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Covalent Crosslinking of Tumor Antigens Stimulates an Antitumor Immune Response

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Abstract

In murine renal cell carcinoma and melanoma models, vaccination with crosslinked tumor antigen in the absence of other adjuvants inhibited the growth of RENCA and B16 tumors. Vaccination with crosslinked antigens (CA9 or gp100) enhanced the cellular immune response as measured by ELISPOT and cytotoxicity assays. Crosslinking antigens enhanced delivery of antigen to bone marrow derived dendritic cells, which were capable of internalizing and processing the antigens. Dendritic cells pulsed with crosslinked antigen were effective in stimulating antigen-specific CD8⁺ T lymphocyte proliferation and interferon- γ secretion. Crosslinking tumor antigens is a simple and effective strategy for enhancing tumor vaccines.

Keywords

crosslinking; immunoadjuvant; tumor vaccine

Introduction

Tumor vaccines have the potential to specifically target malignancies while minimizing clinical toxicity. Tumor specific antigens can direct the immune system to recognize the tumor. However, successful generation of an adaptive immune response requires engagement of the innate immune system, which is commonly accomplished by using immune adjuvants. Alum is an example of an immune adjuvant that is capable of providing the initial danger signal that can activate an adaptive immune response. Although alum is a weak adjuvant, its toxicity is minimal and it remains the standard for human vaccines.[1]

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Effective strategies are needed to stimulate antitumor immune responses. Ideal cancer vaccines should stimulate a vigorous immune response while producing minimal toxicity. If exogenous tumor antigens are used, the vaccine strategy should lead to cross presentation of the antigen and production of a cellular immune response.[2,3] It is known that crosslinking with fixatives and even heat-induced aggregation can enhance immune stimulation. Dearman et al demonstrated that formaldehyde and glutaraldehyde are capable of activating T helper 1 and T helper 2-type cells.[4] Speidel et al showed that heat induced aggregation of antigens can enhance *in vivo* priming of cytotoxic T lymphocytes.[5]

This study assesses crosslinking as a strategy to stimulate an immune response against tumors. We describe a tumor vaccine that uses covalently crosslinked antigens to stimulate an adaptive immune response and inhibit tumor growth in murine models. We show that exogenous crosslinked antigens are cross-presented and lead to activation of antigen specific CD8+ T lymphocytes.

Materials and Methods

Mice, cell lines and DNA constructs

Female C57/BL6 mice and BALB/c mice, 6–8 week old, were purchased from NCI (Frederick, MD) and housed under pathogen-free conditions. All experiments involving animals were approved by the Institutional Animal Care and Use Committee and were in compliance with federal and state standards, which include the federal Animal Welfare Act and the NIH guide for the care and use of laboratory animals.

Gp100 transduced B16 cells (B16-gp100) were kindly provided by Dr Alexander Rakhmilevich (University of Wisconsin, Madison, WI). These cells were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. RENCA cells stably transduced to express CA9 (RENCA-CA9) were a gift from Dr Arie Belldegrun (UCLA, Los Angeles, CA). These cells were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L of L-glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin, 100 µg/mL nonessential amino acids, 100 µg/mL sodium pyruvate, and 100 µL/mL Hepes buffer.

Hsp110, gp100 (a gift from Dr. Nicholas Restifo, National Cancer Institute, Bethesda, MD) and CA9 cDNA (a gift from Dr. Belldegrun) were cloned into pBacPAK-his vector (BD Biosciences Clontech, Palo Alto, CA). Recombinant proteins were expressed using the BacPAK baculovirus system. Proteins were purified using a nickel nitriloacetic acid-agarose column (Qiagen, Valencia, CA). Protein concentrations were measured using a Protein Assay Kit (Bio-Rad, Hercules, CA). Protein purity was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Blue staining. Endotoxin levels in recombinant proteins were assessed using a Limulus Amebocyte lysate kit (Biowhittaker, Walkersville, MD) and were 10-25 endotoxin units/mg protein.

Crosslinking antigen

Antigens were covalently crosslinked by incubating with 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP; Pierce, Rockford, IL) at 20-fold molar excess (0.25-5mM) at room temperature for 30 min. DTSSP reacts with primary amines to form covalent amide bonds. The reaction was stopped by incubating with 39mM Tris, pH 7.5 for 15 min. DTSSP was dialyzed off (Slide-A-Lyzer dialysis cassette; Fisher Scientific, Pittsburg, PA). To confirm successful crosslinking, crosslinked antigens were dissolved in SDS sample buffer (6.25mM Tris, PH 6.8, 10% glycerol, 2% SDS) which did not contain β-mercaptoethanol. The samples were not boiled before resolving by SDS-PAGE. To break

the disulfide bond of the crosslink, antigens were boiled in sample buffer containing 5% β -mercaptoethanol and loaded onto SDS PAGE gels. Monomer forms of the antigen served as controls. Western blotting was performed with mouse anti-human CA9 monoclonal antibody (a gift from Dr Egbert Oosterwijk, University of Nijmegen, Nijmegen, Netherlands).

Tumor prevention study

Female mice were immunized intradermally 3 times, 7 d apart, with 100 μ l of vaccine at 20M concentration, determined prior to crosslinking. The vaccine consisted of crosslinked tumor protein (CA9 or gp100) or MHC class I CA9 epitope (A Y E Q L L S R L with >99% purity by HPLC, synthesized by Alpha Diagnostic international, San Antonio, TX).[6] Control groups were vaccinated with PBS, DTSSP, or crosslinked irrelevant protein (e.g. hsp110). No adjuvants were included in the vaccine. Mice were challenged with 2×10^5 B16-gp100 or RENCA-CA9 cells injected intradermally, 7 d after the last immunization. Tumors were measured every 3 d using an electronic caliper and tumor volume was calculated [$(\text{shortest diameter}^2 \times \text{longest diameter})/2$]. The complete set of experiments was repeated 3 times.

The ELISPOT and ^{51}Cr Release assays have been described.[7] Briefly, for the ELISPOT assay, filtration plates (Millipore, Bedford, MA) were coated with 10 $\mu\text{g/ml}$ rat antimouse IFN- γ (clone R4-6A2; PharMingen, San Diego, CA) at 4°C overnight. Plates were then washed and blocked with culture medium containing 10% FBS. Splenocytes (5×10^5 splenocytes/well) were incubated with the gp100 (20 $\mu\text{g/ml}$) or hsp110 (20 $\mu\text{g/ml}$) at 37°C for 24 hrs. Plates were then washed and incubated with 5 $\mu\text{g/ml}$ biotinylated IFN- γ antibody (clone XMG1.2; PharMingen) at 4°C overnight. After washes, 0.2 unit/ml avidin-alkaline phosphatase D (Vector Laboratories, Burlingame, CA) was added and incubated for 2 hrs at room temperature. Spots were developed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Boehringer Mannheim, Indianapolis, IN) followed by incubation at room temperature for 20 min.

For the ^{51}Cr Release Assay, splenocytes were harvested 2 weeks after immunization and stimulated in vitro with irradiated B16-gp100 cells for 5 days. Splenocytes were then serially diluted in 96-well plates containing ^{51}Cr labeled tumor cells (1×10^4 cells/well) in triplicate with varying E:T ratios. After 5 hrs of incubation at 37°C, supernatant was analyzed for radioactivity using a gamma counter (Packard, Downers Grove, IL). In some experiments, the restimulated effector cells were incubated with anti-CD8 antibodies (20 $\mu\text{g/ml}$) at 4°C for 30 min before performing the cytotoxicity assay.

Binding of crosslinked proteins by dendritic cells (DCs)

To FITC-label CA9, CA9 or crosslinked CA9 was combined with FITC (Sigma, St. Louis, MO) in 0.1 M sodium bicarbonate/carbonate buffer at 20 M excess based on the original CA9 concentration prior to crosslinking. Free FITC was removed with a Sephadex G-25 column (Pharmacia, Piscataway, NJ). Proteins were subjected to SDS-PAGE to confirm FITC conjugation. To assess binding to DCs, FITC-conjugated proteins, at 10 $\mu\text{g/ml}$, were incubated for 20 min on ice with murine bone marrow-derived DCs (BMDCs) at 1×10^6 cells/ml in 100 μL PBS containing 1% BSA. The DCs were washed twice with 1%BSA/PBS. The nucleus was counterstained with DAPI. The cells were fixed with 1% paraformaldehyde (Fisher, Fair Lawn, NJ), and examined by confocal microscopy (Bio-Rad 600, Hercules, CA) and analyzed by flow cytometry (Becton Dickinson, La Jolla, CA) or Western blot analysis. Generation of BMDCs has been described.[8] Briefly, marrows were harvested from murine femurs and tibias and treated with RBC lysis buffer, washed, and plated at a density of 1×10^6 cells/mL in 12-well plates in RPMI 1640 containing 10% fetal

bovine serum and 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (eBioscience). Cultures consisted of 75% to 90% CD11C⁺ cells.

Characterization of DC surface markers and antigen processing

To characterize DC surface markers, BMDCs were pulsed with antigen, crosslinked antigen or LPS for 24hrs. For FACS analysis, cells ($3-5 \times 10^5$) were suspended in 100 μ l PBS with 0.3% BSA and 0.05% sodium azide, and were stained with antibodies for 30 min on ice. After incubation, the cells were washed, and the fluorescence was measured by a FACScan (Becton). For each sample, fluorescence data from 10,000 cells were collected and positive cells were expressed as the percent of total events.

For the antigen processing study, DCs were grown to 90% confluence and treated with 10 μ M MG132 or 10mM NH₄CL for 2 h at 37°C. Untreated cells served as controls. Cells were cooled to 4°C for 30 min before adding CA9 at 10 μ g/ml. The cells were kept at 4°C for 2 hrs and then washed 3 times with cold PBS. The cells were then warmed to 37°C in RPMI 1640 complete medium, and harvest at 0, 1, 3, and 24 hr time points, washed and treated with radioimmune protection assay (RIPA) buffer (Sigma, St. Louis, MO) for 15 min on ice to lyse cells. 20 μ g of lysate was subjected to Western blot analysis. The blots were probed with mouse anti-human CA9 antibody.

T-cells proliferation assay

BMDCs were pulsed for 4 hrs with 10 μ g/ml antigen, with or without crosslinking, and activated with 10 ng/ml LPS for 2 hrs. Cells were washed 3 times with PBS. Lymphocytes were harvested from lymph nodes from Pmel mice or OT-I mice. The cells were labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR), incubated at 37°C for 20 min, washed, and resuspended in complete culture medium (RPMI 1640, 10% fetal calf serum, 2 mmol/l l-glutamine, 100 units/mL penicillin/streptomycin). Antigen pulsed DC and CFSE labeled lymphocytes were mixed at 1:10 ratio and cultured for 48-72 hrs. Lymphocyte proliferation was assessed by incorporation of ³H-thymidine (1 μ Ci/well during the last 16 hrs of culture) or by flow cytometric analysis of CFSE dilution.

For intracellular IFN- γ staining, lymphocytes recovered from culture were restimulated with PMA (20ng/ml) and ionomycin (400ng/ml) for 4 hrs in presence of 10 μ g/ml brefeldin A. Lymphocytes were stained for cell surface antigens (e.g. CD8 or V α -1), fixed, permeabilized, and stained for intracytoplasmic antigens (e.g. IFN- γ). Flow cytometry was performed using the FACScan (Becton-Dickinson, La Jolla, CA) and typically 10,000 live CD8⁺ or V α -1 gated events were analyzed using Winlist software (Verity, Topsham, ME).

Results

Crosslinking produces large complexes with a range of sizes

Tumor antigens were crosslinked by incubating in the presence of 20 molar excess of DTSSP. DTSSP reacts with primary amines to form covalent amide bonds. Therefore, crosslinked antigens are expected to form a range of large molecular-weight complexes. CA9 is a tumor antigen that is highly expressed in renal cell carcinoma. Recombinant CA9, crosslinked with DTSSP, produced a range of large complexes, which appeared as a smear at the top of a stacking gel that was probed with anti-CA9 antibody (Figure 1). To confirm that the smear represents crosslinked forms of CA9, CL-CA9 was boiled in the presence of β -mercaptoethanol, which restored the majority of the monomeric form of CA9.

Immunization with crosslinked tumor antigen prevents tumor growth

To explore the immunostimulatory properties of crosslinked antigens, two syngeneic tumor models were used. In a renal cell carcinoma (RCC) model, vaccination targeted CA9, which is a tumor-associated antigen expressed by the majority of clear cell RCCs. Immunization with crosslinked full-length, recombinant CA9 or crosslinked H2-K^d restricted CA9 peptide prevented growth of RENCA tumors. (Figure 2A) Although CA9 administered without crosslinking also had some antitumor effect, the monomeric CA9 peptide did not. This is not surprising since CA9 has been shown to have chaperoning properties that allow it to stimulate antigen-specific cellular immune responses.[9]

In a melanoma model, vaccination targeted gp100 (Figure 2B). Immunization with CL-gp100 had a strong antitumor effect against B16 tumors. However, crosslinked hsp110 (irrelevant protein) produced no antitumor effect. Other controls included vaccination with PBS, DTSSP, or a mixture of hsp110 and gp100. We've previously shown that hsp110 can be heat-shock complexed to gp100 to produce gp100-specific immunity.(5) However, when these proteins are combined, without heat-shock complexing, no antitumor immunity is observed.

Crosslinked antigens stimulate a specific cellular immune response

Effective antitumor effects against solid tumors require the activation of cellular immunity. To verify that CL-CA9 and CL-gp100 stimulate cellular immunity, ELISPOT and cytotoxic T lymphocyte assays were performed. (Figure 3) CL-CA9 produced a significantly stronger interferon- γ response when compared to control vaccines, while the response due to CL-gp100 approached significance when compared to gp100. Although an interferon- γ response is not specific for a cytotoxic T cell response, immunization with CL-gp100 was shown to produce a strong cytotoxic T cell response. (Figure 3C)

Crosslinking increases antigens bound to dendritic cells

To explore the mechanism of immune stimulation by crosslinked antigens, we examined the binding of CL-CA9 to BMDCs, which is one of the first steps in the activation of adaptive immunity. Crosslinking increased the delivery of CA9 to DCs. (Figure 4) This was qualitatively observed by binding FITC-labeled CL-CA9 to DCs and using confocal microscopy. The increase in binding was quantified by flow cytometry and Western blot assay. Increased binding was associated with a corresponding decrease in CA9 from the culture medium.

Crosslinked proteins bound to dendritic cells were internalized and processed

It is possible that crosslinked antigens activate BMDCs. To assess DC activation, BMDCs were incubated with tumor antigen (gp100 or CA9) or crosslinked forms of the same antigen (Figure 5A). There was no difference in percent of CD11C⁺ cells expressing MHC I, MHC II, CD80 or CD86. LPS served as a positive control. Despite the absence of a change in surface markers, tumor antigens, whether crosslinked or not, were efficiently internalized by DCs and processed through both the proteosomal and endosomal pathways (Figure 5B). Pretreatment of DCs with specific inhibitors for each of the pathways increased the level of intracellular antigen detectable. However, MG132, a proteosomal pathway inhibitor, had the greater effect on processing, indicating that the proteosomal pathway is the dominant pathway for processing crosslinked antigens.

Crosslinking enhancing T cell proliferation and activation

DCs are antigen presenting cell that are capable of activating T lymphocytes, which are effector-cells of cellular immunity. The downstream consequence of binding crosslinked

antigen to DCs was examined using 2 different *in vitro* models. BMDCs were pulsed with gp100 or CL-gp100, and co-cultured with Pmel-1 lymphocytes prelabeled with CFSE. Pmel-1 transgenic mice express CD8⁺ T cells with specificity for a D^b-restricted epitope from the tumor Ag gp100₂₅₋₃₃. [10] CL-gp100 resulted in increased Pmel expansion when compared to noncrosslinked antigen (Figure 6A).

A similar confirmatory assay was performed using cross-linked ovalbumin (CL-OVA) to pulse BMDCs, which were then co-cultured with OT-1 lymphocytes. OT-1 proliferation, monitored by ³H Thymidine incorporation, was increased by crosslinking ovalbumin (Figure 6B). To confirm that OT-1 cells stimulated by CL-OVA are active, the proliferating OT-1 cells were subjected to intracellular interferon- γ staining. BMDCs pulsed with CL-OVA increased interferon- γ in OT-1 cells when compared with controls antigens, including non-crosslinked OVA and CL-CA9 (Figure 6C).

Discussion

RCC and melanoma are classic immunoresponsive malignancies. In RCC for example, patients with metastatic disease have historical 3-year survival rates less than 5%. [11] Of all urologic malignancies, RCC has the highest ratio of disease-related deaths to incidence. The standard treatments for metastatic RCC include immune cytokines and small molecule tyrosine kinase inhibitors (TKIs). TKIs are associated with response rates of 40-44%. [12] However, complete responses are rare, occurring in only 1% of patients. In clinical trials of high dose IL-2, complete responses and durable remissions occurred in 5-10% of patients with metastatic RCC. [11,13,14] This important observation suggests that with more targeted and effective immune stimulation, complete responses can be achieved in greater proportion of patients.

As a general rule, the efficacy and toxicity of an adjuvant are inversely related, and there remains a need for an immune adjuvant capable of stimulating a robust and lasting immune response while producing minimal toxicity. For example, Alum (aluminum salts) is a weak adjuvant that is ineffective in stimulating a cytotoxic T-cell response; however, associated toxicity is minimal and alum remains the standard for clinical use. [1] However, Freund's complete adjuvant is one of the most potent adjuvants available and it has been used extensively for experimental immunology; its toxicity prohibits its use in humans. In this study, crosslinking served as the adjuvant, and no additional immune adjuvants were required. Therefore, vaccination with CL-CA9 is an effective strategy for stimulating an antigen-specific antitumor response, and this approach has the potential to simplify vaccine preparation.

In a RCC model, antitumor effects were seen with CL-CA9 and with a crosslinked MHC class I epitope (A Y E Q L L S R L). [6] The immune stimulating effects of crosslinking were not restricted to the RENCA model. A crosslinked antigen vaccine was also effective in a melanoma model targeting gp100. Crosslinked gp100 prevented growth of B16 tumors. Crosslinked antigens stimulated antigen-specific cellular immune responses. There may be multiple mechanisms through which crosslinked antigens stimulate an antitumor response. However, at least one mechanism appears to involve enhanced delivery of antigens to dendritic cells, which are then able to process and present the antigens to CD8⁺ T lymphocytes.

Crosslinking produced a range of large molecular complexes. It is not known how these large complexes contribute to immune stimulation. There are two competing models that explain the activation of innate immunity through pattern recognition receptors (PRR). Janeway suggested that PRR bind non-self molecules found on harmful pathogens. [15]

Examples of microbial-specific molecules that activate innate immunity include lipopolysaccharide, lipoarabinomannan, and peptidoglycan.[16,17] Alternatively, Matzinger suggested that host factors associated with pathogen-induced injury are responsible for stimulating innate immunity.[18] This second model is supported by a long list of host derived immunostimulatory substances such as hyaluronan[19], heat shock proteins[20], uric acid[21], and surfactants[22]. More recently, Matzinger suggested that the two models can be reconciled by recognizing that PRR respond to hydrophobic regions of ligands, whether pathogen or host-derive.[23]

It is possible that crosslinked antigens produce hydrophobic signals, which are responsible for initiating the immune cascade. Some heat shock proteins have been found to be powerful adjuvants. For example, hsp110 and grp170 naturally exist in high molecular weight complexes in combination with protein antigen.[24] It is possible that the underlying mechanism responsible for the immunostimulatory properties of heat shock proteins results from its ability to crosslink proteins and produce hydrophobic signals. In this study, we document that immune stimulation from crosslinked antigens can generate cellular immune responses that produce antitumor effects that similar to those seen with noncovalent complexes of heat shock protein and tumor antigen.

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Figure 1. Electrophoresis of crosslinked CA9 (CL-CA9)

Crosslinking CA9 produces a range of large molecular complexes as seen on SDS page gel probed with anti-CA9 antibody. Lane 1 (left to right) was loaded with CA9 and lane 2 was loaded with CL-CA9. Samples in both lanes 1 and 2 were not boiled and did not contain β -mercaptoethanol. Most CL-CA9 forms stayed in the stacking gel. Lanes 3 and 4 were loaded with CA9 and CL-CA9, respectively, after boiling in the presence of β -mercaptoethanol to break the disulfide bonds present in the crosslinks; this resulted in the restoration of bands corresponding to CA9 monomer.

Figure 2. Tumor challenge after immunization

CL-antigen prevented tumor growth in murine models. A) CL-CA9 prevented growth of RENCA-CA9. CA9 was covalently crosslinked by incubating with DTSSP. BALB/c mice (5 mice per group) were vaccinated 3 times at weekly intervals with PBS, CA9, CA9 peptide (A Y E Q L L S R L), CL-CA9, or CL-CA9 peptide. 7 days after the 3rd immunization, mice were challenged with 2×10^5 RENCA-CA9. B) Crosslinked gp100 (CL-gp100) prevented growth of B16-gp100. Gp100 was covalently crosslinked by incubating with DTSSP. C57BL6 mice (5 mice per group) were vaccinated 3 times at weekly intervals with PBS, DTSSP, CL-hsp110, CL-gp100, or mix of hsp110 and gp100 (no crosslinking or complexing). 7 days after the 3rd immunization, mice were challenged with 2×10^5 tumor cells. Representative data shown from 2 independent experiments.

Figure 3. Crosslinking elicits a specific IFN- γ and cytotoxic T-cell response

Immunization with crosslinked antigen (CA9 or gp100) elicited an antigen-specific immune response as measured using ELISPOT assay or cytotoxic T lymphocyte assay. A) BALB/c mice (3 mice/group) were immunized intradermally with PBS, DTSSP, CA9, or CL-CA9. The splenocytes were harvested 10d after 2 immunizations performed 10 days apart. B) C57/BL6 mice (3 mice/group) were immunized intradermally with PBS, gp100, or CL-gp100. The ELISPOT assay was performed. C) Specific cytotoxic T-cell response was measured using the ^{51}Cr release assay. The mean + SEM is provided for experiments performed in triplicate.

Figure 4. Binding of CL-CA9 to dendritic cells (DCs)

A) Crosslinking increased CA9 binding to bone-marrow derived DCs. Confocal microscopy showed FITC-labeled CA9 and CL-CA9 bound to the surface of bone-marrow derived DCs at 4 °C. The nucleus was counterstained with DAPI. B) The increase in DC-binding of FITC-labeled CL-CA9 when compared to CA9 was quantified using flow cytometry; the peak fluorescence intensity increased approximately two folds with crosslinking. Bone marrow derived DCs (1×10^6 /ml) were incubated at 4°C with FITC-labeled CA9 or CL-CA9 for 30 min and washed twice with 1% BSA/PBS. C) The increase in DC-binding of CL-CA9 when compared to CA9 was also assessed by Western blotting. Crosslinking increased CA9 binding to DCs and decreased CA9 in the medium. Bone marrow derived DCs (1×10^6 /ml) were incubated at 4°C with FITC-labeled CA9 or CL-CA9 for 30 min. The medium and cell lysate were probed for CA9.

Figure 5. DC activation and processing of CL-CA9

A) Crosslinked tumor antigens did not increase markers of DC activation. Bone marrow derived DCs were incubated with tumor antigens or crosslinked tumor antigens. LPS served as a positive control. There was no statistically significant difference in surface expression of DC markers comparing antigen and crosslinked antigen. B) CA9 and crosslinked CA9 bound to DCs were internalized and processed predominately through the proteosomal pathway. CA9 was allowed to bind DCs for 2 h at 4°C. DCs were washed twice and incubated in fresh medium at 37°C for the indicated time. Cell surface CA9 was washed and intracellular CA9 was monitored by western blotting. CA9 processing resulted in decreasing intracellular CA9 detected by western blotting. Pretreatment of DCs with MG132 (proteasome inhibitor) or NH₄Cl (lysosome inhibitor) for 30 min at 37°C indicated that CA9 was processed by both pathways; however, the proteosomal pathway was dominant.

Figure 6. Bone Marrow derived DCs pulsed with crosslinked-antigen stimulate antigen-specific lymphocytes

A) The downstream consequence of binding crosslinked antigen to DCs was examined. DCs treated with CL-gp100 resulted in increased Pmel-1 expansion when compared to DCs treated with noncrosslinked antigen. BMDCs were pulsed with gp100 or CL-gp100, and co-cultured with Pmel-1 lymphocytes prelabeled with CFSE. Proliferation of Pmel-1 was assessed by gating on CD8 and monitoring CFSE dilution using flow cytometry. B) A similar confirmatory assay was performed using OT-1 lymphocytes. BMDCs were pulsed with CL-OVA or control proteins, and co-cultured with lymphocyte from OT-1 mice. Lymphocyte proliferation was assessed by monitoring ^3H Thymidine incorporation, which was increased by crosslinking ovalbumin. C) CL-OVA resulted in increased activation of OT-1 cells. OT-1 cells stimulated by CL-OVA were subjected to intracellular interferon- γ staining. BMDCs pulsed with CL-OVA were co-cultured with lymphocytes from OT-1 mice, which were then assessed by gating on Va-2 and quantifying percent of cells staining for intracellular Interferon- γ using flow cytometry.