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Apolipoprotein B Binding Domains: Evidence That They Are Cell-Penetrating Peptides That Efficiently Deliver Antigenic Peptide for Cross-Presentation of Cytotoxic T Cells

Norihisa Sakamoto and Amy S. Rosenberg

Low-density lipoproteins (LDLs) are a good source of cholesterol, which is important in cellular homeostasis and production of steroids. Apolipoprotein B-100 (ApoB-100), the sole protein component of LDL, is known to bind to cell surface LDL receptor (LDLR) or cell surface-bound proteoglycans and to be internalized into cells. We found that APCs, consisting of macrophages and dendritic cells, upregulate LDLR on culture in vitro without obvious stimulation. In contrast, T cell populations only upregulate LDLR on activation. Thus, we strategized that targeting immunogens to ApoB-100 might be a useful means to target Ag to APCs. We generated fusion proteins consisting of receptor binding sites in ApoB-100, coupled to OVA peptide (ApoB-OVA), as Ag delivery vehicles and demonstrated that this novel delivery method successfully cross-presented OVA peptides in eliciting CTL responses. Surprisingly, internalization of ApoB-OVA peptide occurred via cell surface proteoglycans rather than LDLRs, consistent with evidence that structural elements of ApoB-100 indicate it have cell-penetrating peptide properties. Finally, we used this strategy to assess therapeutic vaccination in a tumor setting. OVA-expressing EL-4 tumors grew progressively in mice immunized with ApoB-100 alone but regressed in mice immunized with ApoB-OVA fusion protein, coinciding with development of OVA-specific CTLs. Thus, to our knowledge, this is the first article to describe the cell-penetrating properties of a conserved human origin cell penetrating peptide that may be harnessed as a novel vaccination strategy as well as a therapeutics delivery device. The Journal of Immunology, 2011, 186: 5004–5011.

Materials and Methods

Mice

Female C57BL/6 (B6) wild-type and LDLR knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY). Animal care was in accordance with the guidelines of the National Institutes of Health Animal Research Advisory Committee.

Abs and reagents

Mouse isotype control IgG, (MOPC-21), FITC-conjugated mouse isotype control IgG1 (G155-178), FITC-conjugated hamster isotype control IgG1 (A19-3), PE-conjugated rat anti-mouse IgG1, (A85-1), PE- or FITC-conjugated anti-mouse CD3ε (145-2C11), CD4 (L3T4) or CD8 (53-6.7), H-2Kb (APF6-88.5), CD11b (M1/70), CD69 (H.12F3) Abs, PE- or allophycocyanin-conjugated anti-mouse CD11c Ab (HL3), and biotin-conjugated anti-mouse B220 (RA3-6B2), CD5 (53-7.3), CD19 (1D3), CD11b (M1/70), CD11c (HL3), DX5, Gr-1 (RB6-8Cs), TER-119 (Ly-76) Abs, PE- or FITC-conjugated streptavidin were purchased from BD Pharmingen (San Diego, CA). Goat anti-mouse LDLR Ab was purchased from R&D Systems (Minneapolis, MN). Control goat IgG fraction was affinity purified by protein G from goat serum (Sigma-Aldrich, St. Louis, MO). The levels for the complex of H-2Kb and OVA257–264 peptide (H-2Kb-SIINFEKEL) were determined with unconjugated 25D1.16 mAb with secondary Ab as previously described (11), a gift from Dr. J.W. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). PE-conjugated MHC class I pentamer reagents (H-2Kb/SIINFEKEL) were purchased from ProImmune (Bradenton, FL). LPS and heparin were purchased from Sigma-Aldrich. FITC-conjugated 3E8.1 mAb, specific for bovine ApoB-100 and generated in our laboratory, was described previously (12). The following synthetic fusion peptides, ApoB (SVKAPYKKNSDKH-GGG-RLMRKRLGLK) alone, ApoB-SIINFEKEL (OVA257–264), ApoB-OVA257–339 (ISQA VHAHAEINEAGR-), ApoB-Mut1 (FEQNTAQP-) and their biotin conjugates were synthesized and purified by HPLC at the Center for Biologics Evaluation and Research core facility (Rockville, MD). CpG-ODN 1826 (5’-TCC ATG ACG TTC CTC ACG TT-3’) was also synthesized at the Center for Biologics Evaluation and Research core facility. All synthetic peptides and CpG-ODN1826 had <0.1 endotoxin unit of endotoxin per milligram as assessed by a Limulus amebocyte lysate assay (QCL-1000;Lonza, Walkersville, MD).

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Cell lines and culture
EL4 and E.G7-OVA (EL4 cells transfected with OVA cDNA) were purchased from American Type Culture Collection (Manassas, VA). RMA and RMA-S (peptide transporter deficient derivative from RMA) were a gift from Dr. J.W. Yewdall and described previously (11). Cells were cultured with RPMI 1640 medium containing 10% FCS (Hyclone), MEM non-essential amino acids (100 µM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-ME (5µM), and sodium pyruvate (1 mM).

Isolation of lymphocyte subpopulations
Single-cell suspensions of splenocytes were prepared and negatively selected using MACS cell separation columns (Miltenyi Biotec, Auburn, CA) with biotin-conjugated anti-mouse B220, CD19, CD11b, CD11c, and DX5 Abs. A total of 2.5 × 10^6 negatively selected T cell populations were seeded onto the anti-CD3ε Ab (145-2C11)-coated 60-mm dish in medium containing 40 U/ml mouse IL-2 (eBioscience, La Jolla, CA) and cultured for 3 d. For generation of bone marrow-derived DCs (BMDCs), bone marrow progenitors were isolated and cultured as previously described (13) with some modifications. Briefly, after isolation of bone marrow, cells were negatively selected using MACS cell separation column (Miltenyi Biotec) with biotin-conjugated anti-mouse CDS, B220, CD11b, Gr-1, and TER-119 Abs. Negatively selected cells were cultured in 6-well culture plates at 5 × 10^5 cells/ml in medium supplemented with 10 ng/ml recombinant mouse GM-CSF (eBioscience) for 5 d. Cells were cultured with indicated peptides with or without stimulation by LPS (1 µg/ml) or CpG-ODN1826 (2 µM).

Flow cytometry analysis
Cells were washed three times and stained with the indicated Abs in staining buffer on ice and then washed with 2% FCS containing PBS and resuspended in PBS containing 10 µg/ml propidium iodide. Cells were immediately analyzed on a FACS Calibur (BD Biosciences, Mountain View, CA). Data were analyzed using the CellQuest software program (BD Biosciences).

Heparitinase treatment and ELISA
Ninety-six-well ELISA plates (Nunc, Roskilde, Denmark) were coated with hepatic sulfate proteoglycan (HSPG) (Sigma-Aldrich) at 10 µg/ml and incubated at 4˚C for overnight. After washing with PBS, plates were blocked with 0.3% BSA (Roche Diagnostics, Indianapolis, IN) in PBS. For biotinylated ApoB-SIINFEKL, a blocking procedure was not performed because of nonspecific binding to BSA. Heparitinase from Flavobacterium heparinum (NorthStar BioProducts, East Falmouth, MA) was added (5 µg/ml), and the digests were conducted in 100 mM sodium acetate and 10 mM calcium acetate (pH 7.5) at 37˚C for 5 h before addition of biotinylated ApoB-SIINFEKL (50 µM), anti-HSPG mAb (10E4; 0.2 µg/ml), and anti-Δ HSPGs (3G10; 1 µg/ml). 10E4 is specific for an intact hepatic sulfate epitope, and 3G10 reacts with a heparan sulfate neo-epitope that is generated by digesting heparian sulfate with heparitinase. Both Abs were purchased from NorthStar BioProducts. The plates were washed with ELISA washing buffer (R&D Systems) and then probed with HRP-conjugated anti-mouse IgG and IgM Ab (Abcam, Cambridge, MA) or HRP-conjugated streptavidin (BD Pharmingen). The plates were developed using ELISA development system (R&D Systems) and read at 450 nm with SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

Vacccination protocol and tumor inoculation
B6 mice were vaccinated s.c. twice at weekly intervals with the mixture of 100 µg ApoB-SIINFEKL and 100 µg E.G7-OVA (322,130) or 200 µg ApoB alone. Fifty micromicros of CpG-ODN1826 was added in both peptide mixtures as an adjuvant. For tumor inoculation studies, 5 × 10^5 E.G7-OVA cells were inoculated at flanks intradermally on day 0. B6 mice were vaccinated at necks s.c. on days 0 and 7. Tumor size was measured every 2–3 d with the calculation of longest tumor diameter multiplied by shortest tumor diameter.

[51Cr] release assay
Three to 4 wk after the second immunization, spleens were harvested from immunized mice. Single-cell suspensions of splenocytes were prepared and restimulated with irradiated E.G7-OVA (25,000 rad) at the E:T ratio of 1:50 for 4d and then incubated with E.G7-OVA or EL4 target cells (1 × 10^6/cells well) labeled with 100 µCi (1 µCi = 37 kBq) sodium [51Cr]chromate. Target cells were then incubated with effector cells at the indicated E:T ratio. Supernatants were harvested after 4 h, and the radioactivity was measured in a gamma counter WIZARD (Wallac, Turku, Finland). The mean of triplicate samples was calculated, and the percentage of specific lysis equals 100 × (experimental [51Cr] release – spontaneous [51Cr] release)/(maximum [51Cr] release – spontaneous [51Cr] release). The maximum release referred to counts from targets in 2% Nonidet P-40.

Real-time quantitative RT-PCR
Total RNA was isolated with RNAasy Mini Kit (Qagen, Valencia, CA) from harvested tumors and cDNA prepared with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative RT-PCRs were performed according to standard protocols using the TaqMan Gene Expression Master Mix (Applied Biosystems) and a 7900HHT Fast Real-Time PCR System (Applied Biosystmes). The primers/probe set specific for OVA is predesigned TaqMan Gene Expression Assays (Applied Biosystems). Amplification of target genes was normalized by using amplification levels of GAPDH as an endogenous control (mouse GAPDH Endogenous Control VIC/MGB probe; Applied Biosystems). Data analysis and calculation were performed using the 2^(-ΔΔCT) comparative method. The expression level of E.G7-OVA was set as a calibrator (value = 1), and gene expression in all other tumors was calculated as relative-fold expression over the calibrator.

Statistical analysis
Each experiment was repeated at least twice. Data are shown as mean ± SD. The statistical significance of differences was analyzed by using Student t test or two-way ANOVA.

Results
LDLR and ApoB binding increase substantially on resting macrophages and DCs in vitro
LDLR expression and LDL uptake into human lymphocyte subsets have consistently been shown to be enhanced in Ag- or mitogen-stimulated cells (14–17). In our previous studies, we confirmed the generality of this phenomenon by using the bovine ApoB-100 Ab, 3E8.1 (2, 12), to show the marked increase in bovine LDL binding to LDLRs to support rapid proliferation of activated lymphocytes in FCS-containing medium. We confirmed these results in mouse T and B cell subsets (Supplemental Fig. IA–C) and extended these studies by examining the expression of LDLR and uptake of bovine ApoB-100 in APC populations. We isolated and cultured CD11b+ macrophages and CD11c+ DCs with or without LPS stimulation. Macrophages, but more impressively, DCs, exhibited dramatically increased levels of cell surface-bound ApoB-100 and LDLRs on in vitro culture alone (Fig. 1A, 1B). Surprisingly, the level of cell surface-bound ApoB-100 and LDLR expression in DCs cultured without mitogens was only minimally enhanced by stimulation with LPS, indicating that inherent culture conditions have factors or present conditions that readily increase nutritional requirements, perhaps in response to adherence, maturation, and/or cytokines or factors present (18, 19).

A fusion protein consisting of the binding domains of ApoB-100 and an antigenic peptide effectively delivers antigenic peptide for cross-presentation
Because LDLR internalizes exogenous lipid Ags via apolipoprotein binding and deliver them into nonclassical CD1d molecules, to which NKT cells respond (20–22), we wondered whether ApoB internalization could deliver antigenic peptides for crosspresentation. We used an ApoB fragment containing elements known to bind to both the LDLR binding site (residues 3381–3389 [site B]) and to the proteoglycan binding site (residues 3166–3178 [site A]) (23) and conjugated antigenic peptides to this construct at their N-termini (termed ApoB-peptide) (Fig. 2A). The peptides were synthesized and purified by HPLC to ensure the integrity of the fusion protein, thus precluding the direct loading of antigenic peptide fragments into MHC class I molecules. We first evaluated...
the binding of the fusion peptide ApoB-OVA_{257-264} (ApoB-
SIINFEKL) to TAP replete RMA cells (murine T cell lym-
phoma line, H-2b). ApoB and ApoB-fusion peptides bound to
cells at high levels (Fig. 2B, surface binding). We next inves-
tigated whether ApoB-SIINFEKL was processed and presented on
the cell surface, as detected by the mAb 25D1.16 that spe-
cifically recognizes the OVA_{257-264} peptide (SIINFEKL) bound to the
MHC class I (H-2Kb) molecule (11), designated H-2Kb-
SIINFEKL. Mut1 (FEQNTAQF), consisting of aa 52–59 of mu-
tated connexin 37 protein (24), was used as an irrelevant pepti-
de that binds to MHC class I (H-2Kb) but is not detected by 25D1.16.
(Fig. 2B, presentation). Indeed, substantial amounts of H-2Kb-
SIINFEKL were specifically detected on the surface of RMA
cells cultured with ApoB-SIINFEKL (Fig. 2B, presentation). The
requirement for processing of ApoB-SIINFEKL and transport to
MHC class I by TAP was then investigated by evaluating ex-
pression of H-2Kb-SIINFEKL in RMA-S cells, which are Tap2
deficient and lack the ability to load most endogenous pep-
tides onto their MHC class I, resulting in very diminished cell surface
levels of MHC class I compared with the Tapa{sup2} parental RMA cell
line (25). Indeed, in the absence of treatment, we observed ~0.5%
of MHC class I H-2Kb expression in RMA-S cells compared with
the parental RMA cells in which H-2Kb is abundantly expressed
(Supplemental Fig. 2). The expression of H-2Kb-SIINFEKL was
increased in a dose-dependent manner in both RMA and RMA-S
cells cultured with ApoB-SIINFEKL, although the level was much
less on the TAP-deficient cells (Fig. 2C). This finding indicates
that cross-presentation of Ag delivered by ApoB-fusion peptide is
largely TAP dependent (Fig. 2C). Taken together, these results
indicate that the ApoB-binding domain functions as an Ag de-
livery vehicle that efficiently binds to the cell surface, is inter-
alized, and then is processed in a TAP-dependent and, to a much
lesser degree, in a TAP-independent manner to present antigenic
peptide on MHC class I molecules.
To definitively assess the involvement of LDLRs in binding, internalization, and Ag processing/presentation by ApoB-mediated entry, we compared the expression of H-2Kb-SIINFEKL in DCs and in stimulated CD4+ and CD8+ T cells from LDLR knockout mice and their control LDLR-replete counterparts, because stimulated T cells express LDLR on their cell surface in LDLR-replete mice (Supplemental Fig. 1A, 1B). Surprisingly, the expression of H-2Kb-SIINFEKL on the cell surface did not differ in LDLR knockout mice, compared with LDLR-replete mice (Fig. 4A), indicating that the LDLR is not the predominant internalization pathway.

Given that both sites A and B in ApoB are required to bind to cell surface-expressed HSPGs, which are strongly negatively charged molecules, we used heparin (a strongly negatively charged polysaccharide) in an attempt to block ApoB-SIINFEKL from binding to the cell surface. The binding of ApoB-SIINFEKL was significantly blocked, and the expression of H-2Kb-SIINFEKL was reduced in the presence of 100 U/ml heparin (Fig. 4B, 4C).

Furthermore, heparitinase treatment greatly decreased binding of ApoB-SIINFEKL to HSPGs, via removal of the heparitinase-sensitive epitope on HSPGs to which ApoB-fusion peptides bind (Fig. 4D, left panel), as verified by the decreased binding of anti-HSPGs mAb (10E4) (Fig. 4D, middle panel) and increased binding of anti-Δ-HSPGs mAb (3G10) (Fig. 4D, right panel) (31). These results indicate that HSPGs play the major role in ApoB-fusion peptide binding, internalization and Ag presentation.

ApoB-OVA fusion peptides elicit cytotoxic T cells and suppress tumor growth in vivo

Because Ag-fused ApoB peptides are efficiently cross-presented, we next asked whether they elicited CTLs in vivo. First, to ensure that T cell help would not be limiting, we immunized mice with ApoB-SIINFEKL together with ApoB fused to an MHC class II 1Ab-specific OVA peptide (ApoB-OVA323–339) and adjuvanated it with CpG-ODN. Substantial specific kill of OVA-expressing tumor cells was detected in immunized mice (Fig. 5A). We then evaluated the clonal expansion of CD8+ T cells recognizing H-2Kb-SIINFEKL. Following the second vaccine dose, 1–4% of CD8+ T cells in peripheral blood specifically recognized H-2Kb-SIINFEKL, and this population was detectable in peripheral blood for at least 2 wk (Fig. 5B). Control mice immunized without CpG-ODN adjuvant failed to efficiently generate Ag-specific CD8+ T cells (data not shown). Thus, the presence of adjuvant is a requirement for efficient induction of Ag-specific CD8+ T cells. Next, we assessed whether such CTLs were functional in vivo in a tumor model. OVA-expressing E.G7 tumor cells were injected into B6 mice intradermally, and on days 0 and 7, the mice were immunized with ApoB-SIINFEKL and ApoB-OVA323–339 with CpG-ODN adjuvant. We observed tumor shrinkage after the second injection (Fig. 5C), coinciding with detection of CD8+ T cells specific for H-2Kb-SIINFEKL (Fig. 5B). Taken together, these results demonstrate that the ApoB-fusion peptide successfully delivered Ag to DCs and that Ag was cross-presented to elicit Ag-specific CTLs that diminished tumor growth.

Interestingly, although all tumors shrank dramatically following the second vaccine dose, after 3–4 wk, we observed regrowth of some tumors. To address the mechanism by which these tumors regrew, we asked whether they still expressed the target OVA Ag by quantitating OVA gene expression with real time quantitative RT-PCR (Fig. 6). Some tumors that regrew had a higher level of OVA message expression than ex
FIGURE 4. ApoB-fusion peptides bind to and are internalized predominantly through proteoglycans. A, CD11c+ DCs or in vitro-stimulated T cells from LDLR knockout mice (bold solid line) or B6 (thin solid line) were cultured with ApoB-SIINFEKL (25 μg/ml), and then, the expression of H-2Kb-SIINFEKL was assessed by 25D1.16 mAb. H-2Kb panels (at right) show expression levels of mouse MHC class I H-2Kb detected by FITC-anti-mouse H-2Kb Ab. Isotype-matched Ab staining profiles for LDLR knockout cells (dotted line) or B6 cells (dashed line) as shown. PE-anti-mouse IgG 1 was used as secondary Ab for 25D1.16. FITC-streptavidin was used as control for LDLRs. Experiments were performed at least twice with similar results. B, Upper histograms, ApoB-SIINFEKL binding to RMA cells (thin solid line) is blocked by heparin (thick solid line), whereas the binding of H-2Kb–specific Ab is not. PE-streptavidin only or FITC-isotype-matched Ab were used as negative controls (dotted line). Lower bar graphs, The mean fluorescent intensity (MFI) of upper histograms were shown with (+) or without (−) heparin blockade with subtraction of control MFI. ApoB-SIINFEKL was used at the concentration of 25 μg/ml. Data are representative of three independent experiments. C, H-2Kb-SIINFEKL expression is diminished by heparin. RMA cells were cultured with ApoB-SIINFEKL (25 μg/ml) with (thick solid line) or without (thin solid line) heparin, and the expression of H-2Kb-SIINFEKL was assessed by 25D1.16. Lower bar graphs shows MFI with (+) or without (−) heparin blockade with subtraction of control MFI. Data are representative of three independent experiments. D, Abrogation of the ApoB-SIINFEKL binding after heparitinase treatment of HSPGs as detected by ELISA. Left panel, ApoB-SIINFEKL binding treated with (+) or without (−) heparitinase. Middle panel, Anti-HSPGs mAb (10E4) was used to detect intact heparan sulfate. Right panel, Anti–Δ-HSPGs mAb (3G10), specific for a neo-epitope generated by heparitinase digestion of HSPGs, was used to assess extent of removal of HSPGs. Data are representative of four independent experiments. Data are shown as mean ± SD. Statistical differences were assessed with Student t test. **p < 0.001.
vivo-maintained tumor cells. This finding suggests a different mechanism of escape, perhaps involving secretion of soluble OVA decoy molecules and/or development of a regulatory T cell population that might induce tolerance for their survival. Questions remain about the persistence of such Ag-specific CD8+ cytotoxic T cells and how the tumor microenvironment may thwart such killers. Those intriguing questions deserve further study.

Discussion

We developed a vaccine strategy using the well known ability of ApoB-100 to bind to cell surface LDLR, become efficiently internalized into cells, and to deliver lipid Ag to nonclassical MHC class I molecules. Indeed, we found that this strategy led to efficient cross-priming of CD8+ CTLs, via peptide presentation by classical MHC class I molecules, which diminished tumor growth. Unexpected, however, was the finding that uptake of the ApoB-fusion peptide was mediated via cell surface proteoglycans and not through LDLR. The interaction of ApoB-100 with proteoglycans is mediated by sites A and B (5, 24, 33) in a cooperative fashion. Because these binding regions are the two most basic regions of ApoB-100, and are highly enriched in arginine and lysine (SVKAQYKKNSDKH-GGG-RLMRKRGLK), we postulated that our ApoB-fusion peptide functions as a cell-penetrating peptide (CPP). CPPs, of great interest for therapeutic cargo delivery because of their unique property of rapid translocation across the cell membrane (34–36), are cationic, highly enriched in the basic amino acids, lysine (K) and/or arginine (R), and their cell surface binding is blocked by heparin, which is structurally similar to HSPGs, the target binding molecules of CPPs (35, 37–39). Indeed,
our ApoB-fusion peptides possess all of these characteristics, and heparin has been used previously to examine interactions between HSPGs and ApoB-100 (4, 40, 41).

The interaction between polycationic CPPs and negatively charged HSPGs of the plasma membrane has been proposed to be a common internalization mechanism (35, 37, 38) and has been demonstrated for VAT of HIV and penetratin (Antp) of Drosophila antennamedia, both of which are inhibited from internalization by heparin (42–44). Although heparin significantly blocked the cell surface binding and internalization of our ApoB-fusion peptide, the persistence of its binding and internalization in the presence of heparin indicates that other endocytic pathways are likely involved such as macropinocytosis, clathrin-mediated endocytosis, and caveolealidipid raft-mediated endocytosis (35). That the ApoB binding domains were the critical mediators of binding and internalization and not the OVA peptides is indicated by the failure to observe direct binding of SIINFEKL to the cell surface by FACS (data not shown), and because the calculated isoelectric point of OVA peptides is far lower than that of typical CPPs: 6.0–6.9 compared with 11.6 for ApoB fusion protein, 12.7 for VAT, and 12.7 for Antp (45). Also consistent with the CPP activity of our ApoB-fusion peptide is a recent study in which a lentivirus vector containing both heparin and LDLR-binding domains of human ApoB, fused to the lysosomal enzyme glucocerebrosidase, successfully crossed the blood-brain barrier and delivered glucocerebrosidase to CNS neurons and astrocytes (46). Hence, the CPE property of ApoB likely contributed to penetration of the blood-brain barrier. ApoB has one distinct advantage over other identified CPPs in being an endogenous protein and therefore is unlikely to elicit a robust immune response to ApoB-binding domains, which would limit its use as a delivery device.

With regards to Ag processing and presentation, our data indicate that ApoB-fusion peptide was transported efficiently into the MHC class I presentation pathway via the TAP transporter and, to a much lesser degree, independent of the TAP transporter. Although expressed at a low level, the TAP-independent MHC class I presentation suggests degradation of ApoB-fusion peptide by endosomal proteases and loading of the peptides so produced onto recycled MHC class I molecules in the acidic environment of the endosome (47, 48).

Immunization with ApoB-fusion peptides of OVA class I and class II Ags in conjunction with CpG-ODN successfully elicited cytotoxic T cells to OVA Ag, which markedly diminished tumor growth. The effectiveness of this regimen may pertain to the potency of the ApoB “vector” in cross presentation. Indeed, despite the conventional wisdom that CD8α+ DCs more efficiently cross-present than CD8α− DCs (26–28), a similar level of OVA Ag was expressed on CD8α+ and CD8α− DCs when delivered by the ApoB CPP (Fig. 3b), indicating that ApoB-fusion peptides may have unique properties in being equivalently internalized, processed, and cross-presented in both CD8α+ and CD8α− DCs. However, whether such equivalency in Ag expression levels is tantamount to a similar potency in induction of immune responses is not clear. Thus, this ApoB-fusion peptide has potential as a vehicle to deliver Ags in vivo as a vaccine or to deliver therapeutics more efficiently into cells.

We observed successful CTL induction and tumor shrinkage after immunization by ApoB-fusion peptides. However, even though we observed tumor shrinkage, eventually tumor regrowth occurred, with such tumors either having lost OVA expression or expressing much higher levels than the parent tumor (Fig. 6). A previous study reported that tumor escape variants with Ag loss resulted from Ag silencing via an epigenetic mechanism (32). As well, others have reported that higher levels of expressed Ag may be associated with induction of peripheral tolerance with deletion of activated T cells (49). Interestingly, another mechanism of tumor escape with overexpressed Ag was suggested by showing that myeloid-derived suppressor cells induced regulatory T cell expansion through the presentation of tumor-associated Ags (50). These phenomena of Ag loss and overexpression provide support for the immune editing theory, in which immunological forces in the tumor environment continuously shape the immunologic phenotype of the tumor (33, 51, 52).

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Bovine LDL binding and LDLR expression are increased on proliferating T and B lymphocytes.

Mouse spleen cells cultured (A) without or (B) with anti-CD3 antibody and IL-2 were evaluated for binding of bovine LDL from the serum in culture (3E8.1 mAb), LDLR, and activation (by CD69 staining). (C) Mouse spleen cells were evaluated for binding of bovine LDL (3E8.1 mAb) and LDLR expression prior to culture (before culture) and after culture with or without LPS. (A-C) Cells were stained with FITC-3E8.1, FITC-anti-mouse CD69, or biotin-anti-mLDLR and FITC-streptavidin, together with PE-anti-mouse CD4, CD8, or B220 antibodies. Isotype matched control antibodies for 3E8.1 and CD69 staining, and FITC-streptavidin for LDLR staining, were used as negative controls (dotted line). CD4\(^+\), CD8\(^+\), or B220\(^+\) cell populations were gated and analyzed for the binding of bovine ApoB-100 and the expression of LDLR or CD69. These experiments were repeated from four to six times with similar results.

Supplemental Figure 2. RMA cells sufficiently express MHC class I H-2Kb but not on RMA-S cells.

RMA cells or RMA-S cells were stained with FTIC-anti-mouse H-2Kb antibody (thick solid line). Isotype matched antibody was used as negative controls (dotted line). Numbers in histograms show mean fluorescent intensity (MFI) with subtraction of control IgG MFI. Data are representative of two independent experiments.
Supplemental Figure 1.

A. In vitro culture alone

B. Anti-CD3 treatment

C. Splenocyte B220+ gate

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3E8.1 LDLR CD69

CD4+ gate

CD8+ gate

Before culture | No treatment | LPS

3E8.1 LDLR

3E8.1 LDLR

LDLR

LDLR
Supplemental Figure 2.

RMA RMA-S

FL1 - H

136.1 0.66

control IgG

anti-H-2Kb mAb