The Capacity To Induce Cross-Presentation Dictates the Success of a TLR7 Agonist-Conjugate Vaccine for Eliciting Cellular Immunity

Jason Z. Oh and Ross M. Kedl

*J Immunol* published online 15 September 2010
http://www.jimmunol.org/content/early/2010/09/15/jimmunol.1001892

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/09/13/jimmunol.1001892.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Capacity To Induce Cross-Presentation Dictates the Success of a TLR7 Agonist-Conjugate Vaccine for Eliciting Cellular Immunity

Jason Z. Oh and Ross M. Kedl

Covalent conjugation of TLR agonists to protein Ags often facilitates the generation of a CD8+ T cell response. However, mechanisms underlying the efficacy of the conjugate over its unconjugated counterpart have been largely uninvestigated. In this study, we show that conjugation of a TLR7 agonist enhances CD8+ T cell responses without affecting Ag persistence and with minimal impact on cellular uptake of the Ag in vivo. Instead, the conjugated form induced a robust accumulation of dendritic cells (DCs) in regional lymph nodes. Perhaps more importantly, cross-presentation in DCs was detected only when the Ag was delivered in the conjugated form with the TLR7 agonist. Collectively, these data represent the first demonstration that a TLR agonist–Ag conjugate elicits CD8+ T cell responses based not on its capacity to induce DC maturation or Ag persistence and uptake, but on the engagement of DC cross-presentation pathways. The Journal of Immunology, 2010, 185: 000–000.

The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; dLN, draining lymph node; Lm-OVA, Listeria monocytogenes expressing OVA; LN, lymph node; TLR7a, TLR7 agonist.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

Published September 15, 2010, doi:10.4049/jimmunol.1001892

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001892

The Journal of Immunology

The Capacity To Induce Cross-Presentation Dictates the Success of a TLR7 Agonist-Conjugate Vaccine for Eliciting Cellular Immunity

Toll-like receptor 7 is an intracellular receptor that recognizes ssRNA molecules and enables the host to sense RNA viruses such as the Influenza virus (1). However, synthetic compounds agonistic for TLR7 had already been discovered prior to the identification of RNA as the natural ligand (2). Collectively known as imidazoquinolines, these compounds structurally resemble RNA molecules, and similarly to the physiologic ligand, they induce innate immune activation via TLR7 (2–5). Imidazoquinolines upregulate costimulatory molecules such as CD80, CD86, and MHC class II molecules on APCs and induce the localization of dendritic cells (DCs) to the T cell areas of secondary lymphoid tissues (5). In addition, rapid induction of inflammatory mediators, such as TNF-α, IL-12, type I IFNs, and MIP1α were reported in mice treated with TLR7 agonists (TLR7a) (2–5). Given the presence of activated APCs and inflammation, which are necessary conditions for the priming of T cells, targeting TLRs represents a rational approach to designing vaccines that can effectively induce cross-priming of CD8+ T cells.

However, despite their ability to activate the innate immune system, TLR agonists, in general, were demonstrated to be relatively poor inducers of cellular immunity (6). Immunizations using protein Ags mixed with agonists targeting TLRs ranging from TLR2 to TLR9 failed to induce detectable CD8+ T cell responses (7), suggesting that simply the induction of APC activation and inflammatory cytokines is not sufficient to bridge the innate and adaptive immune systems. We previously explored the idea of covalently linking a TLR7a to Ag as a means to mimic the natural recognition of pathogens (6). Using a TLR7a covalently conjugated to the HIV-1 gag protein, we and our collaborators demonstrated that CD4+ and CD8+ T cell responses can indeed be generated in both mouse and nonhuman primates (6, 8). Other studies have shown a similar enhancement of the adaptive response when using Ags conjugated to ligands targeting TLR2, TLR4, or TLR9 (9–16). Thus, the use of chemical conjugation in a vaccine formulation appears to generally enhance the generation of cellular immune responses.

However, mechanisms underlying the efficacy of conjugation remain poorly defined. Several possibilities exist by which conjugation increases the immunogenicity of the Ag and TLR7a. Limited comparisons between free TLR2 and TLR9 agonist vaccinations versus their respective conjugates suggested that mechanisms underly ing the efficacy of the conjugate may be related to increased DC targeting and/or increased Ag uptake (12, 14). Whereas this may be a general mechanism by which all TLR agonist–Ag conjugates can elicit more potent cellular immunity, it is difficult to imagine how agonists targeting TLRs expressed in intracellular compartments may mediate this process. Indeed, a TLR9 agonist–Ag conjugate was reported to be taken up by TLR9-deficient APCs with similar efficiencies as TLR9-sufficient APCs (17), indicating that any increased efficacy of Ag uptake is not related to the presence of the targeted TLR. Another possibility is that the conjugation of Ag and adjuvant insures their co-delivery into a common endosomal compartment, facilitating the efficiency with which the Ag is presented to responding T cells (16, 18). Whereas these and other factors may be necessary, it remains unclear which of these factors truly dictates the success or failure of the vaccination. Comparing these various parameters between conjugated and free TLR agonist-based immunizations provides the opportunity to conclusively determine which of these mechanisms is responsible for facilitating vaccine-induced cellular immunity.

In this study, we examined the possibility that conjugation enhances the stability of the Ag, resulting in prolonged persistence of the Ag. In addition, we also considered that conjugation could result in aggregation of the Ag and hypothesized that it would conse-
quenty affect the efficiency of Ag uptake. Using a TLR7a conjugate to fluorescently labeled OVA protein, we found that conjugation did not elongate the $t_{1/2}$ of the Ag, and had a limited impact on efficiency of Ag uptake. Instead, conjugation resulted in a dramatic increase in the frequency of DCs accumulating in locally draining lymph nodes (LN). Perhaps more importantly, cross-presentation was detected in DCs only when the Ag was delivered as a conjugate to the TLR7a. These results not only clarify the mechanism of action of this particular adjuvant, but more broadly suggest that the timely recruitment of DCs and facilitation of cross-presentation may be the key determinants by which any vaccine adjuvant successfully induces cellular immunity.

Materials and Methods

Mice and immunizations

C57BL/6 and OTI mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at National Jewish Health. Mice were immunized s.c. in the lower/upper flanks or in the footpad with whole OVA protein (OVA; 5–500 μg), or with the TLR7a, 3M012, either in the unconjugated form or as a covalently linked conjugate. OVA was purchased from Sigma-Aldrich (St. Louis, MO), and any contaminating endotoxins were eliminated using Triton X-114 and SM-2 Bio-Beads (Bio-Rad, Hercules, CA), as previously described (19). The TLR7a, 3M012, was obtained through material transfer agreements with 3M Pharmaceuticals (St. Paul, MN). OVA was covalently conjugated to 3M012, as previously described (6, 8). All components injected into mice were tested for LPS contamination using the Limulus amebocyte lysate assay (Lonza, Walkersville, MD) and confirmed to be free of any detectable levels of LPS.

Primary and secondary CD8+ T cell responses

OVA-specific CD8+ T cell responses were measured from PBLs of mice either 7 d (primary) or 5 d (boost) following injections. Boost injections were administered 30–60 d following the primary injection. Cells were then stained with PE-conjugated H-2Kb tetramers loaded with the OV A peptide, SIINFEKL, for 90 min at 37˚C. OVA-specific CD8+ T cells were identified by gating on CD8+ CD4– events that were positive for the activation marker CD44. To monitor cytolysis, PBLs were co-cultured with OV A peptide-pulsed splenic DCs, which were stained with Alexa-488 OV A. The magnitude of uptake on a per cell basis was determined by measuring the mean fluorescence intensity of Alexa-488 in CD11c+ cells.

Protection assay

Mice were immunized with 5 μg OVA–3M012 conjugate in the footpad, as described above. Immunized mice were given the same dose of the conjugate as a boost immunization 30–60 d following the primary injection. Approximately 30–60 d following the boost immunization, mice were challenged i.v. with 2 × 10^7 CFU Listeria monocytogenes expressing OVA (Lm-OVA). Liver and spleen were removed 3 d after challenge, and the harvested tissues were mechanically sheared while resuspended in PBS buffer containing Nonidet P-40 (Sigma-Aldrich). They were then cultured in brain heart infusion plates and incubated overnight at 37˚C. The CFU was calculated the following day. Lm-OVA was provided by M. Bevan (University of Washington, Seattle, WA).

Kinetics of Ag uptake, migration, and activation

Mice were immunized in the footpad with 5 μg Alexa-488–labeled OVA–3M012 conjugate. At the indicated time points following immunization, DCs were isolated from dLNs using collagenase D and DNase, as previously described (5). The capture of Ag by DCs was determined by enzymelinked immunosorbent assay (pDCs) or flow cytometry (CD8+ T cells) (20, 21), our results demonstrate the potency by which conjugation of the TLR7a to protein Ags can establish protective immune responses. We found that the OVA–TLR7a conjugate elicited a substantial OVA-specific CD8+ T cell response (Fig. 1A), similar to previously published results with the HIV-1 gag protein–TLR7a conjugates (6). Moreover, a robust production of IFN-γ by CD8+ T cells was detected upon a boost injection using the same dose as the primary injection. These data suggest the OVA–TLR7a conjugate induces functional CD8+ effector T cells and generates memory CD8+ T cells (Fig. 1B, 1C). In contrast, using a 50-fold higher dose of the unconjugated mixture of the TLR7a and OVA failed to induce any appreciable primary or secondary response (Fig. 1A, 1C), which together highlight the efficiency of conjugation in enhancing cross-priming of CD8+ T cells using minimal amounts of whole protein. Most importantly, mice that received only a single prime and boost injection of the OVA–TLR7a conjugate showed substantial protection against challenge with Lm-OVA. Immunization with the OVA–TLR7a conjugate resulted in ∼1000-fold reduction in the frequency of bacteria in inoculated mice, demonstrating a striking ability of the response to control the infection and lower bacterial burden (Fig. 1D). Given that the relatively low frequency of OVA-specific CD8+ T cells generated in the secondary response to the OVA–TLR7a conjugate (10–15% of CD8+ T cells) when compared with that generated in response to an actual infection such as vaccinia or lymphocytic choriomeningitis virus (20–60% of CD8+ T cells) (20, 21), our results demonstrate the potency by which conjugation of the TLR7a to protein Ags can establish protective immunity.

To determine the mechanism by which conjugation enhances the efficacy of the TLR7a as a vaccine adjuvant, we examined the possibility that conjugation increased the $t_{1/2}$ of the Ag in vivo, resulting in a depot effect of the Ag. We addressed this hypothesis by utilizing the adoptive transfer of OTI TCR transgenic T cells, which express a high affinity transgenic TCR specific for the OVA peptide, SIINFEKL. OTI T cells can respond to concentrations as low as pico- or femtomolar ranges (22–24), allowing them to serve as a functional readout for a level of Ag presentation at which the endogenous OVA-specific T cells may not be responsive. To determine how long Ag persisted in the host in a functionally relevant manner, CFSE-labeled OTI T cells were adoptively transferred into mice at 0, 1, 3, or 7 d after antigenic challenge. The loss of CFSE fluorescence was used as an indicator of OTI T cells encountering Ag, and thus an indication that the Ag injected at a particular time prior to the OTI T cell transfer had persisted in vivo. Interestingly, the OVA–TLR7a conjugate showed a pattern of OTI stimulation similar to the unconjugated TLR7a (Fig. 2). Evidence of cellular division was detected up to, but not beyond 3 d prior to OTI T cell

Results
transfer, suggesting that the conjugated TLR7a-Ag does not persist in vivo to any greater degree than Ag in the presence of the unconjugated TLR7a. Therefore, the enhanced ability of the OVA–TLR7a conjugate to generate CD8+ T cell responses is not due to the establishment of an Ag depot in vivo.

In related experiments, however, it was clear that conjugation increased the sensitivity of the responding T cells to limiting amounts of Ag. OTI T cells were adoptively transferred into naive recipients, followed by injection of limiting doses of the Ag either in combination with, or conjugated to, the TLR7a. The starting dose of OVA (5 μg) elicited very little response from the transferred cells (Fig. 3). Whereas the addition of the TLR7a simply increased the response of the transferred cells to only the highest dose of OVA, the transferred cells responded vigorously to a 10-fold lower dose of the conjugate (Fig. 3). Thus, the differences in the effective dose of the Ag required to stimulate the OTI T cells were exponentially lower for the conjugated than the unconjugated Ag. Collectively, these data indicate that the conjugate facilitates an increased sensitivity to Ag by the responding T cells (Fig. 3) without the formation of an Ag depot (Fig. 2).

**FIGURE 2.** Conjugation of the TLR7a to OVA elicits protective CD8+ T cell responses. A, B6 mice were immunized with either a 50 μg mixture of OVA and unconjugated TLR7a or 5 μg OVA–TLR7a conjugate in the footpad and B, given an equal dose of Ag for a boost injection 30–60 d following the primary immunization. Immunized mice were bled from the tail vein 7 d following primary (A) or 5 d following boost immunization (B). Cells were stained with the SIINFEKL-loaded H-2Kb tetramers to measure the frequency of OVA-specific CD8+ T cells. C and D, Mice were given the indicated doses of OVA and 10 μg unconjugated TLR7a or the OVA–TLR7a conjugate. Thirty days later, immunized mice were injected again with the same dose used for the corresponding primary immunization. C, Spleens from the immunized mice were harvested 6 d later, and cell suspensions were prepared and pulsed with the OVA peptide, SIINFEKL, for 4 h. Cells were then stained for intracellular IFN-γ. Frequency of IFN-γ+ cells shown was gated on live, B220− CD8+ cells. D, Thirty days following the boost injection, mice were challenged i.v. with 2 × 10^5 CFU of Lm-OVA per mouse. On day 3, spleens from mice were harvested and cultured on brain heart infusion plates to assess the CFU of Lm-OVA in the spleen as a measure of protection. Dot plots shown (A) are representative of three mice from each sample group. B and C, Graphed values of the frequency of OVA-specific CD8+ T cells are expressed as the mean ± SEM. Data shown in A–C are representative of four independent experiments, and data shown in D are representative of two independent experiments. Statistical analyses (*) were performed, as described in Materials and Methods.
FIGURE 3. Conjugation increases sensitivity of responding T cells to limiting amounts of Ag. Mice injected with CFSE-labeled OTI T cells were challenged with the indicated amounts of the various forms of Ag, as shown. Three days after transfer of OTI T cells, cells from the dLNs were isolated and the OTI T cells were assessed for the loss of CFSE as an indication of the presence of Ag in the host. The histograms shown were gated on live, CD8+, SIINFEKL-K\textsuperscript{b} tetramer\textsuperscript{+} events. The data shown are representative of three independent experiments.

We (5, 7, 25) and others (4) have demonstrated the potency with which administration of free TLR7a mediates the activation of DCs in vivo. However, it was still possible that the conjugation of the TLR7a enhanced its immunostimulatory properties on the DCs. To test this hypothesis, we compared the level of DC activation induced by the unconjugated and conjugated forms of the TLR7a. Using the level of MHC class II expression as a measure of DC activation, we found that both forms activated DCs equally well (Fig. 4A, 4B). Nearly 80% of the DCs had significantly up-regulated surface expression of I-A\textsuperscript{b} by 18 h following immunization with either the unconjugated or conjugated forms of the TLR7a, a dramatic difference from the phenotype observed in non-dLNs. Similar results were obtained for the expression of costimulatory markers such as CD86 (data not shown) (5, 7, 25).

Therefore, the data suggest that conjugation status of the TLR7a does not influence the maturation state of the DCs in a fashion that is consistent with the binary outcomes in cross-presentation of CD8\textsuperscript{+} T cells (Fig. 1A).

Although the immunostimulatory activities between the two forms are comparable, a possible explanation for the success of the conjugate over the free TLR7a may be related to differences in their capacity to influence overall uptake of the Ag by APCs. To address this, we labeled OVA with the fluorochrome, Alexa-Fluor488, with or without further conjugation to the TLR7a, and tracked its uptake by DCs in immunized mice. Using the labeled OVA, we observed a rapid uptake by DCs, regardless of whether the TLR7a was conjugated, unconjugated, or absent (Fig. 4C, 4D). OVA was taken up by ~30–40% of the DCs present in the dLN. Whereas this percentage was enhanced to some degree in mice injected with the OVA–TLR7a conjugate, the higher level of uptake was observed only at later time points (Fig. 4D). Furthermore, the overall mean fluorescence intensity values for the fluorochrome-labeled OVA in the conjugated and unconjugated forms did not differ in Ag-bearing DCs at all time points examined (Fig. 4E), suggesting that the amount of Ag on a per cell basis is not influenced by the conjugation status of the TLR7a. Interestingly, we found this to be true not only for the bulk population of DCs, but also for the multiple subsets of DCs present in skin dLNs (Supplemental Fig. 1). Collectively, the data suggest that conjugation had a relatively minimal impact on either the proportion of DCs that take up the Ag or the duration of Ag uptake by the DCs when compared with the unconjugated form of the Ag. However, we noticed that conjugation had a more significant impact on the frequency of DCs in the dLNs (Fig. 4F). As much as a 10-fold increase in the number of DCs was observed in mice immunized with the OVA–TLR7a conjugate when compared with either OVA alone or the unconjugated TLR7a (Fig. 4F). Thus, the overall Ag load appears to be higher in OVA–TLR7a conjugate-immunized mice, most likely due more to the sizable increase in the number of DCs than to the relatively minor increase in the proportion of DCs acquiring Ag (Fig. 4F, 4G).

Taken together, the data show that whereas the conjugate has at best a 2-fold enhancement of Ag uptake over time, it enhances both DC accumulation (Fig. 4) and sensitivity of T cells to Ag (Fig. 3) by at least 10-fold. Thus, it was plausible that the increased sensitivity to Ag was simply due to the increased numbers of Ag-bearing DCs. Alternatively, the increased sensitivity to Ag may be due to the capacity of the conjugate to enhance DC Ag processing and cross-presentation on a per cell basis. To test this possibility, we developed an assay to quantitate cross-presentation in vivo following immunization with the conjugate and non-conjugate forms of Ag. Mice were immunized s.c. using OVA either combined with or conjugated to the TLR7a. The DCs from the dLNs were isolated 24 h following immunization and mixed with in vitro-stimulated effector OTI cells, and the magnitude of IFN-\(\gamma\) production by the T cells was measured (Fig. 5A). Because we used effector T cells in this assay, the presentation of Ag by MHC class I is, alone, sufficient for the induction of IFN-\(\gamma\) production by the T cells (Fig. 5B). As such, the response of the T cells can be used as a functional readout of the amount of peptide-MHC complexes on the DC cell surface.

As expected, DCs purified from mice immunized with the OVA–TLR7a conjugate exhibited a high level of Ag cross-presentation (Fig. 5B, 5C). In contrast, the unconjugated mixture of TLR7a and OVA, despite its level of Ag uptake into the DCs (Fig. 4), did not result in cross-presentation. OTI T cells were incubated with a titration of increasing cell numbers of DCs (Fig. 5C), and even the highest titration of DCs used (4 × 10\(^6\) cells) did not stimulate any OTI IFN-\(\gamma\) production (Fig. 5B, 5C). This is significant because we found, at the 24-h time point, relatively similar levels of Ag uptake on a per cell basis between the unconjugated and conjugated forms of the Ag (Fig. 4D, 4E). In addition, when we lowered the amount of conjugate injected to more closely match the amount of Ag uptake observed using the unconjugated form of the Ag, the DCs still cross-presented Ag well above background in response to the conjugate immunization (Fig. 5D).

We make two conclusions from these results. First, the observed failure of DCs to cross-present Ag delivered with the unconjugated
TLR7a is not due to the Ag uptake falling below some critical threshold. Second, the DCs from the conjugate-immunized host have an increased capacity to process and present Ag on a per-cell basis as compared with the DCs from hosts immunized with the unconjugated form of the Ag. The ability to assess DC function on a per-cell basis allows the conclusion that, whereas the conjugate also augments the accumulation of Ag-bearing DCs in dLNs, the critical parameter underlying the efficacy of the conjugated TLR7a is its enabling of cross-presentation. Thus, the phenotype of DC cross-presentation highly mirrors the phenotype of the T cell responses generated by the two forms of vaccination; both functional outcomes (cross-presentation and T cell expansion) display an all-or-none type of a response. The conjugated TLR7a elicited a robust functional response in both assays, whereas the unconjugated form did not elicit any detectable responses.

**Discussion**

Mechanisms by which vaccine adjuvants enhance the immunogenicity of Ags have been examined for several decades. However, a full understanding of the key processes underlying their efficacy remains unclear and under debate. Early studies have implicated the formation of Ag depots as the underlying cause (reviewed in Ref. 26). These findings have led to the proposal that adjuvants enhance the immune response through prolonging the persistence of Ag in vivo. Additionally, Ag depots are thought to form through aggregation, which may affect the mode and efficiency of uptake by APCs (27, 28). However, more recent data suggest that the activation of inflammation via various innate cells and factors is the component dictating the success of an adjuvant (29). To this end, experimental approaches used to identify alternative vaccine adjuvants focus now largely on characterizing innate immune responses.
receptor signaling pathways and what corresponding impact candidate adjuvants targeting these pathways have on the downstream adaptive immune system.

Targeting TLRs through the use of either natural or synthetic agonists have been widely explored as a potential alternative vaccination strategy to induce cellular immunity. This approach has drawn much attention on the basis that TLR stimulation results in robust activation of innate immune cells and the production of proinflammatory cytokines (2–5). However, the assumption that simply inducing these innate immune responses results in cellular adaptive immunity appears to be misplaced. This is particularly evidenced by the failure of TLR agonists to elicit CD8+ and CD4+ T cell responses when administered as a mixture with protein Ag into hosts (6, 7). Therefore, activation of the innate immune system by these agonists alone is insufficient to effectively induce cellular adaptive immune responses.

Previous studies by us (6, 8) and others (9–16) have shown that any demonstration of these agonists to elicit CD8+ T cell responses requires some kind of modification to the way in which the Ag and the TLR agonist are delivered. These include coupling the two components together through chemical conjugation, adsorption by synthetic microparticles, formation of aggregates or Ag depots, or the combinatorial use of anti-CD40 mAbs. However, the current assessment of how these methods elicit cellular immune responses is gravely limited and fails to explain the inability of administering the TLR agonist alone to induce such responses. In the current study, we demonstrated the efficacy of codelivering a synthetic TLR7a with a soluble protein Ag through chemical conjugation as a means to establish cellular immunity. In the process of directly comparing this approach with the unconjugated mixture of the TLR7a, we identified recruitment of DCs and activation of cross-presentation by DCs as two key determinants governing the success or failure of these immune responses.

**FIGURE 5.** Conjugation enhances efficiency of cross-presentation by DCs. A, A schematic outline of the method used to assess the level of Ag cross-presentation by DCs ex vivo. Mice were immunized in the footpad with 50 μg OVA–TLR7a conjugate or the unconjugated mixture of OVA and TLR7a. Twenty-four hours following immunization, popliteal LNs were harvested, the cell suspensions from each mouse group were pooled, and the DCs were purified using anti-CD11c magnetic beads. In some experiments, DCs were purified using a cell sorter (MoFlow). Enriched DCs were incubated at 4.0 × 10^6 cells/well (B) or at the indicated titration of cell numbers (C) with 0.5 × 10^6 effector OTI T cells. The cells were coincubated for 4 h in the presence of brefeldin A and then stained for intracellular IFN-γ. Production of IFN-γ by OTI T cells was assessed by gating on expression of the congenic marker CD45.1. The level of IFN-γ response by the OTI T cells was expressed as a percentage of the maximal production of IFN-γ induced by DCs pulsed with the SIINFEKL peptide. D, Cross-presentation by DCs from mice immunized with either 50 or 25 μg OVA–TLR7a conjugate was assessed as in A–C. The data shown are representative of four independent experiments.
of the conjugated and unconjugated forms of the TLR agonist, respectively. Our data suggest that, although efficient uptake of Ag by APCs and inflammation are necessary, recruiting a sufficient number of DCs and enabling Ag-bearing DCs to cross-present the acquired Ag are paramount to induce CD8+ T cell responses.

DC recruitment and cross-presentation are undoubtedly necessary components mediating CD8+ T cell responses to exogenous Ag. Of all parameters critical for vaccine-mediated immunity, the induction of cross-presentation, in particular, has long been known to hold some level of importance. However, our data elevate this parameter to a previously unappreciated degree of significance. Namely, when all other factors are made equal (Ag duration, Ag uptake, DC phenotype, DC numbers), the simple capacity of a given vaccination method to facilitate cross-presentation is the dominant factor dictating its success or failure. These results therefore provide a new focus for future vaccine adjuvant discovery and development.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. Conjugation does not affect the efficiency of antigen uptake by a particular DC subset.

Mice were immunized in the footpad with 5 μg of Alexa-Fluor488-labeled OVA with the unconjugated TLR7 agonist or with the Alexa-Fluor488-OVA-TLR7a conjugate. At 18 or 30 hours following immunization, popliteal LN draining the foot were harvested, minced, and digested with collagenase/DNase. Cells were then stained with various mAb specific for unique surface markers of DCs. DC subsets were identified using flow cytometry based on the expression of CD11c and a unique combination of CD8α, CD11b, and DEC-205: LCs are DEC-205^{high}CD8α^{+}CD11b^{+}, dermal DCs are DEC-205^{intermediate}CD8α^{+}CD11b^{+}, LN-resident CD8α^{+} DCs are DEC-205^{intermediate}CD8α^{+}CD11b^{+}, and LN-resident CD11b^{+} DCs are DEC-205^{high}CD8α^{+}CD11b^{high}. To confirm our gating strategy based on these phenotypic markers, cells were also stained for Langerin and MHC class II expression and found that indeed only the LC were Langerin^{+}, and both migratory DC subsets (LC and dermal DC) were MHC class II^{high} (data not shown). The percent of DCs harboring the antigen were calculated based on the surface marker profiles and Alexa-Fluor 488^{+} gated events. Statistical analyses were performed as described in Materials and Methods. The summary of P values, as indicated by (*), denote statistical significance of differences between the means of the conjugate and OVA + TLR7 agonist treatments. The data shown are representative of three independent experiments.