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HIV gag protein is efficiently cross-presented when targeted with an antibody towards the DEC-205 receptor in Flt3 ligand-mobilized murine dendritic cells

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Summary

Dendritic cells present exogenous proteins to MHC class I restricted $CD8^+$ T cells. This function does not require endogenous antigen synthesis within DC, providing the potential to elicit $CD8^+$ T cell responses to immune complexes, inactivated microbes, dying cells and proteins like ovalbumin. In mice, the $CD8^+$ or DEC-205^+ DC are specialized for cross-presentation, and this subset can be increased 10 fold in numbers following Flt3L treatment *in vivo*. Therefore we studied cross-presentation by abundant Flt3L DC using HIV gag protein. When enriched by positive selection with anti-CD11c beads, cells from Flt3L mice are not only more abundant but are more highly enriched in CD11c high DC, particularly the DEC-205^+ subset. DC cross-present HIV gag to primed $CD8^+$ T cells, but when the antigen is delivered within an antibody to DEC-205 receptor, cross-presentation becomes 100 fold more efficient than non-targeted antigen. This finding requires gag to be engineered into anti-DEC antibody, not just mixed with antibody. Flt3L DC are a valuable tool to study cross-presentation, since their use overcomes the obstacle posed by the low number of cross-presenting DC in the steady state. These findings support future experiments to use Flt3L to enhance presentation of DC-targeted vaccines.

Keywords

DC; Flt3L; poly IC

Introduction

Induction of strong $CD8^+$ T cell responses is a major goal in the development of preventive and therapeutic vaccines against persistent viruses and tumors. Dendritic cells (DC) can initiate $CD8^+$ T cell responses through either direct priming or cross-priming. Direct priming refers to the generation of peptide-MHC class I complexes from endogenously synthesized proteins, while cross-priming involves processing of exogenous proteins acquired from the extracellular environment. Cross-priming has been shown to be important in initiating MHC class I- restricted responses to tumors, peripheral self, viral and bacterial antigens [1].

The efficiency of cross-presentation can be enhanced when dendritic cells (DC) take up antigens through different receptor-mediated pathways particularly antigen-antibody

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complexes [2-4], dying cells [5,6], and proteins targeted within antibodies to specific receptors like DEC-205 [7], DC-SIGN [8], MMR [9]; Langerin [10], LOX1 [11], and CLEC9A [12]. In our research, we have been studying cross-presentation by genetically engineering the sequences for protein antigens into the heavy chain of monoclonal antibodies (mAb) to DC receptors, particularly DEC-205 [7].

Much of the research in this field has used ovalbumin (OVA) as a tool and the $CD8^+$ OT-I TCR transgenic line specific for OVA presented on $H\text{-}2K^b$ molecules. This is because the SIINFEKL peptide from OVA is presented efficiently, with only picomolar levels of peptide being active when DC are the antigen presenting cells [13]. Nevertheless, DC can cross-present other proteins like malaria circumsporozoite protein [13-16], HIV gag [17,18], HSV and influenza proteins [19-21], and tumor antigens [12,22-24], although this cross-presentation is often studied with TCR transgenic T cells. Vaccine science requires that the cross-presentation pathway be extended to candidate vaccine antigens and non-transgenic T cells.

In mouse lymphoid tissues, a subset of DC, marked by expression of CD8α homodimer and the DEC-205/CD205 endocytic receptor, is more active in cross-presentation [4,6,25]. Here we have studied these cross-presenting DC using mice exposed to Flt3L, which is known to expand several different subsets of DC including a relatively high proportion of $CD8^+$ DC, increasing total numbers of this subset ~ 30 fold [26,27].

With the most commonly studied protein, OVA, concentrations higher than 50 μg/ml of protein are required to detect cross-presentation [9,13,25,28-34]. Here we test whether cross-presentation of an exogenous HIV protein antigen, the gag p24 protein, to primed $CD8^+$ T cells can be enhanced by receptor mediated uptake. We will show that Flt3L DC efficiently cross-present HIV gag via the DEC-205 receptor, about 100 times more effective than non-targeted gag. 0.1 μg/ml of anti-DEC gag is as effective as 25 μg/ml of gag, suggesting that this strategy for increasing DC numbers be used to identify cross-presentation mechanisms and improve protein vaccines.

Results

Expansion and ready enrichment of $CD11c^+$ DC from mice harboring a Flt3L expressing B16 melanoma

We used anti-CD11c coated magnetic beads to enrich splenic $CD11c^+$ DC from CxB6F1 mice injected with B16 Flt3L secreting melanoma cells. As previously reported, Flt3L greatly expanded total DC numbers ~10 fold [26,35,36]. In our experiments 4-5 $\times 10^6$ cells were isolated with anti-CD11c beads from one spleen of an untreated mouse, whereas ~ 60-70 $\times 10^6$ cells could be enriched from the spleen of a mouse harboring a growing B16-Flt3L melanoma. When the DC from untreated and Flt3L mice were compared by flow cytometry in surface antigen expression (Suppl. Fig. 1 for gating conditions), the populations from Flt3L mice were much more enriched in CD11c high cells, ~95% vs. ~25% in untreated mice (Fig. 1). The CD11c-selected population from Flt3L treated mice was PDCA-1^{low}, $B220^{low}$ but $CD11c^{high}$, indicating a paucity of plasmacytoid DC (Fig. 1). The majority of the cells expressed MHC class II molecules, while the representation of the CD8α and DEC-205 subset was increased relative to untreated mice. However in three different experiments we observed that this increase was biased toward $DEC\text{-}205^+$ DC over $CD8^+$ cells, possibly because of the presence of a recently described $CD8\alpha^-$ $CD24^{hi}$ population of precursors to $CD8\alpha^+$ DC [37].

These results extend prior work on Flt3L [27] showing that the expansion induced by this hematopoietin allows for an increase in the purity of CD11c selected cells that are also enriched in DEC-205$^+$ DC.

Spleen CD11c$^+$ DC from Flt3L treated mice are functional antigen presenting cells

Since the DC generated from Flt3L treated mice expressed intermediate levels of I-A^b molecules (Fig. 1), we expected that the cells would be functionally immature. A distinguishing feature of mature DC is their capacity to stimulate naïve T cells in an allogenic mixed leukocyte reaction (MLR). Thus we tested whether DC isolated from Flt3L-treated mice exhibited allo-stimulatory activity, comparing it to untreated spleen DC (Fig. 2A). We evaluated the DC with or without direct *in vivo* activation with poly IC, which is known to mature DC [38]. Accordingly, Flt3L or untreated CxB6 F1 mice were injected with 50 μg of poly IC, and 15 h later, DC were enriched with anti-CD11c beads, fixed with para-formaldehyde to prevent further maturation in culture, washed, and added in graded doses to allogeneic T cells isolated from C57BL/6 mice, which had been labeled with CFSE. In Fig. 2A, we show that both DC populations, upon *in vivo* maturation, stimulated CD4$^+$ and CD8$^+$ T cells to proliferate. We also found that the Flt3L-mobilized DC had a stronger stimulating capacity being about 5 times more active than normal DC. This result can be explained by the enrichment in CD11c^{high} cells in DC preparations from Flt3L treated mice. Populations from Flt3L mice were almost entirely CD11c^{high} and MHC II^{high} DC, whereas populations selected with anti-CD11 beads from normal spleen were roughly 30% CD11c^{high} and MHC II^{high} DC and clearly had contaminating DX5$^+$ and CD19$^+$ NK and B cells (Fig. 1).

To deliver HIV antigens to DC, Trumpfheller et al. cloned HIV gag p24 protein within the heavy chain of a mAb specific for DEC-205 endocytic receptor [17]. We tested the ability of the Flt3L DC to mediate presentation to HIV specific CD8$^+$ and CD4$^+$ T cells after *in vitro* pulsing of DC with anti-DEC-205 HIV gag p24 fusion mAb (anti-DEC-p24) or a control Ig-p24 fusion antibody. Specifically, CD11c$^+$ populations were isolated from Flt3L treated and non treated mice. Then the DC were cultured overnight with poly IC in the presence of the indicated sources of gag antigen, washed to remove excess antigen and added to HIV gag specific T cells for 6 h. We found (Fig. 2B) that both DC populations stimulated IFN-γ production from HIV-gag primed CD8$^+$ and CD4$^+$ T cells isolated from mice primed with Adenovirus-gag p24 and boosted with anti-DEC-p24 and poly IC. Adenovirus-p24 primarily primes CD8$^+$ T cells, while anti-DEC-p24 along with poly IC induces CD4$^+$ T cells as described by Trumpfheller et al. [17]. The response to DC pulsed with anti-DEC-p24 was similar to a pool of pre-processed HIV gag 15 mer peptides, and anti-DEC-p24 antibody resulted in stronger responses than control Ig-p24 (Fig. 2B). There was a small difference between the DC from Flt3L treated mice and those from normal mice in their ability to stimulate antigen-primed T cells. Thus, Flt3L treatment induced high numbers of CD11c$^+$ DC that were functionally comparable to normal DC in their capacity to process and present *in vitro* anti-DEC-p24 mAb.

Altogether these results demonstrate that CD11c selected DC isolated from Flt3L-mobilized mice were functional in inducing MLR and in exogenous MHC class I and class II antigen-presentation pathways.

Cross-presentation of anti-DEC-p24 is promoted by *in vitro* maturation of Flt3L CD11c$^+$ DC and restricted to DEC-205$^+$ DC

Effective T cell stimulation only occurs if in parallel to antigen uptake, DC undergo maturation, a process that can be triggered by different pathogens or mimics of microbial agonists, such as poly IC for double stranded RNA or lipopolysaccaride respectively. *In*

vitro studies have indicated that DC maturation leads to enhanced cross-presentation as well as expression of the costimulatory molecules required for activation of $CD8^+$ T cells [30,39-42]. To confirm the capacity of maturation to promote cross-presentation, we enriched splenic $CD11c^+$ DC from mice treated with B16 Flt3L melanoma cells, added different sources of HIV gag protein or peptides for 5 h, and then cultured the cells overnight without or with 0.1 μg/ml of LPS or 25 μg/ml of poly IC as maturation stimuli. After extensive washing, the cells were added to HIV gag specific T cells in the presence of BFA, and cross-presentation was assessed 6 h later by intracellular cytokine staining for IFN-γ secretion by $CD8^+$ T cells.

In three different experiments (Fig. 3A), LPS or poly IC treatment improved cross-presentation relative to PBS treated DC, although the latter could undergo spontaneous maturation in culture as previously reported [43-46]. Although these results cannot be used to assess if immature Flt3L-mobilized DC have any cross-presenting function for stimulation of IFN-γ production from T cells, the results indicate that anti-DEC-p24 mAb is efficiently cross-presented by more mature Flt3L DC, and all our subsequent studies used poly IC to stimulate the antigen-pulsed Flt3L DC.

Splenic $CD8\alpha^+$ $DEC\text{-}205^+$ DC are specialized to cross-present cell-associated and protein antigens [4,19,21,25,47]. In Fig. 1 we have shown that $CD8\alpha^+$ $DEC\text{-}205^+$ cell numbers expanded considerably after Flt3L treatment with a profound increase skewed toward the $DEC\text{-}205^+$ subset (Fig. 1). To verify that $DEC\text{-}205^+$ DC were responsible for DEC-205 mediated cross-presentation of HIV gag, $CD11c^+$ $DEC\text{-}205^+$ and $CD11c^+$ $DEC\text{-}205^-$ cells were purified and sorted by FACS from the spleens of mice inoculated with B16 Flt3L melanoma cells (Methods and Suppl. Fig. 1). Sorted cells were pulsed with anti-DEC-p24, then poly IC was added. After 15 h, graded doses of antigen-pulsed and matured $DEC\text{-}205^+$ and $DEC\text{-}205^-$ cells were added to HIV gag primed T cells, and IFN-γ secretion was assessed 6 h later.

Both DC subsets were able to stimulate $CD8^+$ T cells to secrete IFN-γ after *in vitro* incubation with a pool of HIV gag p24 15 mer peptides (Fig. 3B). However, when we analyzed cross-presentation of gag protein within anti-DEC-p24 mAb, only the $DEC\text{-}205^+$ DC efficiently cross-presented HIV gag to primed $CD8^+$ T cells over a wide range of DC to T cell doses, 1:10-1:90 (Fig. 3B). In contrast, there was no presentation of control Ig-p24, indicating that the DEC^+ DC subset selectively cross-presents DEC-targeted protein.

With these results, we proceeded to carry out quantitative assays to assess the efficiency of HIV gag p24 cross-presentation by Flt3L-mobilized DC.

DEC-205 greatly increases the efficiency of HIV gag cross-presentation

To better evaluate the efficiency of cross-presentation by Flt3L-mobilized DC, we carried out more detailed studies of antigen and DC dose. When we pulsed Flt3L $CD11c^+$ DC with different doses of anti-DEC-p24 vs control Ig-p24 overnight, along with maturation by poly IC, escalating doses of anti-DEC-p24 resulted in an increased percentage of $IFN\text{-}\gamma^+$ $CD8^+$ T cells, reaching a plateau at only 1 μg/ml of the fusion antibody (Fig. 4A). When used to stimulate HIV gag primed T cells at a DC:T cell ratio of 1:3, the Flt3L DC pulsed with 0.1 μg/ml of anti-DEC-p24 induced higher levels of IFN-γ secretion by $CD8^+$ T cells than DC pulsed with 1 μg/ml control Ig-p24 (Fig. 4A). In Fig. 4B, we displayed the same results but from 4 different experiments.

Next, we assessed the role of DC dose. Flt3L DC were pulsed with a fixed dose of control Ig-p24 vs increasing concentrations of anti-DEC-p24 or gag peptides. After maturation with poly IC, DC were added in graded numbers to HIV gag primed/boosted T cells. *In vitro*

cross-presentation of anti-DEC-p24 by Flt3L DC occurred in a DC-dose dependent manner. Flt3L DC pulsed with anti-DEC-p24 stimulated IFN-γ secretion of $CD8^+$ T cells over a range of DC to T cell doses from 1:3 up to 1:90 (Fig. 4C, left panel). A similar DC dose dependence was obtained when we analyzed stimulation of $CD4^+$ T cells after anti-DEC-p24 *in vitro* pulsing (Fig. 4C, right panel).

Together the results indicate that a low concentration of anti-DEC-p24 antibody mediates presentation of gag protein to both HIV specific $CD8^+$ and $CD4^+$ T cells in a DC-dose dependent fashion.

DEC-205 targeting greatly enhanced cross-presentation of soluble HIV gag p24 protein

Previous studies showed that only relatively high doses of antigens e.g., 50-100 μg/ml OVA, are able to bring about cross-presentation through the exogenous MHC-I pathway. We therefore wondered if DEC targeting could increase presentation of protein that was not coupled to the anti-DEC antibody. We first compared cross-presentation of anti-DEC-p24 mAb with soluble HIV gag p24 protein. In Fig. 5A we found that cross-presentation of soluble HIV gag protein to specific $CD8^+$ T cells by Flt3L DC took place in a dose dependent manner, and that 25 μg/ml of gag was comparable to 1 μg/ml of anti-DEC-gag (or 0.25 μg/ml gag protein within the antibody). These results demonstrate that cross-presentation of anti-DEC-p24 by Flt3L DC improves efficiency almost 100 times relative to non-targeted gag protein.

Then to rule out a boosting effect of the anti-DEC antibody itself, we analyzed $CD8^+$ T cell responses to Flt3L DC pulsed *in vitro* with anti-DEC-p24 fusion mAb, unconjugated anti-DEC-205 mAb, the combination of unconjugated anti-DEC-205 with HIV gag protein, or the soluble HIV protein alone. As shown in Fig. 5B, the combination of unconjugated mAb with soluble HIV protein did not enhance gag presentation.

We conclude that introduction of a protein within anti-DEC-205 antibody greatly enhances the capacity of Flt3L-mobilized DC to cross-present a protective microbial antigen, HIV gag.

Discussion

The limited number of DC isolated from mouse spleen has often restricted their use for functional studies, particularly cross-presentation, where the relevant $CD8\alpha^+$ or $DEC\text{-}205^+$ DC [4,19-21,47] represent a relatively small fraction of CD11c high DC. Flt3L is a regulator of hematopoietic cell development and increases the number of peripheral DC in various tissues of mice [26,36]. It is now evident that the receptor, Flt-3 or Flt-2 or CD135, is a marker for committed progenitors of DC that form in the bone marrow and then continue to respond to Flt3L after migration via the blood into spleen and lymph nodes [48-53]. Interestingly the expansion in DC numbers was skewed towards the expansion of $DEC\text{-}205^+$ cells over $CD8^+$ DC. The $CD8^-$ $DEC\text{-}205^+$ DC may represent precursors to $CD8^+$ $DEC\text{-}205^+$ DC [37]. One emphasis of our current study is that when mice are exposed to Flt3L, it becomes much more feasible to study cross-presentation, since the numbers and purity of cross-presenting $DEC\text{-}205^+$ DC are greatly expanded.

The majority of studies of cross-presentation emphasize the clonal expansion of $CD8^+$ TCR transgenic T cells. Polyclonal T cells from HIV gag primed mice also respond to cross-presenting DC, although the efficiency of presentation was increased by targeting the gag protein to the DEC-205 receptor. Previously we found that the targeting of HIV gag protein to the DEC-205 receptor on monocyte-derived human DC enhanced cross-presentation to gag-specific $CD8^+$ T cells from individuals infected with HIV-1 [18]. In that study, we also

compared different receptors, DEC-205, MMR and DC-SIGN, and obtained evidence that antigen delivery via DEC-205 was superior to the other endocytic receptors for expanding gag-specific $CD8^+$ T cells [18]. Here our current studies were directed to DEC-205 targeting, where DEC-205 represents one receptor that is clearly expressed *in vivo* on most T cell area DC of human lymph nodes [54]. Other DC receptors and surface products might be expected to mediate improved presentation with antibody-targeted antigens. Here we provide quantitative information on the capacity of DC to present antigen via DEC-205, which also takes place in DC preparations that are abundant and highly enriched. Flt3L induced expansion of DC should greatly facilitate studies of cross-presentation and set the stage for the use of Flt3L to enhance the efficacy of DEC-205 targeted vaccines *in vivo*.

Immune responses require DC maturation induced by microbial molecular patterns, in particular by agonists for microbial pattern recognition receptors [55]. These agonists may directly enhance the intracellular mechanism for cross-presentation [29,40,42,56]. The role of maturation is difficult to study with isolated DC because these undergo what is termed “spontaneous” maturation in culture. Nevertheless, we were able to show that maturation of Flt3L DC, either with LPS or with poly IC, improved cross-presentation of anti-DEC-p24.

Traditionally cross-presentation requires high doses of antigens with the most sensitive protein, OVA, typically being used at concentrations of >50 μg/ml or 1 nM to detect a signal even with OT-I TCR transgenic reporter T cells [33,57]. In our prior experience with mouse DC, cross-presentation of OVA to OT-I T cells under similar culture conditions to the gag experiments in our paper required concentrations of 60-250 μg/ml OVA [13]. Here, by using the DEC-205 receptor to target the HIV gag protein to DC, we find that dose as low as 1 μg/ml of anti-DEC-p24 or 5 pM leads to strong presentation to $CD8^+$ T cells from a polyclonal population. Also, relative to non-targeted gag p24 protein, DEC-205 mediated cross-presentation is 100 fold more efficient. The quantitative aspects of our data highlight the potential value that Flt3L mobilization and receptor mediated targeting potentially play in the use of cross-presentation to present non-replicating antigens to $CD8^+$ T cells.

While the goal of this paper is to highlight the usefulness of Flt3L-mobilized DC in cross-presentation of a microbial protein, Flt3L mobilization may have a role in enhancing presentation of DC targeted vaccines. Others have reported that daily injections of Flt3L over a period of 9 days is able to expand DC numbers several fold in humans [58,59]. Further experiments are required to assess if there are ways to combine DC mobilization and DC targeting to improve vaccination.

Materials and Methods

Mice

Balb/c × C57Bl/6 (C × B6) F1 mice from Harlan were maintained under specific pathogen-free conditions and used at 6-8 wk of age in accordance with Rockefeller University Animal Care and Use Committee guidelines.

B16 Flt3L melanoma cells

Melanoma cells expressing Fms-like tyrosine kinase 3 ligand (Flt3L), were established via retroviral gene transfer [60] and generously provided by L. Santambrogio (Albert Einstein College of Medicine, New York, NY). B16 Flt3L melanoma cells were cultured with DMEM containing 10% FBS and 5×10^6 were injected s.c into the belly region of mice. After 15-20 days, all major splenic DC subsets had expanded >10 fold as shown previously [36] and reproduced here.

Antibodies and reagents

We purchased from BD Biosciences FITC-antibodies to CD3ε (145-2C11), B220 (RA3-6B2), MHC-II (IA^b) and DX5, PE- antibodies to CD8α (53-6.7), PDCA-1 and anti-CD11c (HL3 clone), PerCP-anti-CD4 (RM4-5), PerCP-Cy5.5-anti-CD8α, PE-Cy 7 anti-IFN-γ (XMG1.2), APC-antibodies anti-IFN-γ (XMG1.2) and thy 1.2 (CD 90.2, clone 53-2.1), Alexa Fluor 700 anti-CD3 (500A2), APC-Alexa 647-antibodies to DEC-205 and B220 as well as Cytofix/Cytoperm kit and Stabilizing Fixative. Anti-CD11c beads (N418) were from Miltenyi Biotec. Rat anti MHC class II (TIB120, M5/114.15.2) was from ATCC. Anti-rat IgG Dynalbeads and Live/Dead Fixable Aqua vitality dye were from Invitrogen. Polyinosinic-polycytidylic acid (poly IC) was from Thermo Scientific. Other reagents were LPS from *Escherichia coli* 0127:B8 and BFA from Sigma-Aldrich.

Fusion HIV gag mAb

The fusion mAb anti-DEC-p24 and the control Ig-p24 were prepared as described [17] and were characterized by SDS-PAGE and Western Blotting (HRP-conjugated anti-mouse IgG or anti-HIV gag). Binding of the fusion mAb to stable DEC-205 transfected CHO cells was tested by FACS analysis as described [17].

Soluble HIV gag p24 protein

The cDNA for the HIV gag p24 (clade B) was fused to the sequence containing a signal peptide and a FLAG epitope tag. This construct named SF-p24 (GenBank accession number GQ304738) was cloned into pCMV expression vector and stably transfected into CHO cells, in order to produce a soluble, FLAG-tagged (SF) protein of gag p24, which was purified without endotoxin contamination from the culture supernatants of CHO/SF-p24 cells, by anti-FLAG® M1 Affinity Gel (Sigma-Aldrich, St. Louis, MO) following manufacturer's instruction.

HIV gag peptide library

Overlapping (staggered by 4 aminoacids) 15 mer peptides spanning the entire HIV gag p17 or p24 sequence were synthesized by H. Zebroski in the Proteomics Resource Center (The Rockefeller University). HIV gag p24 or p17 peptides were resuspended at 1 mg/ml of each peptides in 100% DMSO and added to DC at 1 μg/ml.

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Cell preparation

Spleens were removed from Flt3L treated mice, cut in small fragments, and digested into single cell-suspensions with 400 U/ml collagenase D (Roche Applied Science) for 25 min at 37°C. After inhibition of collagenase with 10 mM EDTA, the cells were resuspended in PBS in 2 mM EDTA and 2% FCS. $CD11c^+$ DC were enriched by positive selection using anti-CD11c magnetic beads and MACS columns (Miltenyi Biotec). To purify $CD8\alpha^+$ and $CD8\alpha^-$ cells, $CD11c^+$ cells were sorted on a FACSVantage (BD Biosciences) into $B220^-$ $DX5^-$ $CD3^-$ $CD11c^{high}$ $CD8\alpha^+$ and $B220^-$ $DX5^-$ $CD3^-$ $CD11c^{high}$ $CD8\alpha^-$ fractions. The purity of the DC subsets was >95-99%. Antigen primed T cells, which were from F1 mice primed

with Adenovirus gag and boosted 4-6 wks later with anti-DEC-p24 and poly IC, were enriched by excluding MHC class II^+ cells using TIB120/M5/114 rat mAb and anti-rat IgG Dynalbeads.

Mixed leukocyte reaction (MLR) assay for DC maturation and function

B16 Flt3L treated or untreated CxB6 F1 mice were injected i.p. with PBS or 50 μg of poly IC. Fifteen hours later spleens were collected and collagenase digested. CxB6 DC were fixed for 20 min on ice and graded numbers were added to 3×10^5 CFSE labeled (Molecular Probes, Eugene, OR) C57BL/6 T cells. After 4 days of culture, samples were stained with Live/Dead Fixable Violet viability dye (Invitrogen, Carlsbad, CA), Alexa 700 anti-CD3, APC Alexa 780 anti-CD8 and PerCP Cy 5.5 anti-CD4, and acquired on a BD LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo Software (Tree Star, Inc.).

Ag-presentation assay to HIV gag specific T cells

2×10^6 splenic $CD11c^+$ DC, or purified DC subsets, were first pulsed with different antigens (Results) for 5 h in 24-well culture plates in a final volume of 0.5 ml RPMI 1640 containing 5% FCS and the supernatant (3% vol/vol) from J558L cells transduced with murine GM-CSF. Then 25 μg/ml poly IC or 0.1 μg/ml of LPS was added to the cultures for 15-18h. The DC were washed three times with PBS and added to antigen-primed T cells. IFN-γ production during a 6 h DC:T cell coculture in the presence of 10 μg/ml of BFA was monitored by washing the cells, incubating 10 min at 4°C with 2.4G2 mAb to block FcγR, and staining with Live/Dead Fixable Aqua, anti-CD3, anti-CD8 and anti-CD4 mAbs for 20 min at 4°C. Cells were fixed and permeabilized 10 min with Cytofix/Cytoperm and stained with APC-conjugated anti-IFN-γ mAb for 15 min at room temperature and resuspended in stabilizing fixative. 10^5 live-$CD3^+$ events were acquired using a BD LSR II flow cytometer. Data were analyzed with FlowJo Software.

Statistical analysis

Statistical significance was evaluated using two-tailed Student's t test, 95% confidence interval. Results are expressed as means ± SD. In the figures, *p* values of 0.05 are labeled with a single asterisk (*), 0.01 (**) or 0.001 (***). Analysis was performed with a Prism 3 program (Graphpad Sofware Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DC	dendritic cell
OVA	ovalbumin
Flt3L	Fms-like tyrosine kinase 3 ligand
mAb	monoclonal antibody
poly IC	polyinosinic:polycytidylic acid

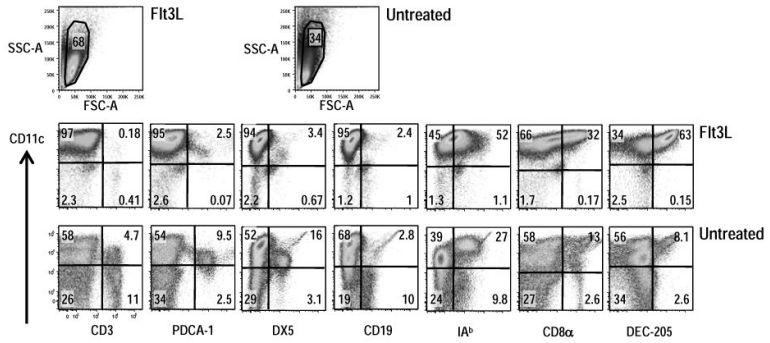

Figure 1. Cell surface markers of splenic CD11c$^+$ DC by flow cytometry

Splenic CD11c$^+$ cells were enriched by positive selection on anti-CD11c beads from CxB6 F1 mice that had been injected with B16 Flt3L melanoma cells or from untreated F1 mice. A six-color flow cytometry panel was used to simultaneously analyze surface markers on CD11c-selected cells. Appropriate isotype-matched, non-binding control Ig's were used to set the quadrant lines (not shown). DC were analyzed by gating against low scatter debris, followed by a live/dead exclusion gate. Analysis is representative of three independent experiments that all examined pooled splenic DC from at least two mice.

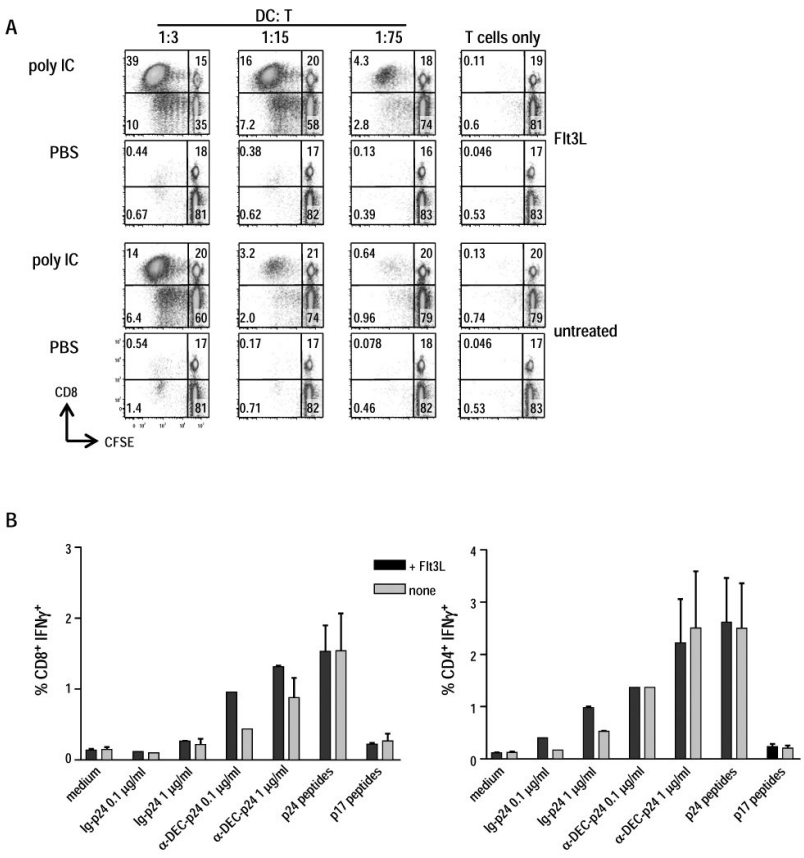

Figure 2. Comparision of antigen-presentation by Flt3L and untreated CD11c$^+$ DC

(A) B16 Flt3L-injected or untreated CxB6 F1 mice were stimulated with 50 μg of poly IC for 15 h and allo-stimulatory capacity of splenic CD11c$^+$ DC was compared. Both populations of DC were fixed with 1% para-formaldehyde, washed 3 times, and cultured at various numbers in the presence of a constant number (3×10^5) of T cells enriched from allogenic C57BL/6 mice. T cell proliferation was detected after 4 days of culture by CFSE dilution of CD8$^+$ and CD4$^+$ T cells. Similar results were obtained using allogeneic T cells from the SJL mouse strain. Data are representative of two independent experiments. (B) Splenic CD11c$^+$ cells purified from Flt3L or untreated F1 mice were pulsed *in vitro* for 4-5 h with anti-DEC-p24, control Ig-p24 at 0.1 and 1 μg/ml, p24 or p17 15-mer peptides mix (1 μg/ml), followed by 25 μg/ml of poly IC. Fifteen hours later, 1×10^6 washed DC were added to 3×10^6 T cells for 6 h in the presence of BFA (10 μg/ml) to detect IFN-γ secretion by intracellular cytokine staining. The T cells were isolated from F1 mice that were primed with Adenovirus gag and boosted with anti-DEC-p24 and poly IC (Methods). Data show mean ± SD of the percentage of CD8$^+$ IFN-γ$^+$ T cells (left) or CD4$^+$ IFN-γ$^+$ T cells (right) representative of two independent experiments.

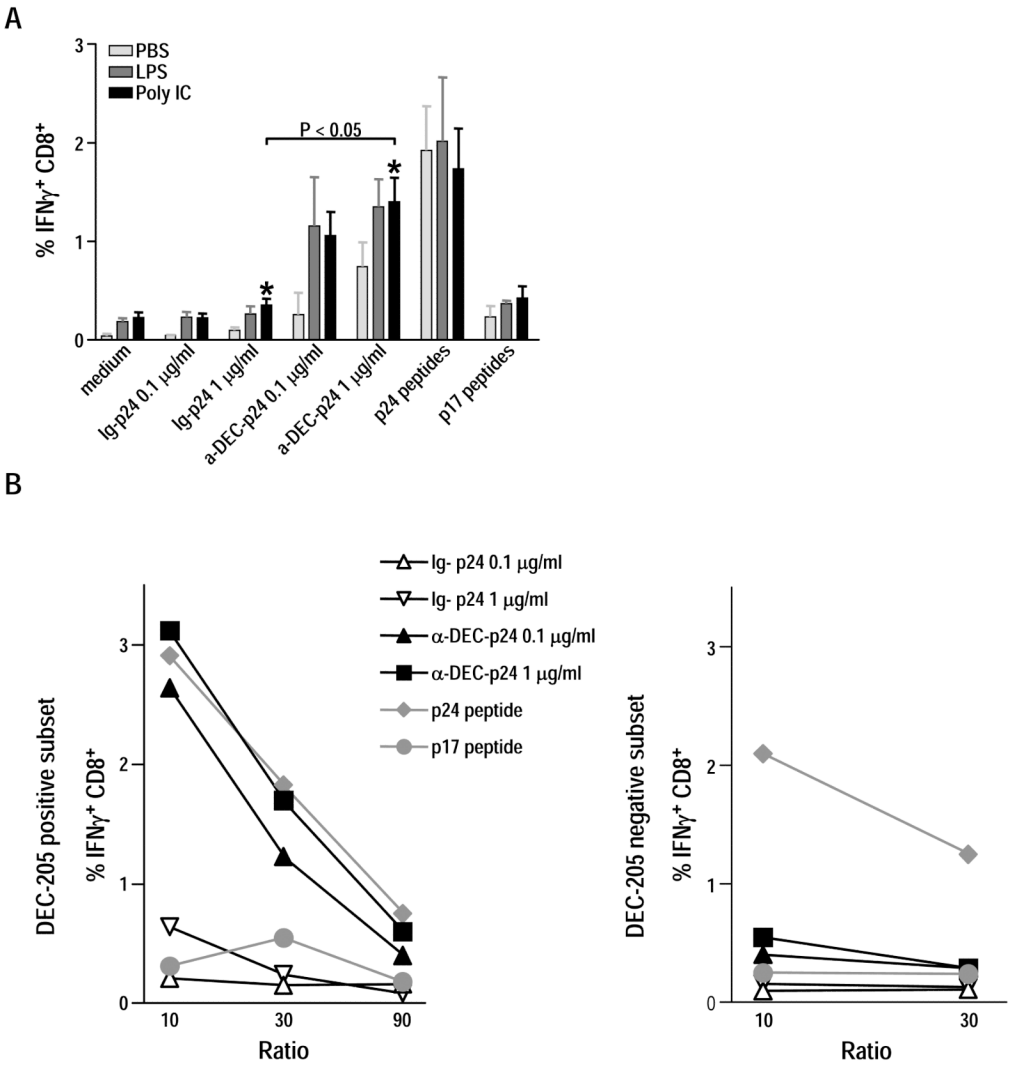

Figure 3. Cross-presentation of anti-DEC-gag p24 is enhanced by poly IC and is restricted to DEC-205$^+$ DC

(A) Splenic Flt3L-mobilized CD11c$^+$ DC were pulsed *in vitro* with 0.1 and 1 μg/ml of anti-DEC-p24, control Ig-p24, or p24 or p17 15-mer peptides (1 μg/ml). Five hours later, medium or LPS (0.1 μg/ml) or poly IC (25 μg/ml) were added. After overnight culture, the yields of cells were comparable. The cells were washed and added for 6 h to HIV gag primed T cells at a ratio of 1:3 with 10 μg/ml BFA. Data show mean ± SD percentage of CD8$^+$ IFN-γ$^+$ T cells pooled from three independent experiments. *p<0.05, paired Student's t-test, 1 μg/ml of anti-DEC-p24 *vs.* 1 μg/ml of control-Ig-p24 after maturation with poly IC. (B) CD11c$^+$ cells from mice injected with B16 Flt3L melanoma cells were enriched by MACS positive selection and then separated into DEC-205$^+$ and DEC-205$^-$ CD11c^{high} DC subsets by FACS. Sorted cells were washed and pulsed with either medium, anti-DEC-p24 vs. control Ig-p24 at 0.1 or 1 μg/ml, or p24 vs p17 peptides at 1 μg/ml. Five hours later, without washing off the antigens, poly IC at 25 μg/ml was added. After 15 h, graded doses of DEC-205$^+$ and DEC205$^-$ cells (x-axis for DC:T cell ratio) were added to 3×10^6 HIV gag primed T cells, and IFN-γ secretion was assessed by intracellular cytokine staining 6 h later. Frequencies of CD8$^+$ IFN-γ$^+$ T cells from two independent experiments are shown after stimulation by DEC-205$^+$ (left panel) and DEC-205$^-$ cells (right panel).

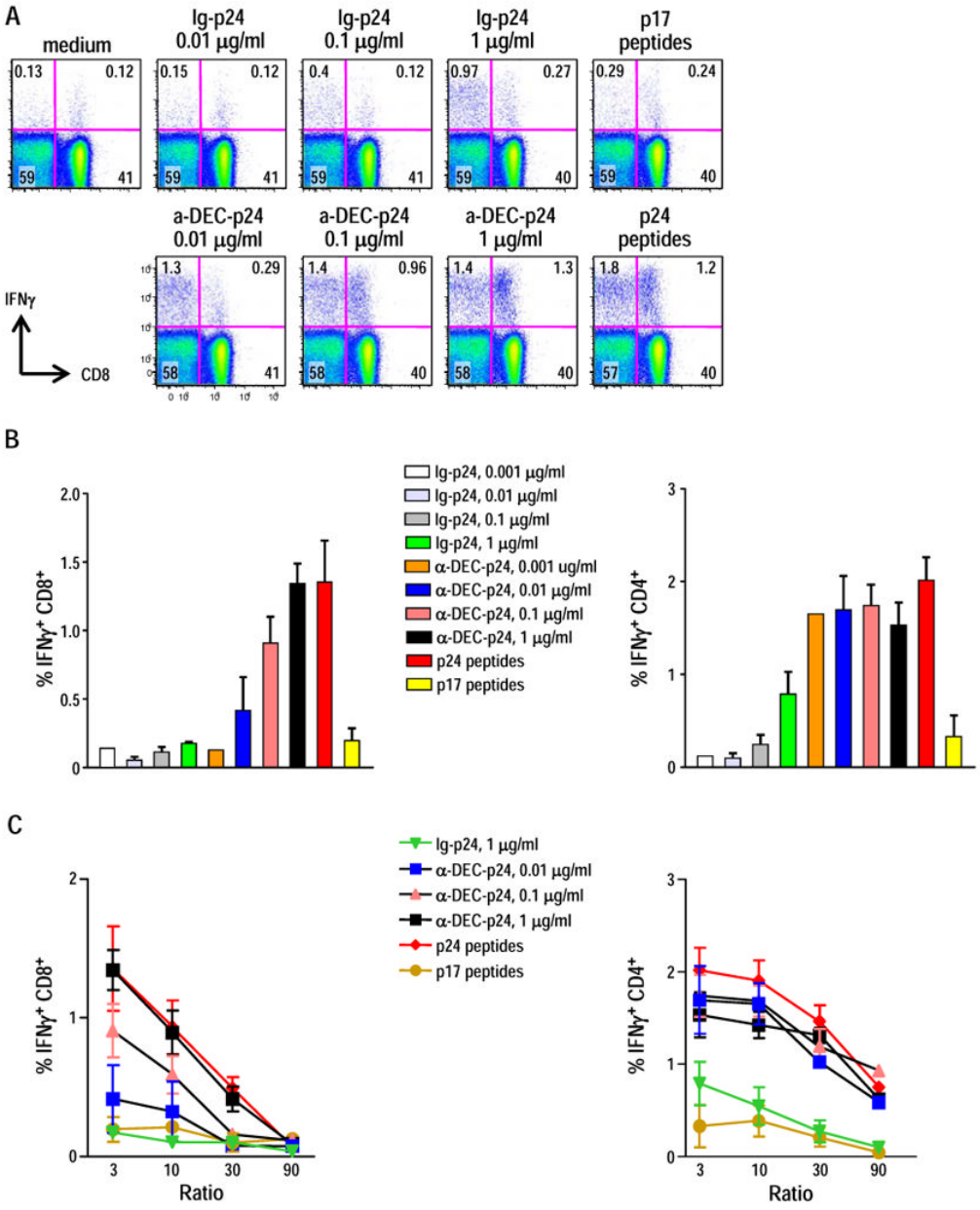

Figure 4. Efficient gag-presentation to CD8$^+$ and CD4$^+$ T cells following *in vitro* targeting of Flt3L DC with anti-DEC-gag p24 mAb

(A) Splenic Flt3L CD11c$^+$ DC pulsed with anti-DEC-p24, control Ig-p24, p24 or p17 peptides at the concentration indicated, matured overnight with poly IC 25 µg/ml were used to stimulate IFN-γ secretion by HIV gag primed T cell in a 6 h assay at 1:3 DC: T cell ratio. Dot plots are representative of three independent experiments. (B) As in A, but Flt3L DC were pulsed with limiting dilutions of anti-DEC-p24 and control Ig-p24, matured with poly IC and cocultured for 6 h with primed HIV gag T cells. IFN-γ secretion from CD8$^+$ T cells and CD4$^+$ T cells was assessed by intracellular cytokine staining. Data show mean ± SD for the percentage of CD8$^+$ IFN-γ$^+$ T cells (left) or CD4$^+$ IFN-γ$^+$ T cells (right) from four independent experiments. (C) Graded doses of Flt3L DC, pulsed and matured as described in B, were cocultured for 6 h with primed HIV gag T cells (x-axis for DC:T cell ratio). IFN-

γ secretion from $CD8^+$ T cells (left) and $CD4^+$ T cells (right) was assessed by intracellular cytokines. Mean ± SD of the frequencies of IFN-γ producing $CD8^+$ and $CD4^+$ T cells in three to four independent experiments are indicated.

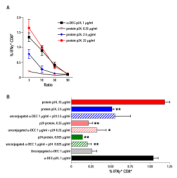

Figure 5. Conjugation of DEC-205 to HIV gag, but not DEC-205 itself, greatly enhances cross-presentation of soluble HIV gag p24 protein

(A) Splenic Flt3L CD11c$^+$ DC were pulsed with 1 μg/ml of anti-DEC-p24 or increasing doses of HIV gag p24 soluble protein. The DC were matured with 25 μg/ml poly IC and added at various doses to stimulate primed HIV gag T cells in a 6 h coculture (x-axis for DC:T cell ratio). IFN-γ secretion from CD8$^+$ T cells was assessed by intracellular cytokine staining. Data show mean ± SD of three independent experiments. (B) Splenic Flt3L CD11c$^+$ DC were pulsed with 1 μg/ml of anti-DEC-p24, or 1 μg/ml of unconjugated anti-DEC, or different combinations of HIV gag p24 protein and unconjugated anti-DEC, or limiting doses of HIV gag p24 protein. As above, the DC were matured with poly IC and added at various doses to stimulate primed HIV gag T cells in a 6 h coculture (x-axis for DC:T cell ratio). IFN-γ secretion from CD8$^+$ T cells was assessed by intracellular cytokine staining. Data show mean ± SD of two independent experiments. *$p<0.05$, **$p<0.01$, paired Student's t-test; 1 ug/ml of anti-DEC-p24 *vs.* other samples.