that $\alpha_2$M*-mediated Ag delivery may enhance CTL immunity in vivo, it is important to determine if a similar response can be observed in naïve mice immunized with $\alpha_2$M*-encapsulated Ag. Toward this end, groups of C57BL/6 mice (n=5) were immunized with OVA or $\alpha_2$M*-OVA, either alone or in combination with CpG 1826, in PBS. Control groups were immunized with PBS, $\alpha_2$M*, or CpG in the absence of the OVA Ag. Mice were subsequently challenged at wk 14 by subcutaneous implantation of MO5 tumors.

Although the focus of this work is on the development of CTL immunity, we found it pertinent to monitor the development of anti-OVA Ab titers prior to tumor implantation in order to validate our work in comparison to previous reports of humoral immune responses to $\alpha_2$M*-encapsulated Ag. The development of anti-OVA IgG Ab was observed in treated mice as early as 8 wks following initial injection. Endpoint titers (Ab titers at the time of tumor implantation; wk 14) are shown in Figure 12. Positive Ab titers were observed for each treatment group. Consistent with previous studies of humoral immunity and $\alpha_2$M*-encapsulated Ag (61, 70, 71), mice treated with a combination of $\alpha_2$M*-OVA and a secondary adjuvant (i.e. CpG 1826) developed the strongest Ab titers, which were significantly greater (more than tenfold) than mice treated with OVA alone.

In order to observe expansion of the Ag-specific CD8$^+$ T cell population in these mice, tetramer staining of PBLs was performed 2 wks following tumor implantation (Figure 13). Both the $\alpha_2$M*-OVA without CpG and the OVA with CpG treatment groups demonstrated a significant expansion of the OVA-specific CD8$^+$ T cell population compared to the control groups. However, as with the Ab titer data, mice treated with a
Figure 12: Immunization with α₂M*-encapsulated Ag induces humoral immunity

End-point titer for each mouse was determined by ELISA and averaged by group. IgG Ab titer was defined as the highest dilution of sera that produced an A₄₅₀ of 0.125. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. The presence of brackets indicates a difference between the indicated treatment groups (*, p<0.05).
Figure 13: Expansion of Ag-specific CD8$^+$ T cells following immunization

Tetramer staining of PBLs from each mouse 2 wks following implantation of MO5 tumors, averaged by treatment group. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. p values (*, p<0.05; **, p<0.005) indicate difference between respective treatment group and each of the three control groups (PBS, CpG, $\alpha_2$M$^*$).
combination of \( \alpha_2M^*-\text{OVA} \) and CpG demonstrated by far the strongest response (more than 17-fold greater than control groups).

### 4.2.3 Immunization with \( \alpha_2M^*-\text{encapsulated Ag} \) induces protection against Ag-presenting tumor

The above Ab titer and tetramer data demonstrate an enhancement of both humoral and cell-mediated immunity with \( \alpha_2M^* \)-mediated Ag delivery. However, observations of MO5 tumor growth are necessary to understand whether these responses actually represent effective anti-tumor activity. Tumor growth for each mouse treatment group is demonstrated in Figure 14. Observable growth of MO5 tumors was delayed in each of the treatment groups. “Survival” curves for each group following tumor implantation were calculated by the Kaplan-Meier method (Figure 15) and statistical significance was determined by log-rank Mantel-Cox analysis. Each of the \( \alpha_2M^*-\text{encapsulated and CpG} \) treatment groups demonstrated improved survival (i.e. prolonged time before tumor volume reached 2cm\(^3\)) compared to the PBS, CpG, and \( \alpha_2M^* \) control groups. Survival of \( \alpha_2M^*-\text{OVA} \)-treated mice was not significantly different from that of mice treated with OVA and CpG. Consistent with the Ab and tetramer data, the treatment group that combined \( \alpha_2M^*-\text{OVA} \) with CpG demonstrated the strongest anti-tumor response and the longest survival; this improvement in survival was statistically significant compared to all other groups (\( p \) values ranging from 0.0018 to 0.029). We propose that this anti-tumor response is largely the result of CTL activity; however, given the significantly enhanced humoral response reported above, we cannot rule out a contribution from Ab-dependent cell-mediated cytotoxicity (ADCC) at this time.
Figure 14: Growth of Ag-presenting tumor inhibited by immunization with \(\alpha_2M^+\)-encapsulated Ag

Tumor growth over days following implantation observed in each mouse, averaged by treatment group. Mice were sacrificed at a tumor volume of \(~2\text{cm}^3\). Values indicate mean ± SEM (n=5 per group). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups as discussed in the text.
Figure 15: Survival of $\alpha_2$M*-OVA-immunized mice presented as Kaplan-Meier plot

Since survival of control groups (PBS, CpG, and $\alpha_2$M*-treated mice) was not significantly different from that of the OVA treatment group, survival data for these groups is omitted from this plot for simplicity. Statistical significance between survival curves was determined by Mantel-Cox log-rank analysis and is discussed in the text.
4.2.3 Immunization with $\alpha_2\text{M}^*$-encapsulated Ag compares favorably to a DC-based vaccine protocol

The previous results indicate that $\alpha_2\text{M}^*$-encapsulation enhances CTL immunity \textit{in vivo} and improves anti-tumor responses to a degree similar to another accepted vaccine adjuvant (CpG 1826). However, in order to examine how $\alpha_2\text{M}^*$-mediated Ag delivery compares with cell-based vaccine technology, additional groups of mice were immunized with peptide-pulsed DCs. For this study, bone marrow was harvested from a C57BL/6 mouse and cultured in the presence of GM-CSF, IL-4, and TNF-\(\alpha\) to induce DC differentiation and maturation. Phenotypic maturation of these cultured DCs was verified by flow cytometry. In addition to CD11c expression, cells demonstrated elevated surface expression of the co-stimulatory molecules CD40, CD80, and CD86, consistent with DC maturation (137-139). These DCs were activated with CpG and loaded with peptide Ag, either SIINFEKL or an irrelevant CTL peptide (LCMV NP205) known to bind H2-K\(^b\) (140).

C57BL/6 mice were injected intradermally with $10^6$ peptide-pulsed DCs each (n=5 per group). At wk 4, $10^4$ MO5 tumor cells were implanted subcutaneously in the left flank of each mouse, as had been done with the previously discussed $\alpha_2\text{M}^*$-OVA-immunized mice. Production of anti-OVA Ab responses were monitored in these mice as with the other treatment groups. Endpoint titers (titers at the time when tumor cells were implanted) are shown in Figure 16. The majority of mice immunized with SIINFEKL-pulsed DCs (4 out of 5) demonstrated elevated anti-OVA Ab titers. However, the average Ab titer in this group was approximately 1/10\(^{th}\) of that elicited in $\alpha_2\text{M}^*$-OVA-immunized mice. Tetramer staining of mouse PBLs 2 wks following tumor implantation demonstrated that SIINFEKL-pulsed DC immunization elicited a \(\sim 70\%\) increase in the SIINFEKL-specific CD8\(^+\) T cell population (Figure 17). However, this
expansion in SIINFEKL-specific CD8\(^+\) T cells was only \(\frac{1}{2}\) of that elicited by immunization with \(\alpha_2M^+\)-OVA and \(\frac{1}{10}\) of that elicited by \(\alpha_2M^+\)-OVA in combination with CpG 1826.

Tumor growth appeared somewhat delayed in mice immunized with SIINFEKL-pulsed DCs compared to those immunized with irrelevant peptide (LCMV)-pulsed DCs (difference was statistically significant for days 18-21), as shown in Figure 18. However, this inhibition of tumor growth was significantly less than that observed with \(\alpha_2M^+\)-OVA mice. Furthermore, survival of mice immunized with SIINFEKL-pulsed DCs was not significantly prolonged compared to mice immunized with LCMV-pulsed DCs (Figure 19). Therefore, it can be safely concluded that the anti-tumor response elicited by this particular protocol with peptide-pulsed DCs is not as robust as that elicited by \(\alpha_2M^+\)-OVA immunization.
Figure 16: Comparison of induced humoral immunity following vaccination with peptide-pulsed DCs and α₂M*-encapsulated Ag

End-point titer for each mouse was determined by ELISA and averaged by group. IgG Ab titer was defined as the highest dilution of sera that produced an A450 of 0.125. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. The presence of brackets indicates a difference between the indicated treatment groups (*, p<0.05).
Figure 17: Comparison of Ag-specific CD8+ T cell expansion with peptide-pulsed DC and α₂M*-encapsulated Ag vaccination

Tetramer staining of PBLs from each mouse 2 wks following implantation of MO5 tumors, averaged by treatment group. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. *p values (*, p<0.05; **, p<0.005) indicate difference between respective treatment group and the control groups (PBS, CpG, α₂M*, LCMV-pulsed DCs).
Tumor growth over days following implantation observed in each mouse, averaged by treatment group. Immunization with SIINFEKL-pulsed DCs is compared to immunization with irrelevant peptide (LCMV NP205)-pulsed DCs pulsed and α2M*-OVA treatments. Mice were sacrificed at a tumor volume of ~2cm³. Values indicate mean ± SEM (n=5 per group). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups as discussed in the text.
Survival of mice immunized with SIINFEKL-pulsed DCs is compared to that of mice immunized with DCs that had been pulsed with an irrelevant peptide (LCMV NP205). Survival of these mice immunized with cell-based vaccines is also compared to that of mice immunized with α2M*-OVA. Statistical significance between survival curves was determined by Mantel-Cox log-rank analysis and is discussed in the text.
4.3 Discussion

The *in vivo* anti-tumor response observed with α2M*-mediated Ag delivery was comparable to that of an accepted vaccine adjuvant (CpG 1826) and, as expected, was further improved by combining α2M*-delivery with that adjuvant. This finding is consistent with previous work that have found α2M*-delivery to elicit humoral responses comparable to or better than CFA or monophosphoryl lipid A (MPL-SE) with GM-CSF, while co-delivery of these adjuvants with α2M*-complexes yields the strongest response (61, 70, 71). This finding validates the investigation of α2M* as a delivery system capable of enhancing immunity by a magnitude similar to other investigative adjuvants. Furthermore, since α2M*, an Ag delivery vehicle, has been shown to elicit even more robust responses when combined with an immunostimulatory adjuvant, vaccine formulations containing combinations of α2M*-encapsulated Ag with such adjuvants may have an improved success rate.

The immune responses elicited with α2M*-OVA immunization also compared quite favorably against a DC-based vaccine protocol. Although there were significant differences in the protein-based and cell-based vaccine protocols described here (3 injections versus 1 injection, for instance), these findings suggest that α2M*-OVA immunization may demonstrate efficacy at least similar to *ex vivo* DC vaccines, while avoiding the cost and technical limitations of cell-based technologies. Preparing complexes of α2M*-encapsulated Ag is significantly less expensive and labor intensive than culturing DCs from patient precursor cells. Furthermore, since preparing *ex vivo* DCs from very ill patients can be impossible (141), α2M*-encapsulated protein vaccines may represent a viable alternative for patients for whom DC vaccination is not an option.
Other characteristics of α₂M* further endorse its candidacy as a Ag delivery vehicle for use in subunit vaccines. Amplification of CTL responses is historically very difficult to achieve using vaccine adjuvants that are safe and cost-effective. Some adjuvants that are known to elicit potent Th1/Tc1 and Th2/Tc2 responses, such as CFA, are too toxic for human use. Others, including liposomal adjuvants, appear to safely enhance humoral and cellular immunity, but suffer from significant stability and manufacturing issues (123, 124). However, no toxic effects of α₂M occur during \textit{in vivo} animal studies. This result is not surprising given its high concentration in plasma [3]. The wide availability of α₂M, either from large-scale protein fractionation or cloning, and its stability in solution at room temperature further increase its appeal as a vaccine adjuvant or delivery vehicle, especially lyophilized and stored for use in developing nations (61, 70, 71).
5. Professional APCs capable of cross-presenting $\alpha_2\text{M}^*$-encapsulated Ag

5.1 Introduction

Over the past few decades, cross-presentation has come to be appreciated as critical to the development of CTL immunity to exogenous Ag (6, 77, 78) as well as to the maintenance of self-tolerance (142, 143). However, the precise intracellular mechanisms as well as the specific cell types involved in the cross-presentation of diverse Ag are still unclear. In the context of vaccine development, understanding the mechanisms and identifying the cell types involved in cross-presentation is vital to designing vaccines that efficiently induce CTL immunity rather than tolerance to exogenous Ag (144).

Although B cells (103), neutrophils (104), and even liver endothelial cells (145) are reportedly capable of cross-presentation, DCs and macrophages are generally considered to be the only cells responsible for significant in vivo cross-priming (105). However, the relative contribution of these two cell types is still a matter of debate. Several groups have identified DCs as the primary cross-presenting cells (84, 101, 106-109, 146). Furthermore, DCs are reportedly 100-1000 times more potent as professional APCs than macrophages in vitro and require 10-fold less Ag to prime CD8$^+$ T cells in vivo (112). Macrophages, however, are much more abundant in sites of infection and inflammation, suggesting that they may indeed be important to T-cell priming (112).

Dendritic cells are a heterogenous and highly plastic group of cells that can be broadly categorized as conventional and plasmacytoid DCs. Although there is evidence that plasmacytoid DCs perform cross-presentation in vitro (147), several studies have concluded that any contribution to cross-presentation by plasmacytoid cells is minimal compared to conventional DCs (148, 149). In mice, three distinct conventional DC
subsets are found in the spleen: the CD4$^+$CD8$^+$, the CD4$^+$CD8$^-$ and the CD4$^-$CD8$^+$ DCs (150).

Several studies have identified the CD8$^+$ DC subset as the principal APC for the cross-presentation of soluble (151), viral (109, 152), bacterial (153), and latex bead-associated (154) Ag and apoptotic debris (155). However, other DC subsets appear to make important contributions to the cross-presentation of some Ags (81). For example, both CD8$^+$ and CD8$^-$ DC subsets are capable of cross-priming immune complexes of Ag (85) which, like $\alpha_2$M*-encapsulated Ags, are thought to undergo receptor-mediated uptake. Also, CD4$^+$CD8$^-$ (double negative, DN) DCs demonstrate a limited ability to cross-present soluble OVA in vivo in the presence of LPS (151), further demonstrating the importance of adjuvants as well as delivery systems in determining the cell types involved.

Furthermore, although it is undisputed that CD8$^+$ DCs are superior to other DC subsets at stimulating cross-priming under many circumstances, reports in the literature differ regarding whether CD8$^+$ DCs are specially equipped to perform cross-presentation or if the DC subset involved in cross-presentation depends more heavily on the form of Ag or the route of uptake employed. In 2002, a report claimed that both CD8$^+$ and CD4$^+$ DCs are capable of efficient cross-presentation but that the former’s superior ability to endocytose dying cells allows it access to certain Ag for cross-presentation that are less available to the other DC subsets (156). The same study also reported that certain Ag, such as bacterial-associated Ag, are actually cross-presented more efficiently by CD4$^+$ DCs compared to CD8$^+$ DCs (156). A subsequent report, however, claimed that the dominance of CD8$^+$ DCs in cross-presenting diverse Ag is not dependent on Ag uptake. This study proposed instead that CD8$^+$ DCs possess
specialized machinery, lacking in CD8⁻ DCs, which account for the superiority of these cells in cross-presentation (154); this hypothesis that has be reiterated in subsequent publications (157).

Yet another study reported that CD8⁺ DCs are more efficient than CD4⁺ DCs at cross-presenting particulate Ag and that the expression of the co-stimulatory molecule CD24 on CD8⁺ DCs seemed to be a determinant of this efficiency (158). Other reports have proposed a discrete functional separation between DCs subsets, suggesting that CD8⁺ DCs efficiently present Ag to CD8⁺ T cells while CD8⁻ DCs (including CD4⁺ DCs) efficiently present Ag to CD4⁺ DCs (85, 120, 151, 154); however, it has also been claimed that CD8⁺ DCs are superior at presenting Ag onto both MHC class I and MHC class II (158).

The work presented in Chapters 3 and 4 strongly suggest that α₂M⁺-encapsulated Ags can indeed be cross-presented both in vitro and in vivo. Identifying the APCs involved in this cross-presentation will help further clarify the mechanism by which α₂M⁺-mediated Ag delivery enhances CTL immunity. Furthermore, detailing the degree to which different APC subsets stimulate cross-priming in response to α₂M⁺-delivered Ag may yield some insight into the influence, if any, of receptor-mediated Ag delivery on determining the relative dominance of different APCs.
5.2 Results

5.2.1 Isolation of DC subsets and macrophages

Successful purification (~95%) of CD11c⁺ DCs and CD11b⁺ macrophages from pooled C57BL/6 murine splenocytes was achieved using Ab-conjugated magnetic beads (Figure 20). Purification (~95%) of CD8⁺ T cells from OT-1 splenocytes was also successfully achieved using similar methods (Figure 20); these cells were purified for use as effector cells in the cross-presentation assays. Purified CD11c⁺ DCs were further separated by magnetic beads into the following subsets: CD8⁺, CD4⁺, DN. Purity of these DC subsets was determined to be at least 75% by flow cytometry (Figure 21).

Figure 20: Purification of CD8⁺ T cells, CD11c⁺ DCs, and CD11b⁺ macrophages

CD8⁺ T cells were purified from OT-1 splenocytes by negative selection using a magnetic bead separation kit. CD11c⁺ DCs and CD11b⁺ macrophages were purified from C57BL/6 splenocytes (pooled from 30 mice) by negative and positive selection, respectively, using Ab-conjugated magnetic beads. Purity of each population following this procedure is estimated to be >95% by flow cytometry.
Isolation of CD8⁺, CD4⁺, and CD8⁻ CD4⁻ (DN) DC subset populations was performed by consecutive staining with ant-CD8 and anti-CD4 conjugated magnetic beads. Purity of each population was estimated to be at least 75% by flow cytometry.

5.2.2 CD8⁺ and CD4⁺ DCs efficiently cross-present α₂M⁺-delivered Ag and stimulate cross-priming

Purified APC subsets (CD8⁺ DCs, CD4⁺ DCs, DN DCs, and CD11b⁺ macrophages) were pulsed for 2h with either OVA or α₂M⁺-OVA, washed to remove unbound Ag, and incubated at a 1:10 ratio (APC:T cell) with CD8⁺ OT-1 T cells. APC subsets that were not treated with Ag were also incubated with CD8⁺ OT-1 T cells as a control.

After 2 days of culture, cell supernatants were collected for detection of IFN-γ by ELISA (Figure 22). All OVA and α₂M⁺-OVA-pulsed APCs elicited some degree of detectable IFN-γ secretion by the effector T cells. However, IFN-γ production in response to α₂M⁺-encapsulated Ag was up to 11-fold greater than with OVA alone. This difference was found to be statistically significant for all APCs, except DN DCs. Among the APC
subsets tested, CD8+ and CD4+ DCs elicited the greatest production of IFN-γ in response to α2M*-OVA (7 to 22-fold greater than DN DCs and macrophages). Although both CD8+ and CD4+ DCs elicited robust IFN-γ responses, the response elicited by CD4+ DCs was approximately 60% greater than that elicited by CD8+ cells (p<0.02).

Additionally, proliferation of similarly prepared CD8+ OT-1 T cells was measured by [3H]thymidine incorporation assay following 3 days of incubation with Ag-pulsed APC subsets (Figure 23). Similar to the above IFN-γ results, detectable responses were observed with all OVA and α2M*-OVA-pulsed APC subsets tested. Proliferation in response to α2M*-encapsulated Ag was up to 6.5-fold greater than with OVA alone. This difference was found to be statistically significant for all APCs, except macrophages.

Consistent with the IFN-γ results above, CD8+ and CD4+ DCs elicited the greatest degree of proliferation in response to α2M*-OVA (2 to 4-fold greater than DN DCs and macrophages). Furthermore, consistent with the IFN-γ findings, both CD8+ and CD4+ DCs elicited robust responses, although proliferation elicited by CD4+ DCs was approximately 35% greater than that elicited by CD8+ cells (p<0.05).

These results indicate that both CD8+ and CD4+ DCs are capable of stimulating cross-priming to α2M*-delivered Ag with high efficiency. Furthermore, the CD4+ DC subset may actually be somewhat superior to CD8+ DCs in presenting α2M*-encapsulated Ag onto MHC class I and stimulating Ag-specific CD8+ T cells. To a lesser extent, Macrophages also elicited some degree of IFN-γ and proliferation responses. Although DN DCs appeared to elicit small IFN-γ and proliferation responses, it is difficult to determine if these results indicate true cross-presentation by this subset, albeit less efficient than with CD8+ and CD4+ DCs, or are the result of low-level contamination of this subset with more efficient DCs.
Effector OT-1 CD8^+ T cells were incubated at a 1:10 (APC:T cell) ratio with the following APC subsets: CD8^+, CD4^+, and DN DCs and CD11b^- macrophages (MΦ). APCs had been pulsed with either OVA, α_2M*-OVA, or no Ag. Secretion of IFN-γ was measured after 48h by ELISA. Each condition was assayed in triplicate; values are mean ± SD. Results representative of two experiments. *, p<0.05; **, p<0.005; ***, p<0.0001, between α_2M*-OVA and OVA treatments, as calculated by the Student’s t test. Statistical differences between APC subsets determined by ANOVA and are discussed in the text.
Effector OT-1 CD8\(^+\) T cells were incubated at a 1:10 (APC:T cell) ratio with the following APC subsets: CD8\(^+\), CD4\(^+\), and DN DCs and CD11b\(^+\) macrophages (M\(\Phi\)). APCs had been pulsed with either OVA, \(\alpha_2\text{M}^*-\text{OVA}\), or no Ag. Cell proliferation was measured after 3 days by \(^3\text{H}\)thymidine proliferation. Each condition was assayed in triplicate; values are mean ± SD. Results representative of two experiments. *, \(p < 0.05\); **, \(p < 0.005\), between \(\alpha_2\text{M}^*-\text{OVA}\) and OVA treatments, as calculated by the Student’s \(t\) test. Statistical differences between APC subsets determined by ANOVA and are discussed in the text.
5.3 Discussion

The observations of increased in vitro and in vivo CTL responses to α2M*-encapsulated Ag discussed in Chapters 3 and 4 indicate that α2M*-delivered Ag are processed and cross-presented onto MHC class I. However, prior to these studies, there was little information to suggest which professional APCs might engage in the cross-presentation of α2M*-delivered Ag. Both DCs (54, 55) and macrophages (52, 53) are known to express LRP-1/CD91+, the α2M* uptake receptor, and are capable of rapidly internalizing α2M* via receptor-mediated endocytosis (59-61). Likewise, both DCs (84, 101, 106-109, 146) and macrophages (82, 102) are capable of cross-presentation, although their relative contributions in vivo are still not fully understood (6, 112, 159).

The findings presented here demonstrate that DCs and macrophages, to a lesser extent, are capable of cross-presenting α2M*-delivered Ag and priming Ag-specific CTLs. However, CD8+ and CD4+ DCs appear to do so much more efficiently than either DN DCs or CD11b+ macrophages. Both CD8+ and CD4+ DCs, when pulsed with α2M*-encapsulated Ag, elicit robust CD8+ T cell responses, as measured by IFN-γ secretion and [3H]thymidine incorporation. These responses were as much as 13-fold greater than those elicited by macrophages and 22-fold greater than those elicited by DN DCs. Since the elicited T cell responses observed with DN DCs were modest and the purity of this subset may have been less than 80%, it is unknown if these responses were the result of less-efficient cross-presentation with this subset or contamination with some CD8+ and CD4+ DCs.

The finding that certain DC subsets (CD8+ and CD4+) elicit cross-priming in response to α2M*-encapsulated Ag more potently than macrophages is consistent with a previous report that identified lin’ CD11c+ DCs, isolated from human PBMCs, as the
chief cells responsible for inducing T cell activation and proliferation in response to $\alpha_2M^*$-encapsulated Ag (55). Furthermore, this finding is consistent with numerous reports indicating that, while macrophages are capable of cross-presentation (82, 102), they are typically far less efficient than DCs at stimulating cross-priming (81).

The observation that CD4+ DCs cross-present $\alpha_2M^*$-delivered Ag and elicit CD8+ T cell responses comparable to or greater than CD8+ DCs is significant given the ongoing debate regarding cross-presentation with these DC subsets. CD8+ DCs have repeatedly been identifying as a superior APC for the cross-presentation of most Ags, including soluble Ag (151), viral Ag (109, 152) and apoptotic debris (155). Some reports have contended that, since CD8+ DCs cross-present several forms of Ag and CD8+ DCs (including CD4+ DCs) do not (120), CD8+ DCs must possess specialized intracellular machinery (154) or co-stimulatory molecules (158) that make this cell subset uniquely equipped for cross-presentation. Other reports, however, have suggested that the superiority of CD8+ DCs in cross-presenting certain Ags and stimulating cross-priming of Ag-specific CTLs is related to its superior ability to endocytose dying cells (155, 156). Furthermore, there is some evidence that CD4+ DCs may cross-present certain Ags as well or better than CD8+ DCs, depending on the mechanism involved (155, 156). For instance, immune complexes of Ag, thought to undergo receptor-mediated uptake, are reportedly cross-presented by both CD8+ and CD8 DC subsets (85).

The results reported here support the hypothesis that Ag uptake, rather than functional differences between CD8+ and CD4+ subsets, is a principal determinant of which DC subsets are responsible for cross-presentation. Based on these findings, if CD8+ and CD4+ DCs indeed possess comparable cross-presentation machinery, it seems likely that receptor-mediated uptake by LRP-1/CD91 is similarly efficient with both
CD8+ and CD4+ DCs. However, further studies of LRP-1/CD91 expression as well as α2M*-mediated Ag uptake with these DC subsets would be needed to confirm this.
6. Future Directions and Concluding Remarks

In these studies, we have verified that $\alpha_2M^*$-mediated Ag delivery promotes cross-presentation and enhances CTL immune responses, both in vivo and in vitro. Furthermore, we have demonstrated that both DCs and macrophages are capable of cross-presenting $\alpha_2M^*$-encapsulated Ag and priming CD8+ T cells, although CD8+ and CD4+ DCs do so most efficiently. Considered in the context of previous work, these findings suggest that $\alpha_2M^*$-mediated Ag delivery may have significant implications for the development of effective subunit vaccines as well as further study of the mechanisms of cross-presentation. However, there are still several issues regarding possible applications of $\alpha_2M^*$ and the mechanism of these enhanced biological responses that merit investigation.

6.1 Co-delivery of $\alpha_2M^*$ with Unbound Ag

For each of the studies described previously in this dissertation, $\alpha_2M^*$-encapsulated Ag was prepared by incubation of amine-activated $\alpha_2M^*$ with a 40-molar excess of Ag in vitro at 37°C for 18h. This $\alpha_2M^*$-Ag complex was then purified by size-exclusion chromatography to remove unbound Ag. Although this process is neither particularly difficult nor time-consuming, it would be a considerable advancement if we could promote $\alpha_2M^*$-encapsulation of Ag in vivo without the added steps of in vitro incorporation and isolation.

Although it was reported that maximal incorporation of Ag into $\alpha_2M^*$ occurs at 24h of 37°C incubation (67), some incorporation of Ag certainly occurs at much earlier time points. While it is believed that $\alpha_2M^*$-encapsulation occurs naturally in vivo as a mechanism of targeting Ag for uptake, processing, and presentation by professional
APCs (61), it is unknown whether in vivo α2M*-encapsulation could be promoted in the setting of a vaccine that co-delivers α2M* with unbound Ag. A relatively high local concentration of Ag may be necessary to drive incorporation into α2M*. Since most routes of injection would result in somewhat rapid dissipation of Ag, conditions that create a depot effect (e.g. intradermal injection, alum absorption) should promote α2M*-encapsulation in vivo.

As a preliminary study to determine if co-delivery of Ag with unbound α2M* could enhance CTL immunity, groups of naïve C57BL/6 mice (n=5) were immunized intradermally with OVA and unbound α2M* (1.35µg and 6µg, respectively) with or without the addition of CpG 1826 (10µg) according to the same immunization scheme (boosts at days 35 and 63) described in 2.2.2. Separate unbound α2M* and OVA preparations were kept on ice and combined immediately prior to injection in order to minimize the possibility of α2M*-encapsulation occurring in vitro. The immunization and tumor challenge of these mice was performed simultaneously with the groups discussed in 4.2.2 and 4.2.3. As with the α2M*-OVA groups discussed previously, the development of anti-OVA IgG Ab was first observed in these mice 8 wks following initial injection. End-point titers (Ab titers at the time of tumor implantation; wk 14) are shown in Figure 24. Mice immunized with OVA and unbound α2M*, with or without the addition of CpG 1826, produced anti-OVA Ab titers comparable to those produced with OVA and CpG 1826. Although the mean Ab titers produced by conjugated α2M*-OVA, with or without CpG 1826, were greater than those elicited with OVA and unbound α2M*, the difference between these groups was not found to be statistically significant.

As with the immunization groups discussed previously, OVA-expressing MO5 tumor cells (10⁴ cells per mouse) were implanted subcutaneously at wk 14. Tetramer
staining of PBLs was performed 2 wks following tumor implantation in order to observe expansion of the Ag-specific CD8\(^+\) T cell population in these mice (Figure 25). Although the OVA with unbound \(\alpha_2M^*\) 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 \(\alpha_2M^*\) alone). Mice treated with OVA and unbound \(\alpha_2M^*\) with the addition of CpG, however, did elicit an expansion of the OVA-specific CD8\(^+\) T cell population that was significant compared to the control groups \((p<0.05)\). Furthermore, although conjugated \(\alpha_2M^*\)-OVA treatment groups appeared to elicit greater expansion of Ag-specific CTLs than the OVA with unbound \(\alpha_2M^*\) groups, neither the difference between \(\alpha_2M^*\)-OVA and OVA with unbound \(\alpha_2M^*\) nor the difference between \(\alpha_2M^*\)-OVA with CpG and OVA with unbound \(\alpha_2M^*\) and CpG was found to be statistically significant.

The above Ab titer and tetramer data demonstrate that the co-delivery of \(\alpha_2M^*\) with unbound Ag can enhance both humoral and cell-mediated immunity to a degree that is not significantly different from conjugated \(\alpha_2M^*\)-Ag treatment. Furthermore, anti-tumor responses elicited by the co-delivery of \(\alpha_2M^*\) with unbound OVA were also found to be similar to those observed with \(\alpha_2M^*\)-OVA. Observable growth of MO5 tumors over time is shown in Figure 26. Mice immunized with OVA and unbound \(\alpha_2M^*\), with or without CpG, demonstrated delayed tumor growth compared to each of the control groups. Furthermore, MO5 tumor growth for these groups was not significantly different from the \(\alpha_2M^*\)-OVA treatment groups. Survival of mice treated with co-delivered \(\alpha_2M^*\) and unbound OVA, either with or without CpG, was significantly prolonged \((p<0.005)\) compared to OVA treatment alone (Figure 27). However, survival
of these mice did not differ significantly from that of mice treated with conjugated α₂M*-OVA, with or without the addition of CpG.

Figure 24: Co-delivery of α₂M* with unbound Ag induces humoral immunity

End-point titer for each immunized mouse was determined by ELISA and averaged by group. IgG Ab titer was defined as the highest dilution of sera that produced an A450 of 0.125. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. The presence of brackets indicates a difference between the indicated treatment groups (*, p<0.05).
Figure 25: Expansion of Ag-specific CD8⁺ T cells following immunization with co-delivered α₂M* and unbound Ag

Tetramer staining of PBLs from each immunized mouse 2 wks following implantation of MO5 tumors, averaged by treatment group. Values indicate mean ± SEM (n=5). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups. *p values (*, p<0.05; **, p<0.005) indicate difference between respective treatment group and each of the three control groups (PBS, CpG, α₂M*).
Figure 26: Growth of Ag-presenting tumor inhibited by immunization with co-delivery of $\alpha_2M^*$ and unbound Ag

Tumor growth over days following implantation observed in each immunized mouse, averaged by treatment group. Mice were sacrificed at a tumor volume of $\sim2\text{cm}^3$. Values indicate mean $\pm$ SEM (n=5 per group). ANOVA was performed followed by multiple pairwise comparisons (Tukey) to determine significant differences between groups as discussed in the text.
Figure 27: Survival data of mice immunized with co-delivered $\alpha_2M^*$ and unbound OVA presented as Kaplan-Meier plot

Since survival of control groups (PBS, CpG, and $\alpha_2M^*$-treated mice) was not significantly different from that of the OVA treatment group, survival data for these groups is omitted from this plot for simplicity. Statistical significance between survival curves was determined by Mantel-Cox log-rank analysis and is discussed in the text.
In summary, these preliminary findings suggest that co-delivery of \( \alpha_2 \text{M}^* \) with unbound Ag can enhance humoral and cell-mediated immunity, resulting in improved anti-tumor responses, to similar degree as \( \alpha_2 \text{M}^* \)-Ag complexes prepared \textit{in vitro}. We propose that the enhanced immune responses reported here with \( \alpha_2 \text{M}^* \) and OVA co-delivery are the result of \textit{in vivo} encapsulation of Ag. The conditions of Ag delivery, including the depot effect caused by intradermal injection and the \(~37^\circ\)C environment of the mouse, are similar to the conditions used to successfully incorporate Ag into \( \alpha_2 \text{M}^* \) \textit{in vitro} \((67)\). However, further studies need to be conducted to verify that the enhanced response observed is secondary to \( \alpha_2 \text{M}^* \)-encapsulation and not ligation of the \( \alpha_2 \text{M}^* \) receptor, LRP-1/CD91. If subsequent studies indeed confirm that \( \alpha_2 \text{M}^* \) co-delivery with unbound OVA can enhance humoral and CTL immunity, this finding could be a significant advancement in the use of \( \alpha_2 \text{M}^* \) as an Ag delivery vehicle.

\textbf{6.2 Mechanism of anti-tumor response following immunization with } \( \alpha_2 \text{M}^* \)-encapsulated Ag

The anti-tumor response, demonstrated by delayed tumor growth and prolonged animal survival, induced by \( \alpha_2 \text{M}^* \)-Ag immunization and discussed in Chapter 5 was presumed to be primarily the result of increased CTL activity. However, the increased production of anti-OVA Ab observed in 4.2.2 raises the possibility that Ab-dependent cell-mediated cytotoxicity (ADCC) may also contribute to this anti-tumor response. Therefore, in order to fully understand the enhanced anti-tumor response elicited by \( \alpha_2 \text{M}^* \)-mediated Ag delivery, we need to explore the possible contribution of ADCC to this response.

In a preliminary effort to determine if the Abs produced by \( \alpha_2 \text{M}^* \)-OVA are capable of binding MO5 tumor cells and initiating ADCC, sera collected from the \( \alpha_2 \text{M}^* \)-
OVA-immunized mouse with the strongest Ab response (titer ~ 1:120,000) was incubated with both B16 and OVA-expressing MO5 tumor cells. A FITC-conjugated ant-mouse secondary Ab was used to assess binding by flow cytometry (Figure 28). Although Abs from the α2M*-OVA-immunized mouse sera appeared to bind MO5 cells to a somewhat greater extent than non-immune control sera, similar binding was also observed against B16 melanoma cells that do not express OVA. These results suggest that the anti-OVA Ab detected by ELISA may not be capable of binding OVA-expressing MO5 tumor cells and, therefore, likely do not contribute to ADCC. Additional sera samples from α2M*-OVA-immunized mice should be analyzed, however, to confirm this finding. Furthermore, if subsequent sera samples demonstrate significant binding to MO5 cells, cell-mediated cytotoxicity assays should be performed to verify that the binding of these Abs can indeed initiate ADCC.

Since Ab and tetramer data confirmed that both humoral and CTL immunity are enhanced by α2M*-mediated Ag delivery, the relative contribution of these responses to tumor inhibition is somewhat unclear. These preliminary results suggest that ADCC may play a minimal role in tumor inhibition compared to CTL activity but further study is warranted to verify this conclusion.
Figure 28: Anti-OVA Ab produced by $\alpha_2M^*$-OVA-immunized mice does not appear to bind Ag-presenting tumor cells

Sera from the $\alpha_2M^*$-OVA-immunized mouse demonstrating the highest wk 14 Ab titer was incubated at a 1:200 dilution with both B16 and OVA-presenting MO5 tumor cells. Binding was detected by flow cytometry. No statistical difference was found between Ab binding to MO5 and B16 tumor cells.
6.3 Summary

The studies reported in this dissertation establish the ability of α₂M*-mediated Ag delivery to enhance specific CTL responses both in vitro and in vivo. This enhancement in CTL immunity was found to contribute to anti-tumor activity, as demonstrated by delayed tumor growth and prolonged animal survival. Also, α₂M*-encapsulation elicited CTL responses as great or greater than those elicited with a well-studied vaccine adjuvant (CpG 1826) or with cell-based vaccine methods. Furthermore, uptake of α₂M*-Ag complexes by the α₂M* receptor, LRP-1/CD91, appears to promote efficient cross-presentation by mouse DCs, and macrophages to a lesser extent, and priming of Ag-specific CD8+ T cells. These findings support the future investigation of α₂M* as an Ag delivery vehicle that may have substantial utility for the development of effective subunit vaccines.

Currently, there are 31 vaccines licensed in the United States for the prevention of infectious diseases (160, 161). The development of vaccines for the prevention of acute infectious disease was one of the greatest scientific successes of the 20th century (162). However, progress in the development of vaccines for chronic infectious, such as HIV and tuberculosis, and noninfectious diseases, including cancers and Alzheimer’s disease, has been slow due to the inherent complexity of these disorders (162). Furthermore, numerous barriers still exist to the development and global distribution of effective vaccines.

Many vaccine candidates lack sufficient immunogenicity or fail to promote cross-presentation of Ag, making the development of new vaccine adjuvants and Ag delivery systems critical (6, 30, 163). However, toxicity (8) and manufacturing issues (164) have complicated the production and FDA approval of new vaccine adjuvants (123). As a
well-conserved serum protein, α₂M* does not seem to induce toxic effects in animal studies; α₂M*-conjugation has actually been found to shield cells from the toxicity of bound molecules, such as polymyxin B (165). Furthermore, the finding that α₂M*-encapsulation promotes cross-presentation of Ag and priming of Ag-specific CTLs makes a strong case for α₂M* as an Ag delivery system with great potential for enhancing the immunogenicity of subunit vaccines. Its cost-effectiveness, wide availability, and thermal stability (70), further endorse α₂M* as an Ag delivery vehicle that may be particularly advantageous in developing nations (61, 70, 71).

The introduction of new vaccine technologies, including adjuvants and Ag delivery systems, is critical to the development of effective vaccines against complex pathogens and chronic diseases. However, these technologies only address some of the obstacles preventing vaccines from effectively targeting human disease. The World Health Organization (WHO) estimates that more than 2.5 million children under the age of 5 died in 2002 from diseases for which preventative vaccines are already available (Table 3). Clearly, new public health initiatives, collaboration between governments, advanced technologies, and improved education are all essential in realizing the full potential of vaccines to reduce global illness.
Table 3: Estimated number of deaths in 2002 from vaccine-preventable illnesses among children aged < 5 years

<table>
<thead>
<tr>
<th>WHO region</th>
<th>No. of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>African</td>
<td>1,113,000</td>
</tr>
<tr>
<td>American</td>
<td>44,000</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>353,000</td>
</tr>
<tr>
<td>European</td>
<td>32,000</td>
</tr>
<tr>
<td>South East Asian</td>
<td>757,000</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>251,000</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2,550,000</strong></td>
</tr>
</tbody>
</table>

Adapted from (166).
References


and A. Sher. 1997. Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. *J Immunol* 159:2409-2417.


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Biography

Edith Villette Bowers was born in Charleston, South Carolina on September 15th, 1981 to Karen Aageson Bowers and John Calvert Bowers. She has two older sisters, Cynthia and Patricia. In 1999, she graduated from Stafford Senior High School in Fredericksburg, VA. As an undergraduate at The College of William and Mary in Williamsburg, VA, she worked for four academic years and three summers in the laboratory of Dr. Deborah C. Bebout in the Department of Chemistry. Her research was funded in part by Howard Hughes Medical Institute undergraduate research fellowships. She was named a Beckman Scholar in 2001 and received the Gladys Anderson Emerson Scholarship from Iota Sigma Pi National Honor Society for Woman in Chemistry in 2002. She was inducted into Phi Beta Kappa in the fall of 2002. In 2003, she received her Bachelor of Science degree from The College of William and Mary, graduating summa cum laude with a double major in Chemistry and Physics and receiving Highest Honors in Chemistry for her thesis: “Investigations of Hg(II) and Cd(II) Coordination with Dipodal and Tripodal Ligand Systems.” Under the supervision of Dr. Hans Christian von Bayer, she also completed a senior thesis in Physics entitled “Constructing a Comprehensive Bibliography of Physics Popularizations.”

In 2003, she entered the Medical Scientist Training Program at Duke University in Durham, NC. In 2005, after completing the first two years of medical school, she began graduate studies in the Department of Pathology under the mentorship of Dr. Salvatore V. Pizzo. In 2007, she married her college sweetheart, Robert William Owen. Her doctoral thesis is entitled “Receptor-Mediated Antigen Delivery by α2-Macroglobulin: Effect on Cytotoxic T Lymphocyte Immunity and Implications for Vaccine Development.” Upon completion of her graduate training, she plans to work as
a postdoctoral fellow in the laboratory of Dr. Pizzo until May 2010 when she will return to her final year of medical school. She plans to pursue a career in academic medicine.

Publications


Abstracts and Presentations

1. **Bowers, E.V.,** Horvath, J.J., Bond, J.E., Cianciolo, G.J., and S.V. Pizzo. Toward a Better Vaccine: antigen delivery by \( \alpha_2 \)-macroglobulin enhances cell-mediated
immunity. Oral presentation at the 17th Annual Biological Sciences Graduate Student Symposium, Duke University, Durham, NC, November 13-14, 2008.


