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# Induction of Innate and Adaptive Immunity In Vivo by Selective Antibody Targeting of Poly dA:dT and Antigen to Dendritic Cells

Scott A. Barbuto

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**INDUCTION OF INNATE AND ADAPTIVE  
IMMUNITY *IN VIVO* BY SELECTIVE ANTIBODY  
TARGETING OF POLY dA:dT AND ANTIGEN TO  
DENDRITIC CELLS**

A Thesis Presented to the Faculty of  
The Rockefeller University  
in Partial Fulfillment of the Requirements for  
the degree of Doctor of Philosophy

By  
Scott A. Barbuto  
June 2012



# INDUCTION OF INNATE AND ADAPTIVE IMMUNITY IN VIVO BY SELECTIVE ANTIBODY TARGETING OF POLY dA:dT AND ANTIGEN TO DENDRITIC CELLS

Scott A. Barbuto, Ph.D.  
The Rockefeller University 2012

Dendritic cells (DCs) have been shown to be efficient and specialized in antigen presentation, but innate stimuli have not been selectively delivered to determine if these cells alone can integrate innate and adaptive immunity *in vivo*. Here we illustrate a novel method using expressed protein ligation (EPL) to attach the immune stimulant, poly dA:dT, to the DC uptake receptor, DEC205 (DEC). We will show that inoculation of  $\alpha$ -DEC-poly dA:dT fusion monoclonal antibodies (mAbs) is sufficient to induce rapid innate cytokine production by DCs. Adaptive T cell immunity is also elicited when the fusion mAb is given in combination with a DC-targeted antigen. Forced entry is not required because  $\alpha$ -DEC fusion mAbs deliver the poly dA:dT to its cytosolic targets.



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## List of Abbreviations

APC: Antigen Presenting Cell

BSA: Bovine Serum Albumin

CDP: Common-DC-Progenitors

CFSE: 5-(6)-carboxyfluorescein diacetate succinimidyl diester

CHO: Chinese Hamster Ovary

CLEC: C-type Lectin-Like Receptor

CLR: C type-Lectin Receptor

CuAAC: Copper(I)-catalyzed Huisgen 1,3 dipolar cycloaddition of azides and alkynes

Cys-DNA: N-terminal cysteine DNA

Cy5: Cyanine 5

DC: Dendritic Cell

DCM: Dichloromethane

DEC: DEC205/CD205

DIC: Diisopropylcarbodiimide

DMF: Dimethylformamide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

ED-B: Extra Domain of Fibronectin (ED-B)

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-linked Immunosorbent Assay

ER: Endoplasmic Reticulum

EPL: Expressed Protein Ligation

FCS: Fetal Calf Serum

FITC: Fluorescein Isothiocyanate

FMOC: Fluorenylmethyloxycarbonyl

GFP: Green Fluorescent Protein

GM-CSF: Granulocyte-macrophage Colony-stimulating Factor

HAV: Hepatitis A Vaccine

HBSS: Hanks Balanced Salt Solution

HBV: Hepatitis B Vaccine

HEK: Human Embryonic Kidney

HI: Hemagglutination inhibition

HIS-Tag: Poly-histidine peptide

HIV: Human Immunodeficiency Virus

HPV: Human Papillomavirus

HRP: Horseradish Peroxidase

Ig: Immunoglobulin

IKK: I $\kappa$ B (inhibitor of NF- $\kappa$ B) kinase

IL: Interleukin

i.p.: Intraperitoneal

LPS: Lipopolysaccharides

MBP: Maltose Binding Protein

MDP: Macrophage and Dendritic Cell Progenitor

MESNA: Mercaptoethanesulfonic acid

MHC: Major histocompatibility Complex

MMR: Macrophage Mannose Receptor

MoDCs: Monocyte Derived Dendritic Cells

MyD88: Myeloid Differentiation Primary Response Gene 88

Mxe GyrA: *Mycobacterium xenopi* DNA gyrase

NOD: Non-obese diabetic

NTA: Nitrilotriacetic acid

OVA: Ovalbumin

PBS: phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDC: Plasmacytoid DC

PDL-1: Programmed cell death ligand- 1

PE: Phycoerthrin

PerCP: Peridinin Chlorophyll A Protein

PFTase: Protein-farnesyl Transferase

RIG-I: Retinoic Acid-Inducible Gene I

RPM: Revolutions Per Minute

SE: Standard Error

SPPS: Solid Phase Peptide Synthesis

TBK1: NF- $\kappa$ B activator (TANK)-binding kinase 1

TEAA: Triethylamine/acetate

TFA: Trifluoroacetic Acid

TIS: triisopropylsilane

TLR: Toll-Like-Receptor

TNF: Tumore Necrosis Factor

TRIF: TIR Domain-containing Adapter-inducing Interferon  $\beta$

WT: Wild Type

# **Chapter I**

## **Introduction**

Since the human immunodeficiency virus (HIV) was discovered to cause Acquired Immunodeficiency Syndrome (AIDS) in 1984, there has been an urgency to create a vaccine to prevent the spread of disease. Despite adequate information on the HIV genome and its proteins, researchers have failed to create candidate vaccines that induce the broad and long-lasting T cell-mediated immunity necessary for protection (Mascola et al., 2001). Better vaccine delivery and vaccine adjuvants, or enhancers of immunity, are required to elicit vaccination.

In contrast to humoral immunity which relies on B cells and their antibody products to react directly with native antigens, T-cell mediated immunity requires recognition of fragmented antigens displayed on infected cells' surface membranes (Pamer et al., 1998). To elicit T cell immunity, vaccine antigens must be captured, processed, and bound to antigen presenting molecules, typically those of class I or class II MHC (Steinman et al., 2002). For this purpose, dendritic cell (DC) physiology can be exploited to improve vaccine antigen presentation.

DCs are professional antigen presenting cells, necessary for maintaining tolerance and initiating immune responses (Steinman et al., 2002).. It has been shown that a peptide sequence delivered specifically to dendritic cells is displayed 100 to 1000 times more effectively than a non-specific adjuvant (Hawiger et al., 2001). Vaccine antigens can also

be targeted to specific antigen uptake receptors on DCs. In this way, the vaccine can access the DC's most efficient antigen processing receptors, in particular, receptors that can process the delivered antigen on both class I and class II MHC (Steinman et al., 2002).

However, to induce immunity and not tolerance, DCs must be activated when the specific antigen is delivered, making the use of adjuvants critical. In this thesis, I develop a method to ligate poly dA:dT, an immune stimulant, to an antibody to the C-type lectin receptor, DEC205. In this way, the poly dA:dT is delivered directly to the DCs that need to be stimulated for immunization of an antigen. The anti-DEC205-poly dA:dT antibodies were examined for their capability to induce innate and adaptive immune responses *in vitro* and *in vivo*. The implications of directly targeting the immune stimulant to the DCs are discussed.

## **Adjuvants**

Vaccination remains the most valuable tool for preventing infectious diseases. The use of well-defined antigens for the generation of subunit vaccines has led to products with an improved safety profile. However, purified antigens are usually poorly immunogenic, making essential the use of adjuvants (Ebensen et al., 2008). However, only a handful of vaccine adjuvants are licensed for human use. Thus, the development of new adjuvants, which are able to promote broad and sustained immune responses, remains a major goal in vaccinology (Ebensen et al., 2008).

For more than 70 years, alum (aluminum salts) has been licensed as a vaccine adjuvant for prophylaxis in humans. Today, alum is the most widely used adjuvant and is found in numerous vaccines, including HAV, HBV, HPV, Diptheria and Tetanus (DT), Haemophilus influenza type B (HIB), and pneumococcal conjugate vaccines (Mbow et al., 2010). Alum appears to work by increasing antigen uptake and stability at the site of injection (Morefield et al., 2005). In addition, alum induces a local pro-inflammatory reaction that can increase immunogenicity (Goto et al, 1982 and 1997). Several independent studies have shown that alum also activates NALP3, a component of the inflammasome complex, implicated in the induction of several proinflammatory cytokines (Eisenbarth et al, 2008, Kool et al., 2008, and Li et al., 2008). However, it has also been demonstrated that the NALP3 pathway is dispensable for alum-mediated adjuvant activity *in vivo* (Franchi et al., 2008 and McKee et al., 2009). Thus, it appears

that redundant pathways modulate the activity of alum in the host. Although alum is the most widely used adjuvant, pre-clinical and clinical studies have shown that alum is often less potent than other adjuvants. In addition, alum is a poor inducer of protective TH-1 associated immune responses. TH-1 response, or cell-mediated immunity, is characterized by the production of cytotoxic T-lymphocytes, macrophages, NK cells and cytokines, which are crucial for the development of vaccines against intracellular pathogens (Li et al., 2008). This is in contrast to TH-2 response, or humoral immunity, which involves the production of antibody molecules in response to antigen and is mediated by B-lymphocytes.

The squalene-based oil-water emulsion, MF59, has been licensed in Europe for adjuvanted Flu vaccines since 1997. This emulsion has been shown to increase hemagglutination inhibition (HI) titers and cross protection in both the elderly and the young (Podda et al., 2001). In 2009, a clinical trial demonstrated that Avian H5 pandemic flu vaccines containing MF59 were superior to alum-adjuvanted and non-adjuvanted vaccine; MF59 containing vaccine had increased seroconversion and cross-presentation, promoted CD4<sup>+</sup> T cell response that predicts long term persistency of protective antibodies, and allowed for better recall responses in individuals boosted many years after their primary vaccination (Clark et al., 2009). The mechanism of action for MF59 is to induce a local immunostimulatory environment at the injection site characterized by up-regulation of cytokines, chemokines, and other innate immunity genes (Mosca et al., 2008). MF59 is also known to enhance antigen uptake by dendritic cells (Dupuis et al., 1998).



More recently, a new class of vaccine adjuvants have been designed that target the Toll-Like-Receptor (TLR) pathways. TLR receptors are essential to the role in the innate immune response. They are single, membrane-spanning receptors that recognize structurally conserved molecules derived from microbes. Activation of TLRs leads to the transcriptional activation of genes encoding for pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which subsequently control the activation of immune responses (Didierlaurant et al., 1998). Table 1 summarizes the main antigens for the TLRs as well as some other important immune sensors.

The TLR4 agonist, AS04 (3-O-desacyl-4'-monophosphoryl lipid A/MPL), is currently the only adjuvant of this type to be licensed; it has been approved for use against human papilloma virus and hepatitis (Harper et al., 2009). Studies indicated that AS04 produced a transient and local activation of NF-kB activity and cytokine production, thus providing an innate immune signal for activation of antigen presenting cells (Didierlaurant et al., 1998). AS04 is adsorbed to alum, yet no synergistic effect between the two compounds was noted. It appears, however, that the alum prolongs the cytokine response of AS04 at the injection site.

There are numerous TLR agonists in pre-clinical and clinical trials for use as vaccine adjuvants (Mbow et al., 2010). A TLR9 agonist, 1018 ISS, has been effective in boosting the recombinant hepatitis B surface antigen vaccine (Barry et al., 2007). Another TLR9 agonist based adjuvant, IC31, consists of a cationic peptide KLKL(5)KLK vehicle and

**Table 1: PRRs and their ligands.** Pattern recognition receptors (PRRs) are proteins expressed by cells to identify pathogen-associated molecular patterns (PAMPs). The table lists three types of PRRs: Toll-Like Receptors (TLRs), Retinoic acid-inducible (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs).

**Table 1**

<b>PRRs</b>	<b>Localization</b>	<b>Ligand</b>	<b>Origin of Ligand</b>
<b>TLR</b>			
TLR1	Plasma Membrane	Triacyl Lipoprotein	Bacteria
TLR2	Plasma Membrane	Lipoprotein	Bacteria, virus, parasite, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma Membrane	LPS	Bacteria, virus, self
TLR5	Plasma Membrane	Flagellin	Bacteria
TLR6	Plasma Membrane	Diacyl Lipoprotein	Bacteria, virus
TLR7	Endolysosome	ssRNA	Virus, bacteria, self
TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
<b>RLR</b>			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA virus, DNA virus
MDA-5	Cytoplasm	Long dsRNA	RNA virus
<b>CLR</b>			
Dectin-1	Plasma Membrane	$\beta$ -Glucan	Fungi
Dectin-2	Plasma Membrane	$\beta$ -Glucan	Fungi
DEC205	Plasma Membrane	Unknown	Unknown

the immunostimulatory oligodeoxynucleotide ODN1a sequence. The complex between the bacterial peptide and ODN results in prolonged retention of antigen-adjuvant formulation and subsequent amplification of immune response. This adjuvant is currently undergoing human clinical trials in a vaccine against TB (Agger et al., 2006 and Aagaard et al., 2009). The small molecules imiquimod (TLR7 agonist) and resiquimod (TLR 7/8 agonist) were shown to improve the immunogenicity of a variety of vaccine adjuvants if adequately formulated or directly conjugated to protein antigens (Wille-Reece et al., 2005)

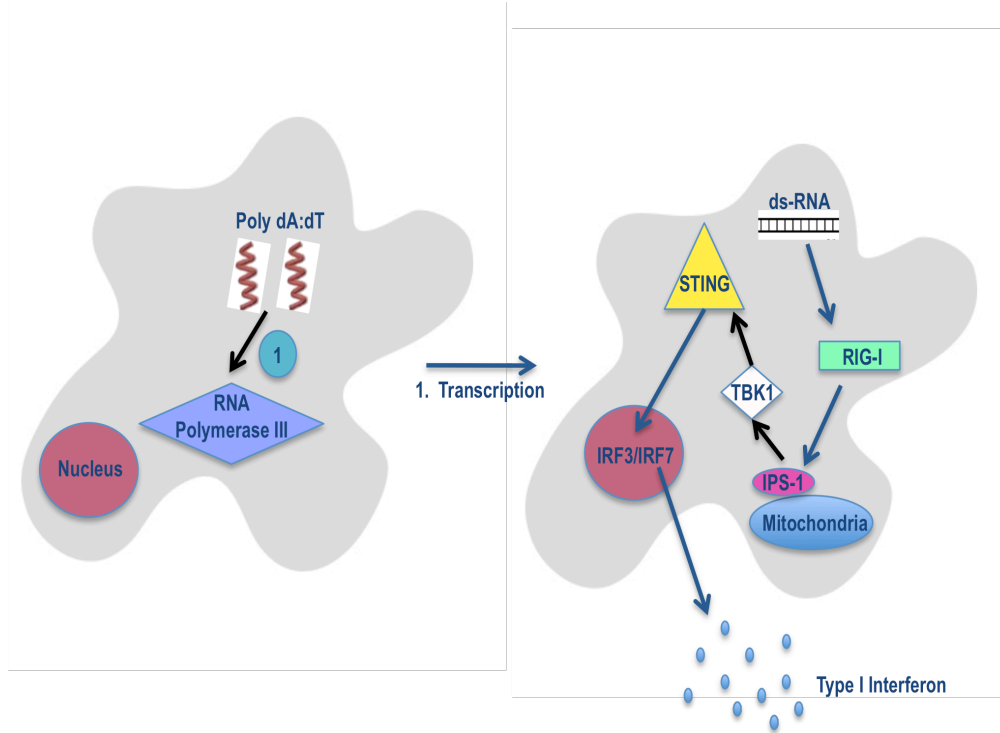
In addition to the TLR pathway, there are other innate pathways that could be exploited to design effective and potentially safe vaccine adjuvants. The intracellular innate receptor, retinoic acid inducible gene I (RIG-I), is one possible candidate. This receptor recognizes double-stranded RNA and initiates signaling cascades that lead to the activation of the protein kinases IKK $\alpha\beta$ , TBK1, and IKK $\epsilon$  and subsequent activation of the transcription factors NF-kB and IRF3 (Sen et al., 2005). The activation of these transcription factors leads to the production of interferon alpha/beta and tumor necrosis factor alpha (TNF- $\alpha$ ). RIG-I is known to strongly bind poly I:C, poly A:U, and 5' and 3' untranslated regions of hepatitis C virus genomic RNA. It does not bind dsDNA, poly(rA), or yeast tRNA (Yoneyama et al., 2007).

Recently, Ablasser *et. al* described a novel pathway where poly dA:dT activated RIG-I *in vitro*. In one key experiment, it was shown that knockdown of RIG-I significantly decreased the production of IFN- $\alpha$  in human monocyte derived dendritic cells (MoDCs)

when lipofectamine-transfected with poly dA:dT (Ablasser et al., 2009). Furthermore, it was determined that RNA polymerase III was also essential for cytokine production in this assay. It was concluded that RNA polymerase III transcribes poly dA:dT to poly (rA:rU), which then activates RIG-I (Ablasser et al. and Chiu et al., 2009). This activation was abolished when RNA 5'-polyphosphatase was present, indicating the importance of 5'-triphosphorylation. Of note, the pathway appears to be different in humans and mice; knockdown of RIG-I does not significantly reduce the amount of IFN- $\alpha$  in mouse dendritic cells (Ablasser et al., 2009). Figure 1 depicts the pathway described above.

**Figure 1: Poly dA:dT Activation Pathway.** Poly dA:dT enters into the cytoplasm of a cell with the help of lipofectamine. It is transcribed into poly (rA:rU) with 5' triphosphorylation by RNA polymerase III. The ds-RNA activates RIG-I, which in turn, activates IPS-1 on the mitochondria. This activates the molecule TANK-binding kinase 1 (TBK1) which interacts with STING to lead to gene transcription of IRF3 and IRF7 in the nucleus. Type I interferon is produced and secreted from the cell.

**Figure 1**



One of the goals of this project was to produce a potential vaccine adjuvant that would activate RIG-I. Since RIG-I is intracellular, delivery of poly dA:dT to antigen presenting cells is difficult. To circumvent this problem, poly dA:dT was linked to the DEC205 antibody. DEC205 is a receptor on DCs that is primed for antigen presentation. It is able to present both MHC class I and class II molecules; thus, it is said to be able to cross-present. As a result of this, it was speculated that poly dA:dT would be delivered to the cytoplasm of the immune cells when linked to the DEC205 antibody.



## **Dendritic Cells**

DCs were first recognized in 1973 for their unique morphology (Steinman et al., 1973).

It took many years to convince the scientific community that DCs were distinct from macrophages, the best antigen presenting cell known at the time. Now, DCs are known to be the most potent stimulators of immune reactions and effector T cell responses, orders of magnitudes better than macrophages (Steinman, R.M., 2007 and Nussenzweig et al., 1980).

DCs can be found in both lymphoid and non-lymphoid tissues. Although there is not a single unique feature that can distinguish all DCs, they are characterized by expression of CD11c and high levels of MHCII. DCs consist of a heterogeneous population that can be divided into several subsets (Edelson et al., 2010).

Like all other immune cells, DCs originate from hematopoietic stem cells (Figure 1). Their half-life is short, and DCs are replaced by progenitors from the blood within 14 days (Liu et al., 2007). Although closely related, the development of DCs, monocytes, and macrophages is distinct. In the bone marrow, macrophage and DC progenitors differentiate into either monocytes or common-DC-progenitors (CDPs) (Naik et al., 2007). CDPs no longer give rise to monocytes or macrophages, and they further differentiate into pre-DCs, losing the potential to produce plasmacytoid DCs (PDCs) (Naik et al., 2007). Pre-DCs leave the bone marrow, circulate in the blood, and seed lymphoid and non-lymphoid organs. It is outside the bone marrow where these cells

differentiate into subsets of conventional DCs (Liu et al., 2007 and Bogunovic et al., 2009).

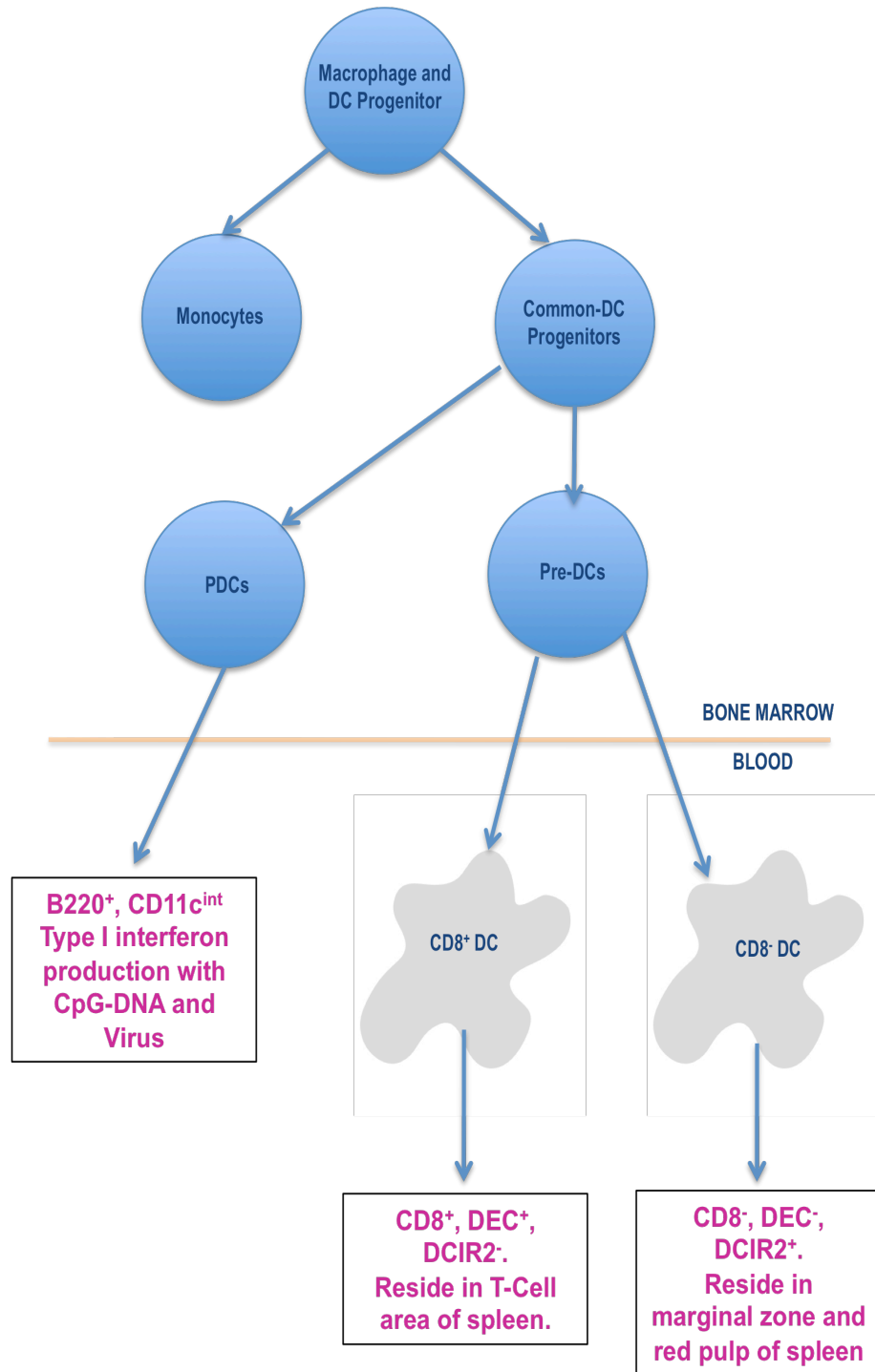
PDCs are very distinct from other DCs, known as conventional DCs. PDCs are B220<sup>+</sup> and CD11c<sup>int</sup> and produce large amounts of type I interferon upon CpG DNA or viral exposure. PDCs are long-lived, do not display dendritic-like morphology, and finish their development in the bone marrow (Gillet et al., 2008 and Liu et al., 2005).

Conventional DCs can be divided into two major subsets based on their expression of CD8. The CD8<sup>+</sup> subset is also positive for DEC205. The CD8<sup>-</sup> DC subset includes a major CD4<sup>+</sup> (CD11b<sup>+</sup> and DCIR2<sup>+</sup>) population and a minor CD4<sup>-</sup> (double negative) population. CD8<sup>-</sup> DCs reside in the marginal zone and red pulp of spleen whereas CD8<sup>+</sup> DCs localize in the T cell area (Naik et al., 2007 and Arvadin et al., 2003).

CD8<sup>-</sup> and CD8<sup>+</sup> DCs both function in similar ways. In the steady state, they both capture antigens and present them to T cells, ensuring tolerance by promoting regulatory T cell development or deletion and anergy of self-reactive T cells (Steinman et al., 2003). They also have an innate sensing function, reacting to potential pathogens and changing the context in which they present antigens to T cells in order to initiate adaptive immune responses (Joffre et al., 2009). Thus, CD8<sup>-</sup> and CD8<sup>+</sup> DCs can both induce tolerance or immunity depending on the way in which they capture antigens.

**Figure 2: Dendritic Cell Morphology.** In the bone marrow, macrophage and DC progenitors differentiate into either monocytes or common-DC-progenitors (CDPs). CDPs further differentiate into pre-DCs, losing the potential to produce plasmacytoid DCs (PDCs). Pre-DCs leave the bone marrow, circulate in the blood, and seed lymphoid and non-lymphoid organs where they differentiate into subsets of conventional DCs.

**Figure 2**



## **DEC205 Receptor**

DCs are both efficient and specialized in antigen presentation, and in addition, they control the magnitude, quality, and memory of the ensuing immune response (Steinman et al., 2002). Receptors involved in the recognition and uptake of antigens into DCs are crucial for establishing a balanced immune response. In particular, pattern recognition receptors such as C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) have an important role in the recognition of antigens. CLRs are important for the recognition and internalization of glycosylated antigens into intracellular compartments present in DCs, leading to processing and presentation on MHC molecules. CLRs do not differentiate between glycans of self and non-self origin (van Kooyk, Y., 2008). In contrast, TLRs are key receptors for the induction of intracellular signaling cascades after recognition of pathogens; TLR ligation leads to DC maturation and activation, often resulting in robust immune responses and induction of effector T-cells (Figdor et al., 2002). CLR ligation does not necessarily result in the induction of effector T-cells, but facilitates the antigen-presentation capacity of DCs. Thus, uptake of antigen by CLRs without any TLR ligation may induce antigen-specific tolerance (van Kooyk, Y., 2008).

DEC-205/CD205 (DEC) is a CLR with multiple external and contiguous carbohydrate recognition domains (Steinman et al., 2005). It is a 205 kDa protein and a homologue of the macrophage mannose receptor (MMR) (Mahnke et al., 2000). The amino termini of both MMR and DEC205 have two domains, one cysteine rich and the next fibronectin like, followed by 10 external contiguous carbohydrate recognition domains. Both

receptors also have cytosolic domains with motifs for localization to coated pits and rapid entry into the cell (Jiang et al., 1995).

Despite their similar structures, MMR and DEC205 were found to have different intracellular trafficking patterns. DEC205 does not recycle through peripheral endosomes, like most recycling endosome receptors, but instead moves through late endosomes or lysosomes that are rich in MHC class II. This feature of DEC205 was attributed to a triad of acidic amino acids in the cytosolic domain, and was shown to cause more efficient antigen presentation on MHC class II products relative to ligands for MMR (Figure 3) (Mahnke et al., 2000).

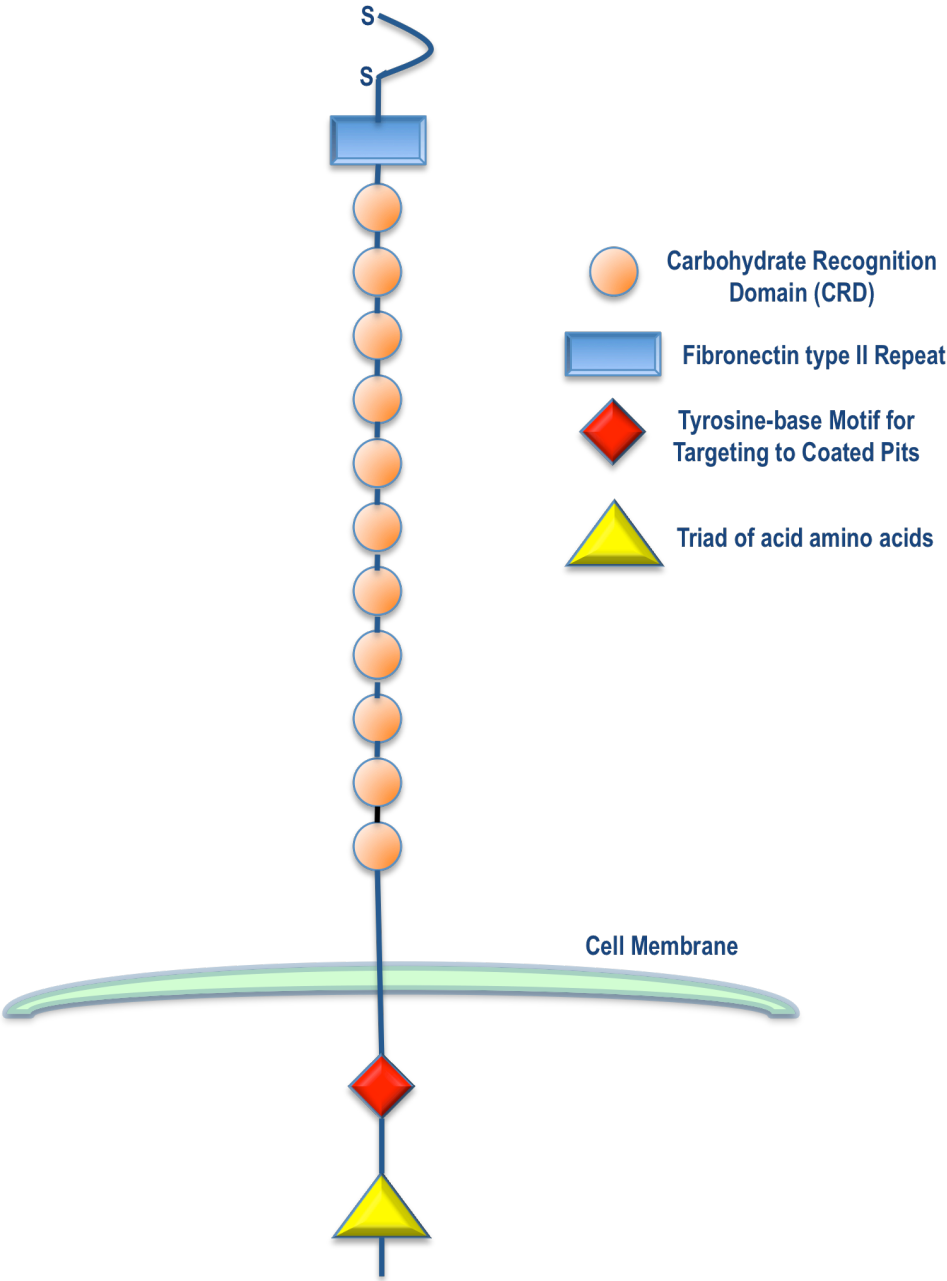
In both human and mouse, DEC205 is expressed by thymic cortical epithelium and DC, but can also be detected on lymphocytes (Butler et al., 2007). T cells, B cells and CD14<sup>+</sup> monocytes were all characterized to have some level of expression for DEC205. All subsets also carried mRNA for DEC205 (Kato et al., 2006). Thus, DEC205 is widely expressed amongst hematopoietic subsets, but higher levels tend to be detected on cells with antigen presenting capability. Although present on most leukocytes, DEC205 showed low level of expression on monocytes, increased expression on immature DC, and greatly up-regulated expression on mature DC. As it is an antigen uptake receptor, monocytes and immature DC possessed extensive intracellular compartments containing DEC205. However, an analysis of mature DC revealed DEC205 staining to be predominantly at the cell surface with little intracellular staining. This seems to indicate

that, although mature DC has increased expression of DEC205, there is a down regulation of DEC205 mediated endocytosis (Butler et al., 2007).

**Figure 3: Structure of DEC205.**



**Figure 3**



The natural ligands for DEC205 are currently unknown, but antibodies have been used as surrogate ligands to perform a variety of studies. In one of the first studies, ovalbumin (OVA) protein was linked to inter-heavy chain thiol groups on a DEC205 monoclonal antibody generated by mild reduction conditions. After determining that the antibody was still functional, it was shown that OVA was presented by CD11c<sup>+</sup> lymph node DCs to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Presentation was at least 400 times more efficient than unconjugated OVA. Furthermore, targeting anti-DEC205-OVA antibody to DCs in the steady state initially induced 4-7 cycles of T cell division, but the T cells were then deleted and the mice became specifically unresponsive to rechallenge with OVA in complete Freund's adjuvant. Thus, the anti-DEC205-OVA antibody induced tolerance when given in the steady state. In contrast, when anti-DEC205-OVA antibody was simultaneously delivered with a DC maturation stimulus, strong immunity was induced. The CD8<sup>+</sup> T cells responding to the antigen produced large amounts of IL-2 and IFN- $\gamma$ , acquired cytolytic function *in vivo*, and responded vigorously to OVA rechallenge (Bonifaz et al., 2002).

After the initial success of antigen delivery to dendritic cells via the DEC205 receptor, other antigens have been linked to the DEC205 antibody. To induce tolerance, superagonist peptide MimA2 was linked to the DEC205 antibody. MimA2 is an insulin peptide that is recognized by diabetogenic CD8<sup>+</sup> T cells (Mukhopadhyaya et al., 2008). The anti-DEC205-MimA2 antibody delivers MimA2 to DCs for presentation on class I MHC both *in vivo* and *in vitro*. By selectively removing DCs from a transgenic non-obese diabetic (NOD) mouse using diphtheria toxin, the group was also able to show that

DCs were required for this presentation. Next, it was shown that the transferred  $\beta$  cell-autoreactive CD8<sup>+</sup> T cells were eventually deleted in response to the delivery of the MimA2 peptide. Finally, it was shown that the anti-DEC205-MimA2 antibody induced peripheral CD8<sup>+</sup> T cell tolerance in the NOD mice; the immunized mice did not undergo an immune response when challenged with a large dose of MimA2 peptide given in conjunction with complete Freund's adjuvant (Mukhopadhyaya et al., 2008).

In 2006, Trumpfheller *et. al.* reported the effects of a DEC205 antibody with Gag p24 fused to its heavy chain's C-terminus. The article indicated that such an antibody, when delivered with an adjuvant, was over 100 times more effective in inducing an immune response in mice when compared to other methods of using Gag as an antigen. The anti-DEC205-Gag p24 antibody also caused high frequencies of polyfunctional T cells, induced long-lived T cell memory, and elicited protective immunity at mucosal surfaces in mice (Trumpfheller et al., 2006). In 2007, Bozzacco *et. al.* reported that the anti-DEC205-Gag p24 antibody stimulated proliferation and IFN- $\gamma$  production by CD8<sup>+</sup> T cells isolated from the blood of HIV-infected donors (Bozzacco et al., 2007). These findings have prompted the preparation for a Phase I clinical trial studying DEC205-Gag p24 antibody in the prevention of HIV transmission.

Most recently, the human cancer antigen, mesothelin, was linked to the DEC205 antibody. Mesothelin, which is expressed on normal mesothelial cells, is overexpressed in several cancers, including mesothelioma, ovarian cancer, pancreatic adenocarcinoma, lung adenocarcinoma, uterine serous carcinoma, and acute myeloid leukemia (Hassan et

al., 2008). Targeting human mesothelin to DEC205 receptor, in the presence of an adjuvant, induced stronger CD4<sup>+</sup> T-cell responses compared to high doses of mesothelin protein. CD4<sup>+</sup> T cells were primed to produce IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and IL-2. Targeting also resulted in cross-presentation to CD8<sup>+</sup> T cells. Thus, anti-DEC205-mesothelin antibody could be valuable for enhancing immunity to mesothelin in cancers where this nonmutated protein is expressed (Wang et al., 2009).

## **Cross-Presentation**

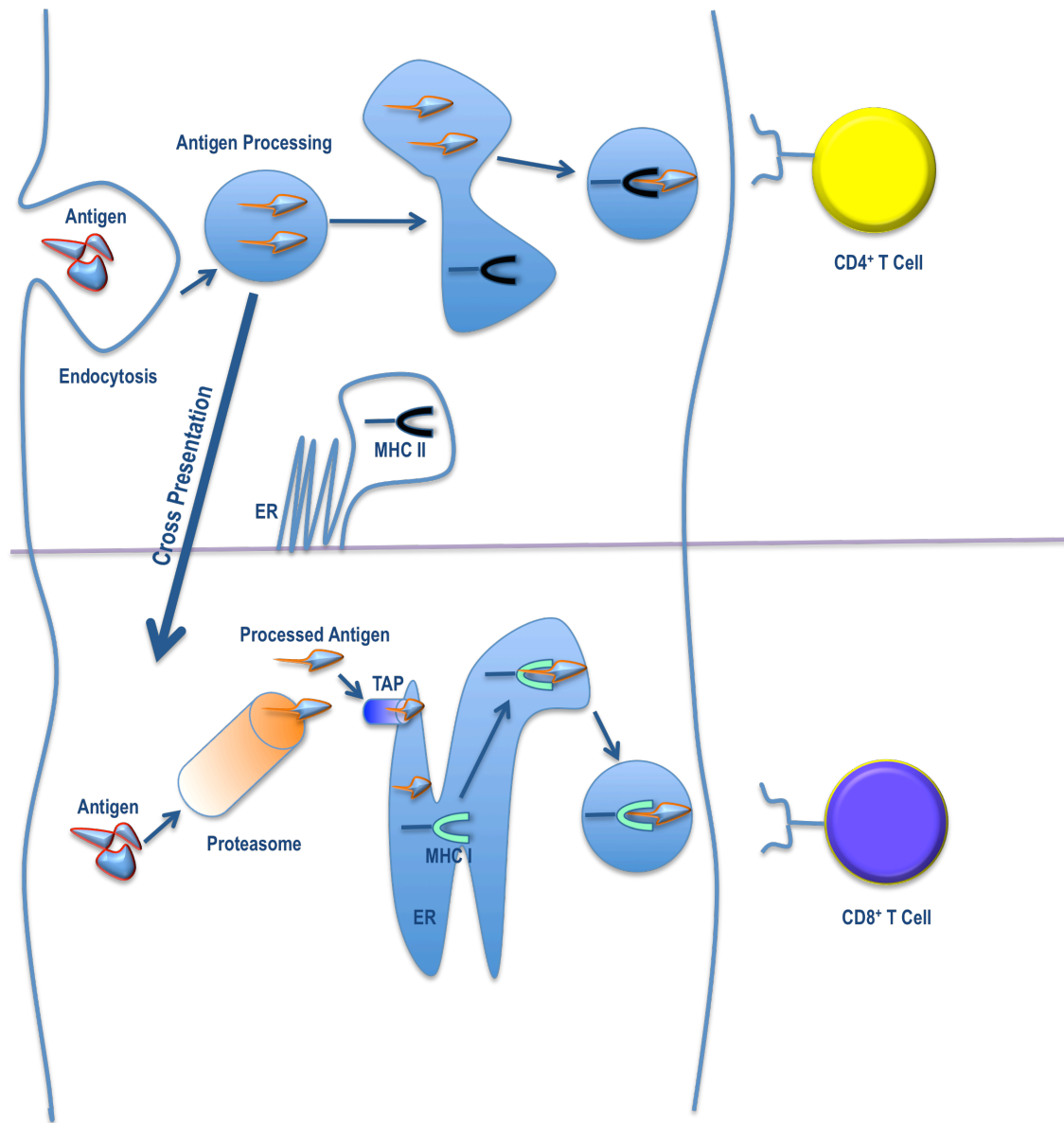
Cross-presentation was discovered in the 1970s, when antigens from injected cells ‘crossed’ into the MHC class I pathway of host antigen-presenting cells (APC), for naïve CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) priming (Figure 4) (Brevan et al., 1976). Since its discovery, it has been determined that cross presentation is required for the defense against many viruses and tumors and is essential for vaccinations with protein antigens (Huang et al., 1994 and Sigal et al., 1999).

DCs have been identified as the most relevant cross-presenting cells through experiments where *in vivo* depletion and functional inhibition of DCs compromises cross-priming (Kurts et al., 2001 and Jung et al., 2002). Furthermore, the transcription factor, BATF3, which is expressed by CD8<sup>+</sup> and CD103<sup>+</sup> DCs, has been shown to be necessary for cross-presentation in mice (Hildner et al., 2008). Researchers have discovered that only a handful of endocytosis receptors are capable of cross-presentation: namely, Fc and certain CLRs, such as CLEC9A, CLEC7A, DC-SIGN, DEC205, and mannose receptor 1 (Kurts et al., 2010).

There have been two mechanistic explanations as to why a cell can cross-present. The first hypothesis proposed that only cross-presenting DCs possess the antigen processing machinery that leads endocytosed antigen onto MHC class I molecules (Dudziak et al., 2007). Thus, only certain DCs have the ability for antigen escape from endosome into the cytoplasm to gain access to the proteasome and TAP transporters (Bryant et al., 2004). The second hypothesis proposed that cross-presenting DCs have specialized

**Figure 4: MHC I and II Processing and Cross-presentation. Top:** MHC II processing. The antigen is brought into the cell via endocytosis and processed into peptides. The endosome then fuses with another endosome carrying the MHC II machinery produced in the ER. **Bottom:** MHC I processing. The antigen is in the cytoplasm. It is degraded into peptides in the proteasome. The processed antigen is transported to the ER where it enters through TAP. Here, it combines with MHC I machinery. Cross-presentation occurs when the antigen “crosses” from the MHC II pathway into the MHC class I pathway.

**Figure 4**



phagosomes that contain MHC class I machinery. Hence, the endocytosed antigen would not have to compete with loading of endogenous peptides in the ER, but instead would be brought to these phagosomes for MHC class I peptide presentation (Lin et al., 2008 and Villadangos et al., 2005). It is still debated, however, how the loading machinery reaches the phagosomes (Kurts et al., 2010).

Cross-priming CTLs specific for peripheral tissue antigens involves the cooperation between the different DC types. Tissue DCs transport antigen from tissue to secondary lymphoid organs where resident CD8<sup>+</sup> DCs cross-prime CTLs (Honey, K., 2005). This antigen transfer mechanism allows for wider distribution of antigen and makes it possible for the signal to reach the draining lymph nodes; cross-presenting DCs are killed by the CTLs whereas the non-cross-presenting DCs can spread the message (Kurts et al., 2010).

The activation of certain receptors, such as TLRs, leads to the upregulation of co-stimulatory molecules and enhances cross-priming. This is particularly true for TLR3 and TLR9 activation (Wei et al., 2010). Furthermore, activation of RIG-I and the inflammasome boost CTL responses, and indirect evidence suggests that these cytoplasmic sensors can stimulate cross-priming (Wei et al., 2010). DCs also require specific T<sub>H</sub> cells for immunogenic cross-priming. CTLs activated without T cells help lack expression of specific anti-apoptotic molecules, have a short life-span, and cannot carry out cytotoxic effector functions (Jung et al., 2002). It remains unclear to what extent signaling events induced by T<sub>H</sub> cells or by TLRs overlap in cross-priming DCs. Some differences must exist, however, because T<sub>H</sub> cells and pattern-recognition



receptors synergistically activate DCs and both are necessary for optimal cross-priming (Kurts et al., 2001).

## **DNA-Protein Conjugates**

For years, researchers have developed methods to create DNA-protein conjugates (Figure 5). The generation of such semisynthetic conjugates is desirable because it allows the unique structure-directing properties of DNA to be combined with the almost unlimited variety of protein functionality. DNA is readily accessible by synthetic chemical means and has high physicochemical stability. DNA also possesses great mechanical rigidity, and nature provides numerous highly specific DNA-modifying enzymes that allow for processing and manipulation of the DNA with atomic precision (Niemeyer, C., 2010). Proteins have been tailored to perform highly specific functions, such as catalytic turnover, energy conversion, and translocation of components across membranes. Combined together, DNA-protein conjugates have a broad range of applications (Niemeyer, C., 2010).

One of the first methods developed to generate semisynthetic DNA-protein conjugates is based on the high affinity of biotin-streptavidin binding. Streptavidin has extreme chemical and thermal stability, making it possible to either chemically or genetically link it to a protein of interest (Wilcheck et al., 1990). Biotinylated oligonucleotides are easily synthesized and commercially available. Thus, the DNA-protein conjugate can be prepared by simple mixing the biotinylated DNA and the protein of interest conjugated to streptavidin. Due to the high affinity of streptavidin-biotin interaction, dissociation is not an issue for this method. However, streptavidin is tetrameric in nature, making it difficult

to control the stoichiometry of the respective DNA-protein conjugates (Wilcheck et al., 1990).

Another method for the generation of DNA-protein conjugates uses the specific interaction of a poly-histidine peptide (His-tag) with nickel(II) ions complexed by nitrilotriacetic acid (NTA) ligands. With this approach, a hexahistidine tag is incorporated to the N- or C-terminus of the protein of interest (Meredith et al., 2004). Then, oligonucleotides are synthesized containing one, two, or three NTA groups. The two compounds are then mixed together to create the DNA-protein conjugate. Dissociation constants of 120 and 6 nM are reported for the bisNTA- and trisNTA- DNA respectively (Goodman et al., 2009). The benefit of this approach is that the linkage is relatively specific (note, as is often observed with His-tag based affinity purifications, non-specific binding will occur to some extent), and reversible; the linkage can easily be broken by a nickel chelator such as EDTA or imidazole. However, this approach is not recommended when dissociation of the DNA from the protein of interest is not desirable.

To make DNA protein conjugates, other affinity tags can be tethered to the DNA by solid-phase synthesis. These tags can then be used as a hapten to specifically bind immunoglobulin G (IgG) antibodies raised against it (Kadkol et al., 1999). For example, the chromophore fluorescein can be attached to DNA oligonucleotides to generate probes for the *in vitro* detection of complimentary nucleic acid sequences, by using anti-Fsc-IgG conjugated to a chromophore, fluorophore, or reporter protein. Other hapten-antibody

pairs that have proven useful for this approach include biotin, dinitrophenol, digoxigenin, and short peptides such as FLAG tag (Knappik et al., 1994).

A semisynthetic approach to DNA conjugation has also been developed for enzymes that contain nondiffusible cofactors. In this case, the cofactors are extracted from the protein to yield the respective apoenzyme. Then, the DNA moiety is attached to the cofactor, and the modified cofactor is reinserted into the apoenzyme (Niemeyer, C., 2010). This approach was done with both apo-myoglobin and apo-horseradish peroxidase; the heme was modified with DNA, and the reconstituted enzymes were found to be fully functional and capable of specific hybridization with complementary nucleic acids immobilized on a range of surfaces (Fruk et al., 2006).

To circumvent obstacles that result from the dissociation of noncovalent, reversible interactions, researchers have developed methods to covalently link the DNA to the protein of interest. The most common approach involves maleimide coupling (Niemeyer et al., 1994). A single cysteine is incorporated into the protein of interest. Then, the protein of interest is reacted with a cross-linker bearing a maleimide functionality. After purification, the maleimido-activated protein is treated with thiol-modified DNA oligonucleotides. This results in the desired DNA-protein conjugate. Of course, not all proteins of interest can be manipulated so that there is only one cysteine in its sequence. Hence, this approach is not always applicable (Joerger et al., 1995 and Kukolka et al., 2004).

Expressed protein ligation offers a way to create DNA-protein conjugates without modifying the protein of interest's sequence. In this method, the protein of interest is genetically modified at its C-terminus with an intein domain. Addition of low-molecular-weight thiol compounds, such as mercaptoethanesulfonic acid (MESNA), leads to the cleavage of the intein from the protein of interest. This leaves the protein of interest with a reactive  $\alpha$ -thioester at its C-terminus. It can then be ligated with a nucleic acid conjugate that has been engineered to have an N-terminal cysteine. The result is the desired DNA-protein conjugate linked via a stable amide bond (Lovrinovic et al., 2003).

Copper(I)-catalyzed Huisgen 1,3 dipolar cycloaddition of azides and alkynes (CuAAC) has also proven useful in the production of DNA-protein conjugates (Gramlich et al., 2008). In this approach, the enzyme protein-farnesyl transferase (PFTase) is used to specifically attach an azido-modified isoprenoid to a short recognition sequence that was fused to the protein of interest through genetic modification. The azido groups are then coupled with alkyne-modified oligonucleotides in the presence of copper to generate the desired DNA-protein conjugate (Duckworth et al., 2007).

Another approach to create DNA-protein conjugates uses the enzyme transglutaminase from *Streptomyces mobaraensis*, an enzyme that specifically catalyzes the acyl transfer reaction between a primary amine and the  $\gamma$ -carboxyamide group of glutamine (Tominaga et al., 2007). The oligonucleotide was modified with carbobenzyloxyglutaminyglycine which functions as an acyl donor. The protein of interest was genetically modified with a short peptide tag containing acyl-acceptor lysine

residues. The DNA-protein conjugate was created when the modified DNA and protein were combined in the presence of the microbial enzyme (Tominaga et al., 2007).

In yet another method, human O<sup>6</sup>-alkylguanine DNA-alkyltransferase is fused by genetic modification to the protein of interest. This moiety is then treated with benzylguanine-modified DNA oligonucleotides to produce the desired DNA-protein conjugate. This approach has the advantage that no purification is required for the modified protein of interest. The enzyme modified protein of interest was able to be conjugated to the DNA within crude *E. coli* lysate (Jongsma et al., 2006).

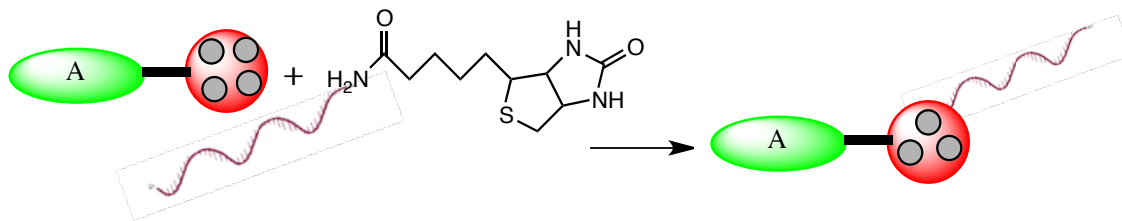
At the outset of these studies, we expected that the best approach for the production of the anti-DEC205-poly dA:dT antibody is expressed protein ligation. With this approach, a covalent bond is created between the DEC205 antibody and the poly dA:dT. Thus, the dissociation between the antibody and poly dA:dT is not a factor. In addition, the product should have well-defined stoichiometry and the linkage will be regiospecific. The DNA will always be attached to the heavy chain of the antibody's C-terminus, and since there are two heavy chains per antibody, the DNA to antibody ratio will be 2:1 (assuming the reaction goes to completion). Finally, and perhaps most importantly, there is no extraneous sequence in between the poly dA:dT and the antibody. Thus, any immune response elicited will be due to the poly dA:dT; the DEC205 antibody has been shown not to induce an immune response.

**Figure 5: Production of DNA-Protein Conjugates.** Strategies to link DNA to protein

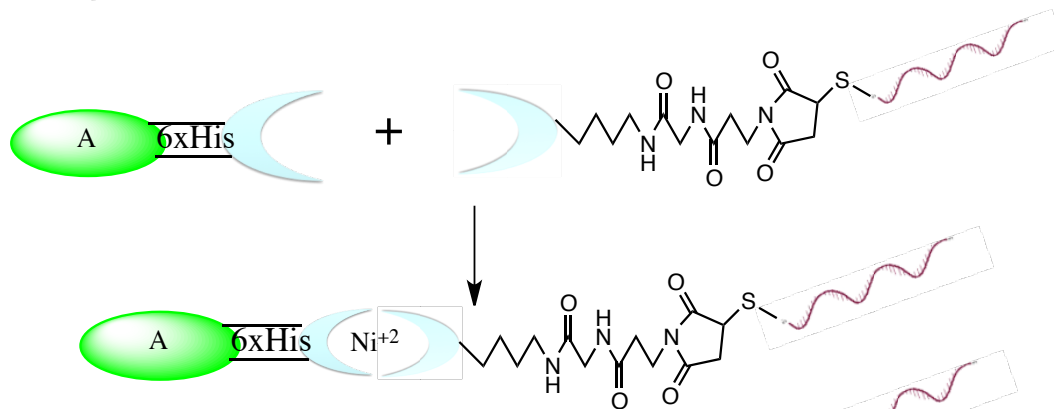
A. **A.** Biotin-streptavidin coupling. **B.** Ni-NTA-His<sub>6</sub> conjugation. **C.** Antibody-hapten interaction. **D.** Cofactor reconstitution of apoenzymes. **E.** Maleimide Coupling.

**Figure 5**

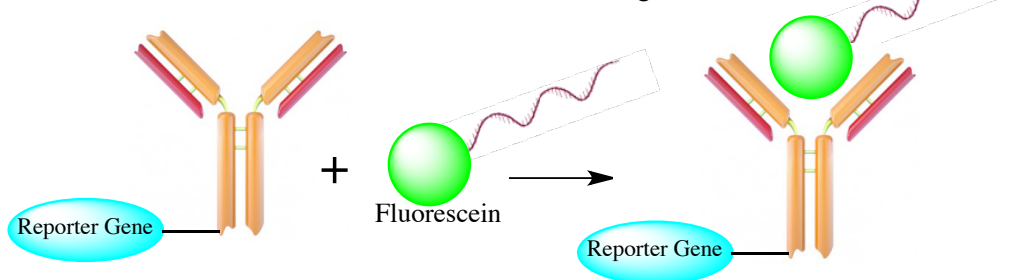
A



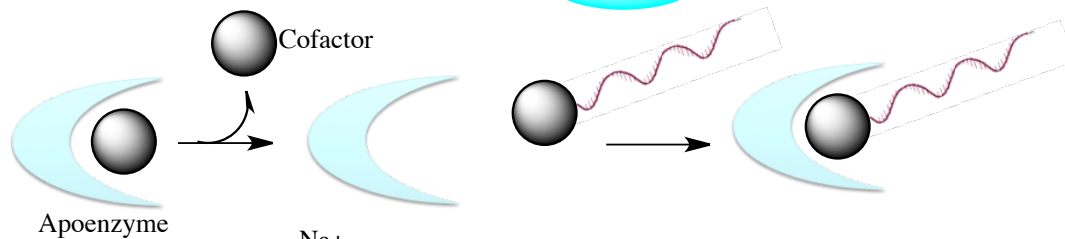
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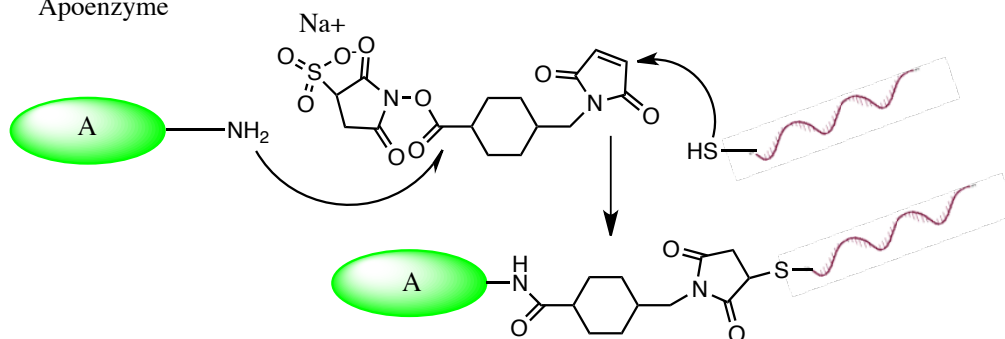
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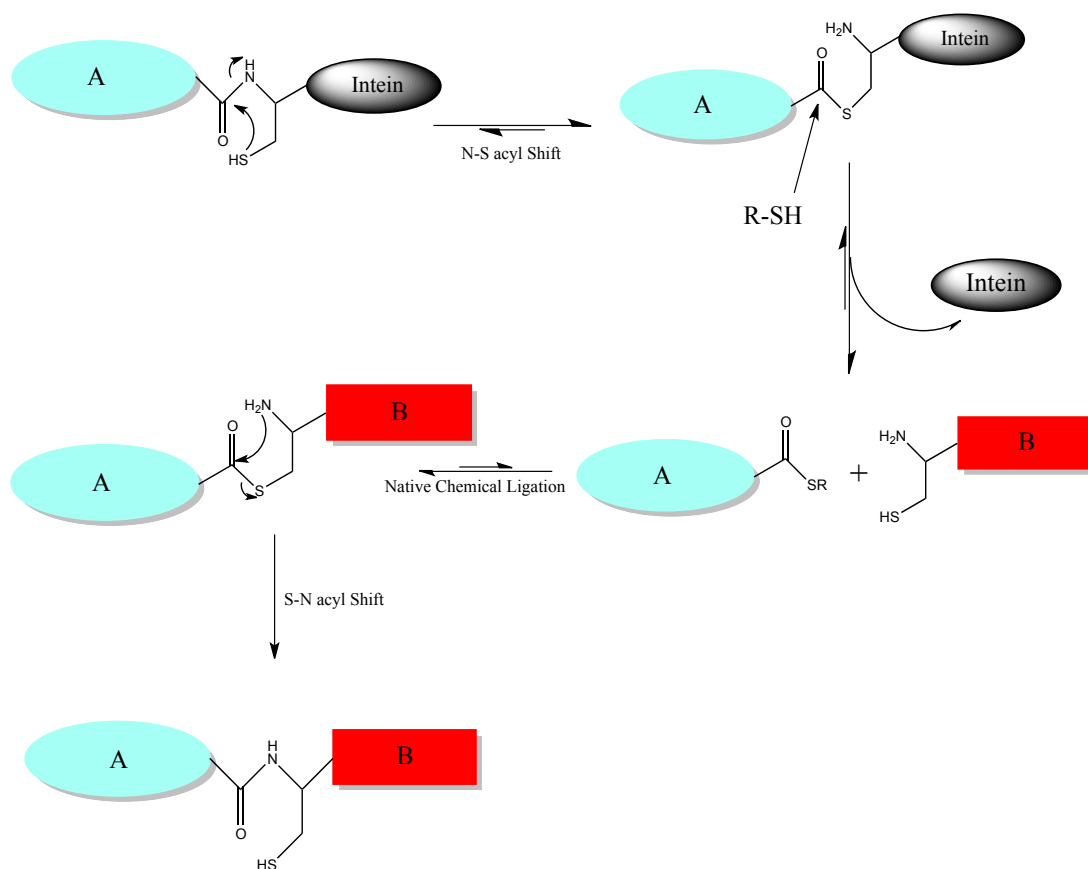
## Expressed Protein Ligation

In 1998, the method of expressed protein ligation (EPL) was introduced. This approach requires the use of an intein, a segment of a protein that is able to excise itself and rejoin the remaining portions, termed the exteins, with a peptide bond (Muir, T.W., 2003). A full length mutant intein (the mutant intein can only undergo the first step of protein splicing) is fused to the C-terminus of a recombinant protein. After expression, the mutant intein is cleaved from the protein using a reactive thiol; this generates an  $\alpha$ -thioester at the C-terminus of the protein of interest. This  $\alpha$ -thioester can then undergo a chemoselective reaction with another protein where the N-terminal amino acid is a cysteine, i.e. native chemical ligation. There is an initial transthioesterification reaction followed by a spontaneous intramolecular S-N acyl shift to generate an amide bond between the two proteins (Figure 6) (Muralidharan et al., 2006).

Several modified inteins can be used for the purpose of EPL. One of the most commonly used inteins is the *Mycobacterium xenopi* DNA gyrase intein (Mxe GyrA). This intein has several features that make it ideal for this method: it is relatively small and can be expressed efficiently in *E. coli*; it does not have special sequence preferences for the last residues of the N-extein fragment; the thiolysis reaction can be performed in the presence of detergents, organic solvents, and moderate amounts of denaturing agents; the GyrA intein can be efficiently refolded thus allowing recovery of intein-fusion proteins from *E. coli* inclusion bodies (Muir, T.W., 2003).

**Figure 6: Mechanism of Expressed Protein Ligation (EPL).** The protein of interest (A) is expressed with a full length mutant intein attached to the C-terminus. A reactive thiol is added to remove the intein, leaving A with a reactive  $\alpha$ -thioester bond. Protein A can then undergo native chemical ligation to a molecule (B) that has an N-terminal cysteine.

**Figure 6**



Expressed protein ligation has been applied to a wide variety of protein engineering problems. In the simplest case, EPL allows the semisynthesis of a target protein from two polypeptide pieces. This permits the incorporation of probes within the flanking regions of a protein (Muralidharan et al., 2006). The approach can also be used to ligate a synthetic peptide to a recombinant protein. This approach has been widely used to incorporate unnatural amino acids, post-translational modifications, and spectroscopic probes into many classes of proteins (Muir, T.W., 2003 and Schwarzer et al., 2005). Less commonly, EPL has been used to link together two recombinant proteins; this approach has proven useful in the production of cytotoxic proteins from innocuous fragments (Evans et al., 1998 and We et al., 2002).

It was recently demonstrated that protein-intein fusions can be expressed in not only bacteria, but also a variety of eukaryotic cell lines. Many proteins of interest, such as antibodies, require post-translational modifications that bacteria are not able to perform. Prior to the research of Singla *et. al.*, it was unclear whether Mxe gyrA intein would be able to undergo thiolysis after protein-intein expression in systems other than bacteria. The group demonstrated that the extracellular domain of the Eph receptor with the Mxe gyrA intein attached was able to be expressed in both transfected mammalian HEK293 cells and baculovirus infected insect cells (Singla et al., 2008). In both cases, the fusion protein was secreted into the growth medium. Critical to this result was mutation of cysteine 79 and 114 to serine in the Mxe GyrA intein sequence. This was done to prevent formation of non-native disulfide bonds between the intein and the protein of interest – something that might occur in the oxidizing milieu or the ER/Golgi and the medium.

Thus, these mutations improved the fraction of correctly folded protein and thereby increased the cleavage efficiency of the intein when a reactive thiol was added (Singla et al., 2008).

In 2003, EPL was proven effective in ligating nucleic acids to proteins of interest (Lovrinovic et al., 2003). In these cases, nucleic acids are engineered so that they possess an N-terminal cysteine. The nucleic acid is then ligated to the protein of interest that has a C-terminal  $\alpha$ -thioester (Niemeyer, C., 2010). In 2007, Lovrinovic *et. al.* published a paper monitoring the ligation of maltose binding protein (MBP) to cysteine-modified DNA oligomers. Their results indicated that a pH of 8 to 8.5, an oligonucleotide/protein ratio of 1:4, and a reaction time of at least 12 hours were optimal for ligation. Furthermore, the reaction seemed unaffected by the amount of NaCl and  $Mg^{2+}$  in the ligation buffer (Lovrinovic et al., 2007).

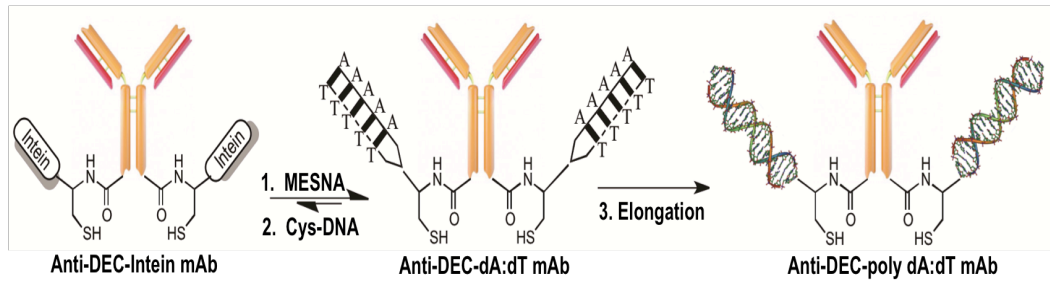
The main application of the resultant DNA-protein conjugates has been to perform immuno-PCR, a modification of the conventional enzyme-linked immunosorbent assay (ELISA). In this procedure, the protein part of the DNA-protein conjugate is used to detect some type of antigen. Then, the DNA moiety of the hybrid is amplified by PCR. The almost exponential amplification power of PCR results in the immuno-PCR method being about 1000-10000 fold more sensitive than ELISA (Burbulis et al., 2007).

In the following research, EPL was used to make DNA-protein conjugates for a novel purpose; it was used to specifically target dendritic cells with an immune activating

agent. Figure 7 shows the scheme in which anti-DEC205-poly dA:dT antibodies were made. Briefly, the DEC205 antibody is expressed with an intein attached to the C-terminus of its heavy chain. The reactive thiol, sodium 2-mercaptoethanesulfonate (MESNA), is then used to remove the intein and leave the antibody with a reactive  $\alpha$ -thioester at its C-terminus. Next, the antibody is allowed to react with short double stranded oligonucleotides that have an N-terminal cysteine to produce the antibody-DNA conjugates. Finally, the DNA is elongated using klenow fragment.

**Figure 7:** Schematic of ligation procedure. The DEC205 mAb is expressed with an intein attached to its heavy chain C-terminus. The intein is then removed using MESNA. A 20 nucleotide oligomer of dA:dT with an N-terminal cysteine (cys-DNA) is ligated to the mAb's activated C-terminus. Klenow fragment is used to elongate the ligated DNA, producing the anti-DEC205-poly dA:dT mAbs.

**Figure 7**





## **Material and Methods**

### **Reagents**

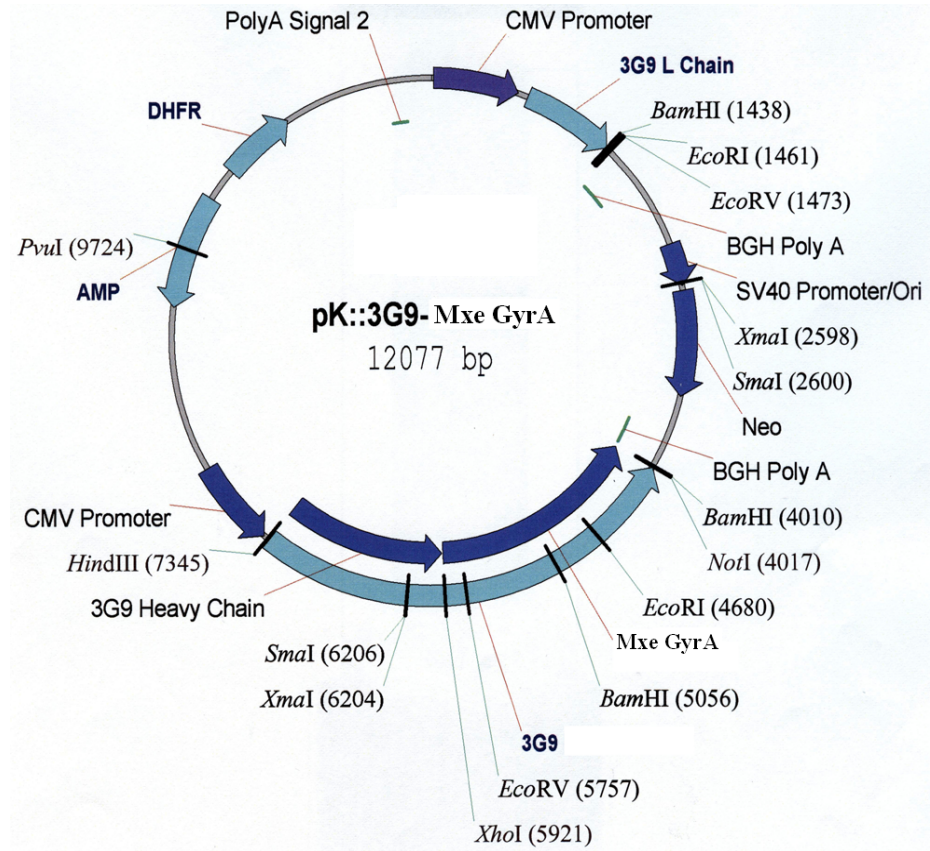
Chloroquine and LPS were from Sigma-Aldrich (St. Louis, MO). Human interleukin 4 (IL-4) was from R&D systems (Minneapolis, MN). Granulocyte-macrophage colony stimulating factor (GM-CSF) was from Berlex (Berkeley, CA). Poly IC, Resiquimod, and CLO were from InVivogen (San Diego, CA). Klenow Fragment was from New England BioLabs (Ipswich, MA). Amine-DNA oligomers were purchased from Fisher Scientific (Pittsburg, PA).

### **Production of Fusion mDEC205-gyrA and hDEC205-gyrA Antibodies**

*Mycobacterium xenopi* gyrase A intein (gyrA) was cloned in frame after the C-terminus of anti-DEC205 heavy chain, as done previously for immune antigens such as HIV Gag p24 (Hawiger et al., 2001). Briefly, the GyrA intein was cloned from a plasmid containing the gene for *Mycobacterium xenopi* gyrase A. Quikchange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) was used on this plasmid to obtain GyrA intein with cysteine 79 and 114 converted to serine. Utilizing XhoI and NotI restriction sites incorporated into oligonucleotides primers, the fragment containing the Mxe GyrA (C79S C114S) was cloned into pK::hDEC205, a mammalian expression vector with both the heavy and light chain of human DEC205 antibody encoded within it. The resultant vector produced DEC205 antibody with GyrA fused to the C-terminus of that antibody's heavy chain (Figure 8). The identity of the cloned fragment was confirmed by DNA sequencing (data not shown). To determine if the anti-DEC205-Mxe GyrA (C79S

**Figure 8: Plasmid map of the anti-DEC205-Mxe GyrA intein antibody.** Mxe GyrA intein was cloned in frame to the DEC205 heavy chain's C-terminus using restriction sites, *XhoI* and *NotI*. The resultant plasmid, when incorporated into HEK293T cells, produces the DEC205 antibody with the Mxe GyrA intein attached to it.

**Figure 8**



C114S) intein antibody was expressed, the recombinant vector was used to transiently transfect HEK 293T cells via calcium phosphate in serum-free DMEM media supplemented with Nutridoma SP (Roche, Indianapolis, IN) (Wang et al., 2009). After the 293T cells were allowed to express the anti-DEC205-Mxe gyrA (C79S C114S) intein antibody for three days, the medium was collected and filtered with Steritop vacuum filter cups (Millipore, Billerica, MA). The medium was then loaded onto a column containing 1 mL of protein G sepharose beads (GE Healthcare, Piscataway, NJ). The beads were washed with 200 mL of Protein G binding buffer (Pierce, Rockford, IL). The anti-DEC205-Mxe gyrA (C79S C114S) intein antibody was then eluted off the beads using Protein G elution buffer (Pierce, Rockford, IL). The antibody was dialyzed against phosphate buffered solution (PBS) and concentrated using amicon concentrators (Millipore, Billerica, MA). The integrity of the purified proteins was determined by SDS-Page Coomassie blue and western-blot analysis using rat anti-human IgG Ab conjugated with HRP (Jackson ImmunoResearch, West Grove, PA). The typical yield for the preparation ranged from 1.25 to 2.5 mg/L.

Mxe GyrA (C79S C114S) intein was cloned in frame to a mammalian expression vector that encoded the heavy and light chain of anti-mouse (m) DEC205 antibody. The resultant vector produced mDEC205 antibody with GyrA fused to the C-terminus of the antibody's heavy chain. Anti-mDEC205-Mxe gyrA (C79S C114S) intein antibody was then produced and purified in the same manner as described above for anti-human (h)DEC205-Mxe gyrA (C79S C114S) intein antibody. The integrity of the purified proteins was determined by SDS-Page Coomassie blue and western-blot analysis using

goat anti-mouse IgG Ab conjugated with HRP (Jackson ImmunoResearch, West Grove, PA). The typical yield for the preparation ranged from 1.5-3 mg/L.

Control antibodies were also produced. Mxe GyrA (C79S C114S) intein was cloned in frame to a mammalian expression vector that encoded the heavy and light chain of anti-mouse IgG (mControl) antibody and anti-human IgG (hControl) antibody. The resultant vectors were used to produce and purify anti-mControl and anti-hControl Mxe GyrA (C79S C114S) intein antibodies, respectively. The integrity of the purified proteins was determined by SDS-Page Coomassie blue and western-blot analysis using either rat anti-human IgG or goat anti-mouse IgG Ab conjugated with HRP (Jackson ImmunoResearch, West Grove, PA). The respective yields of anti-mControl and anti-hControl gyrA (C79S C114S) antibodies ranged from 1.0-2.5 and 1.0-2.0 mg/L).

Mxe GyrA (C79S C114S) intein was also cloned in frame to a mammalian expression vector that encoded the heavy and light chain of anti-DCIR2 antibody. The resultant vector was used to produce and purify anti-DCIR2 Mxe GyrA (C79S C114S) intein antibodies. The integrity of the purified protein was determined by SDS-Page Coomassie blue and western-blot analysis using goat anti-mouse IgG Ab conjugated with HRP (Jackson ImmunoResearch, West Grove, PA). The respective yield of anti-DCIR2 Mxe gyrA (C79S C114S) intein antibody was 1.25 mg/L.

## **Production of Short Fluorescent Peptide**

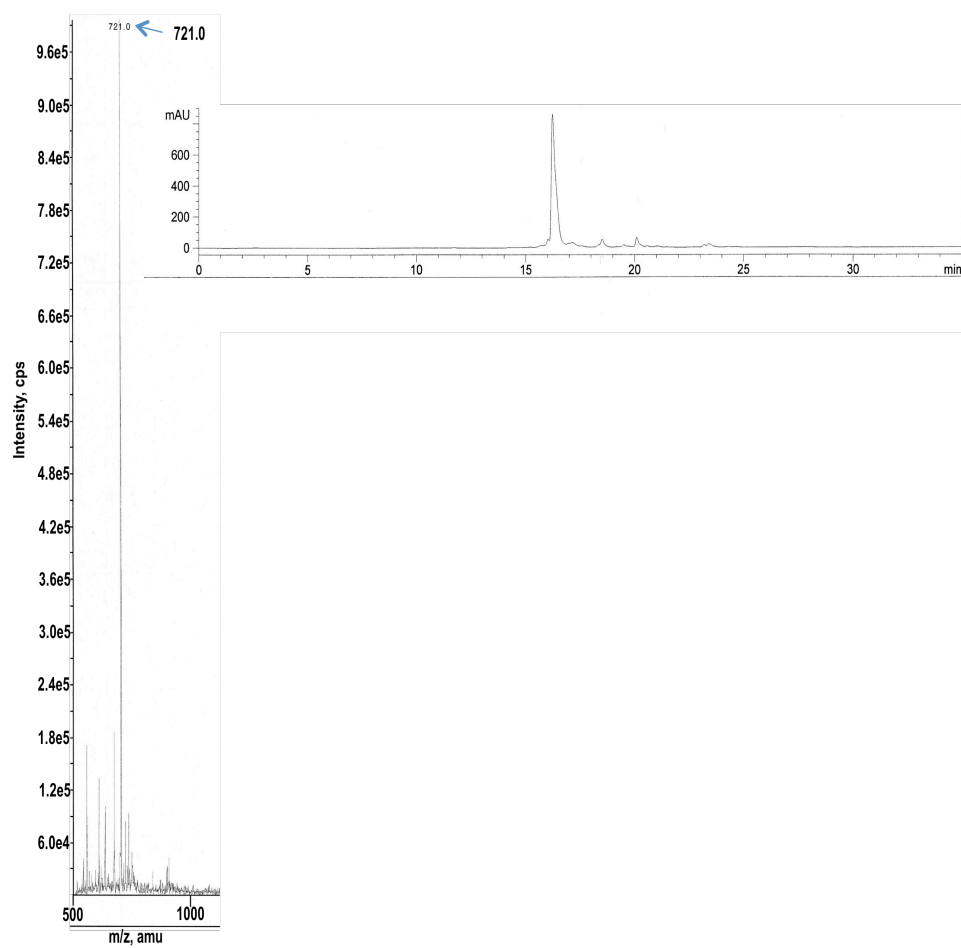
Fmoc-based solid phase peptide synthesis (SPPS) was used to produce a peptide of sequence H-Cys-Gly-Lys(Fluorescein)-Gly-NH<sub>2</sub>. The following amino acids were used: Fmoc-Gly-OH, Fmoc-Lys(Mmt)-OH, and Fmoc-Cys(StBu)-OH (Sigma Aldrich, St. Louis, MO). The sequence was assembled on a Rink amide resin (Merck, Whitehouse St., NJ) using HBTU (Sigma Aldrich, St. Louis, MO) as the activating agent and 20% piperidine/dimethylformamide (DMF) for Fmoc deprotection. Following chain assembly, the lysine side-chain was deprotected with 5x3 minute treatments of 1% trifluoroacetic acid (TFA), 5% N,N'-Diisopropylcarbodiimide (DIC), and 94% Dichloromethane (DCM). Carboxyfluorescein was then coupled to the lysine using DIC/N-hydroxysuccinimide activation. Next, the Fmoc and StBu protecting groups on the cysteine were deprotected using 20% piperidine/DMF and phosphine hydrochloride, respectively. Finally, the peptide was cleaved off the resin using 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% H<sub>2</sub>O solution. The peptide was purified by reverse-phase HPLC (Figure 9). Mass spectrometry was used to identify the fraction with the correct peptide (Figure 9).

ESI-MS: 721.42 (Calculated); 721.0 ± 1.0 (observed).

HPLC retention time: 16.4 minutes (C18 Yvdac column, Buffer A: 0.1% TFA in water, Buffer B: 90% CH<sub>3</sub>CN, 0.1% TFA in water, 0-73% solution B over 30 min, 1 mL/min).

**Figure 9: Production of short fluorescent peptide.** A. A peptide with a sequence of H-Cys-Gly-Lys(Fluorescein)-Gly-NH<sub>2</sub> was synthesized using Fmoc-based solid phase peptide synthesis (SPPS). After production, the sample was then run through a C18 Yvdac column. (Right) The 280 nM absorbance trace on the HPLC. The main peak was collected and analyzed via ESI-Mass Spectrometry (Left).

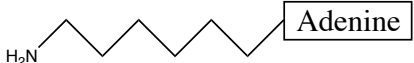
**Figure 9**





## Production of Cys-DNA

A DNA oligonucleotide with an N-terminal amine group was purchased from Fisher (Pittsburg, PA). The sequence is (AminoC6)AAAAAAAAAAAAAAAAAAAAA where

aminoC6 is . Fmoc-Cys(StBu)-OH (Sigma, St. Louis,

MO) was activated with N-hydroxysuccinimide (Fmoc-Cys(StBu)-OSU) using DIC.

Next, the amino-DNA was reacted with 50 fold excess Fmoc-Cys(StBu)-OSU in a 1:1 solution of 50 mM borate buffer pH 8.5 and dimethylformamide (DMF) (Figure 10A).

The protected Cys-DNA was purified by HPLC using a C18 Vydac column where buffer A was 10 mM triethylamine/acetate (TEAA) in water and buffer B was 80% CH<sub>3</sub>CN in water. A gradient of 0-50% B over 30 minutes was used for purification (Figure 10B).

Next, the protected Cys-DNA was incubated overnight in 30% ammonium hydroxide to remove the Fmoc group on the cysteine. Finally, the sample was placed in 1 M dithiothreitol (DTT) to remove the t-butyl group that protected the side chain group of the cysteine. The deprotected Cys-DNA was purified using the same HPLC gradient as before. The desired product was confirmed by MALDI mass spectrometry (Figure 11).

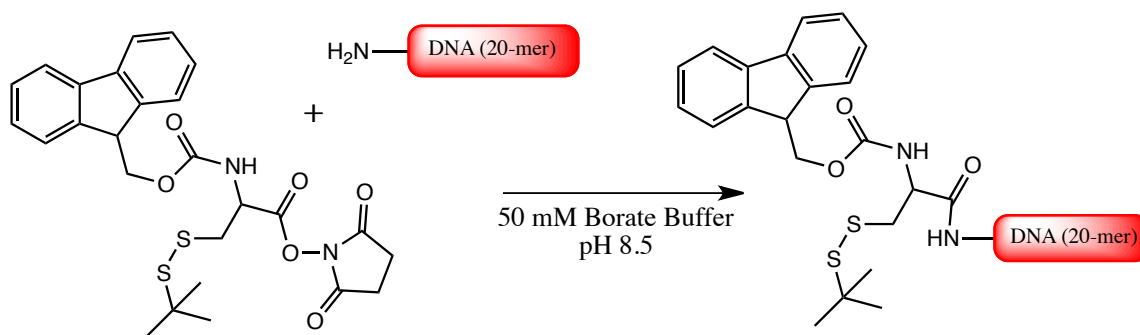
HPLC Retention Times: unmodified DNA oligonucleotide (22.8 minutes), Fmoc-Cys(StBu)-OSU (41 minutes), Cys(StBu)-OSU hydrolysis (35 minutes), protected Cys-DNA (30.2 minutes), deprotected Cys-DNA (21.2 minutes).

MALDI-MS for deprotected Cys-DNA: 6483.4 (calculated); 6495.73 ± 83.66 (observed)

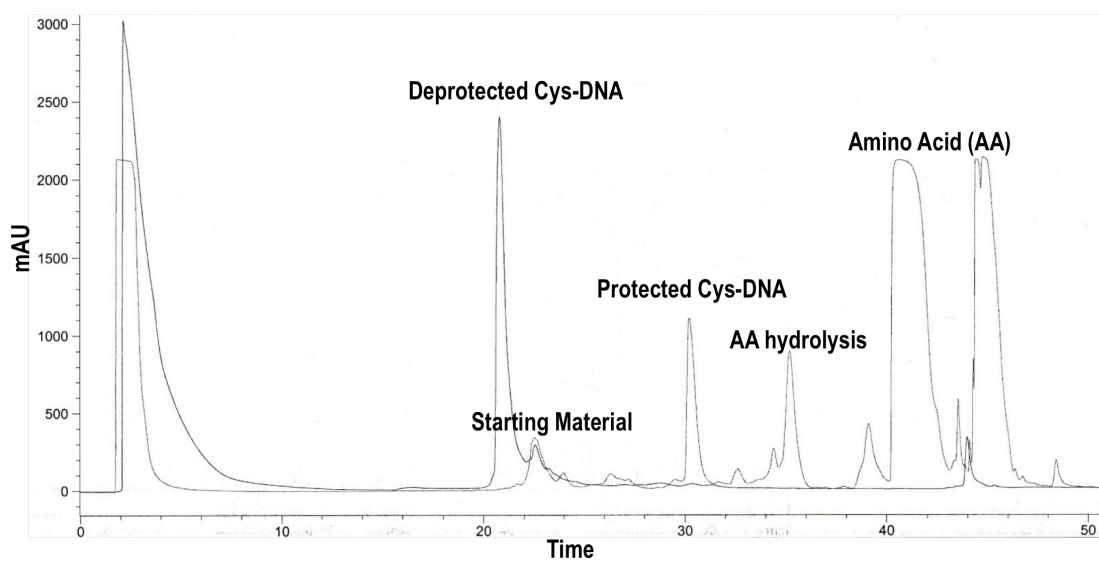
**Figure 10: Production of Cys-DNA.** **A.** Schematic of reaction to produce protected cys-DNA. Protected cysteine was activated with N-hydroxysuccinimide (Fmoc-Cys(StBu)-OSU). Amino-DNA was reacted with 50 fold excess of this moiety in a 1:1 mixture of 50 mM borate buffer, pH 8.5 and DMF. **B.** HPLC trace of Cys-DNA purification. Buffer A: 10 mM triethylamine/acetate (TEAA). Buffer B: 80% acetonitrile (Acn). A gradient of 0-50% B over 30 minutes was used for purification. Peaks are labeled accordingly. Fmoc-Cys(StBu)-OSU is Amino Acid.

**Figure 10**

**A**

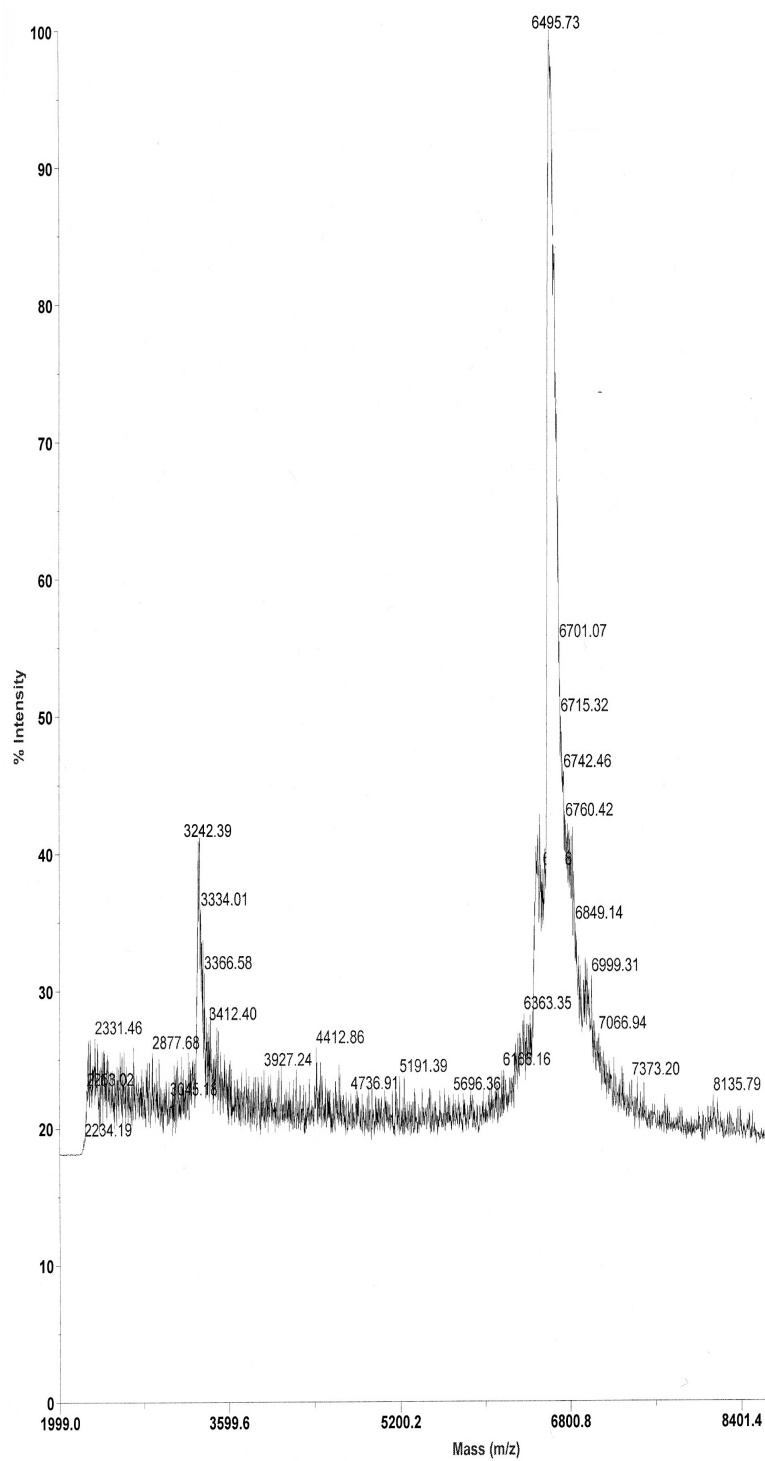


**B**



**Figure 11: MALDI Spectrum of Cys-DNA.** The matrix for the sample was 10:1 50 mg/mL hydroxy-picolinic acid (HPA) to 50 mg/mL ammonium citrate. MALDI-MS for deprotected Cys-DNA: 6483.4 (calculated);  $6495.73 \pm 83.66$  (observed)

**Figure 11**



## **Expressed Protein Ligation**

An oligonucleotide of sequence TTTTTTTTTTTTTTTTTTTT was purchased from Fisher (Pittsburgh, PA). It was combined in a 1:1 ratio with Cys-DNA in water and then annealed to the Cys-DNA by heating to 95°C for 5 minutes and allowed to gradually cool to room temperature for 1 hour. Fusion antibodies were activated by the addition of 100 mM sodium 2-mercaptoethanesulfonate (MESNA) (Sigma, St. Louis, MO) for 18 hours in PBS. At a concentration of 50  $\mu$ M, the activated antibody was reacted with 500  $\mu$ M of double stranded (ds)-Cys-DNA in ligation buffer (1x PBS, 400 mM NaCl, 6 mM TCEP, 1 mM EDTA, 100 mM MESNA, pH 7.5). The sample was allowed to react at room temperature overnight. Reaction progress was monitored by gel electrophoresis using 2-20% TBE native gels (Bio Rad, Hercules, CA), and then staining with ethidium bromide (InVitrogen, Carlsbad, CA) to check for ligation. After ligation, the sample was dialyzed 5 times against PBS for at least 2 hours each time at 4°C using Slide-A-Lyzer Cassettes, 20,000 molecular weight cut-off (MWCO) (Pierce, Rockford, IL). 100,000 MWCO microcon concentrators (Millipore, Billerica, MA) were then used to concentrate the sample. Trace amounts of cys-DNA were usually still seen at this point as visualized on the 2-20% TBE native gels stained with EtBr. If necessary, the samples were diluted to 1 mL and reconcentrated (5 mg/ml) until no Cys-DNA was visible on the EtBr gel (usually 2 times). Approximately 30% of the antibody (.3 mg from 1.0 mg) is lost from this purification procedure.

## **Elongation**

3.2  $\mu$ M of purified DNA-antibody conjugates were reacted with 5  $\mu$ M Klenow fragment (New England BioLabs, Ipswich, MA) in the presence of 0.3 mM dATP and dTTP for 90 minutes in NEB buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol). Aliquots of the antibodies were then removed and treated with 0.1  $\mu$ g/mL proteinase K (Qiagen, Valencia, CA) for 1 hour and run on a 2% agarose gel stained with EtBr to check for elongation. For preparative scale, 1 mL (320  $\mu$ g of antibody) reactions were used. The sample was then purified and concentrated using the 100,000 MWCO microcon concentrators (Millipore, Billerica, MA).

## **CHO Staining**

CHO cells stably transfected to produce hDEC205 or mDEC205 receptors as well as naive CHO cells (CHO NEO) were plated. Cells were spun down and placed into 50  $\mu$ L of FACS buffer (PBS pH 7.4 containing 2% fetal calf serum (FCS) and 0.02% NaN<sub>3</sub>) with 2  $\mu$ M EDTA. Then, 2  $\mu$ g, 0.2  $\mu$ g, and 0.02  $\mu$ g of the anti-DEC poly dA:dT mAb was added independently to both cells types. After a 20 minute incubation at 4 °C, the cells were spun down and washed three times with FACS buffer with 2  $\mu$ M EDTA. An hIgG-PE or mIgG-PE antibody was then diluted 1:250 into the cells. After another 20 minute incubation at 4 °C, the cells were washed twice with FACS buffer with EDTA. Flow cytometry was then performed on the cells to determine the extent of hDEC205 and mDEC205 binding to its corresponding receptors.

## **Human Cell Isolation and Culture**

Human PBMCs were isolated from whole blood of healthy volunteers by density gradient centrifugation using Ficoll-Hypaque (GE healthcare, Piscataway, NJ). Human monocytes were isolated from PBMCs with anti-CD14 paramagnetic beads (Miltenyi Biotec, Auburn, CA) and were differentiated for 5 days into MoDCs in the presence of IL-4 (20 ng/mL) and GM-CSF (20 ng/mL). Primary cells were cultured in RPMI (GIBCO, Carlsbad, CA) media supplemented with 2% pooled human serum (GemCell, Pennant Hills, NSW). 293T cells were cultured in DMEM (GIBCO, Carlsbad, CA) with 10% FCS (Omega Scientific, Tarzana, CA), L-glutamine (GIBCO, Carlsbad, CA), and HEPES (Lonza, Walkersville, MD).

## **Mice**

C57BL/6 mice were from Harlan (San Francisco, CA). DEC205 KO and human DEC transgenic mice were obtained from M. Nussenzweig (The Rockefeller University, New York). CD11c-DTR mice were produced as described previously.<sup>80</sup> To produce chimeras, B6 or DEC205 KO mice were irradiated twice 3 hours apart with 550 rads for 4 minutes. 3 hours after the last irradiation, mice were given either DEC205 KO or B6 bone marrow intravenously. The mice were given sulfatrim and antibiotic water (Neomycin) for at least two weeks until use. Mice were maintained under specific pathogen-free conditions and used at 7-8 week of age according to Institutional Animal Care and Use guidelines.



### **In vivo Cytokine Secretion**

Mice were injected i.p. with poly I:C or anti-DEC205-poly dA:dT mAb, and serum was collected after 6 hours. Production of IFN- $\alpha$  (PBL Interferon Source, Piscataway, NJ) was determined by ELISA following manufacturer's instructions.

### **Cell Sorting**

Spleens were cut in small pieces and incubated at 37 °C for 30 minutes in Hank's medium supplemented with 400 U/mL Collagenase D (Roche Diagnostics, Indianapolis, IN). 5 mM EDTA was added for the last five minutes. Cells were washed and then resuspended in 1x RBC lysis buffer (eBioscience, San Diego, CA). After 1 minute, the cells were washed and stained with CD8-APC, CD11c PE-Cy7 (eBioscience, San Diego, CA), and Live/Dead Fixable Aqua viability dye (Invitrogen, Carlsbad, CA). Different populations were isolated by sorting on a FACS Aria with DiVa configuration. Three populations (CD11c<sup>low</sup>, CD8<sup>+</sup> DCs, and CD8<sup>-</sup> DCs) were then plated at 300,000 cells/mL in RPMI supplemented with 5% FCS. The next day, the supernatant was collected and an IFN- $\alpha$  ELISA was performed following manufacturer's instructions.

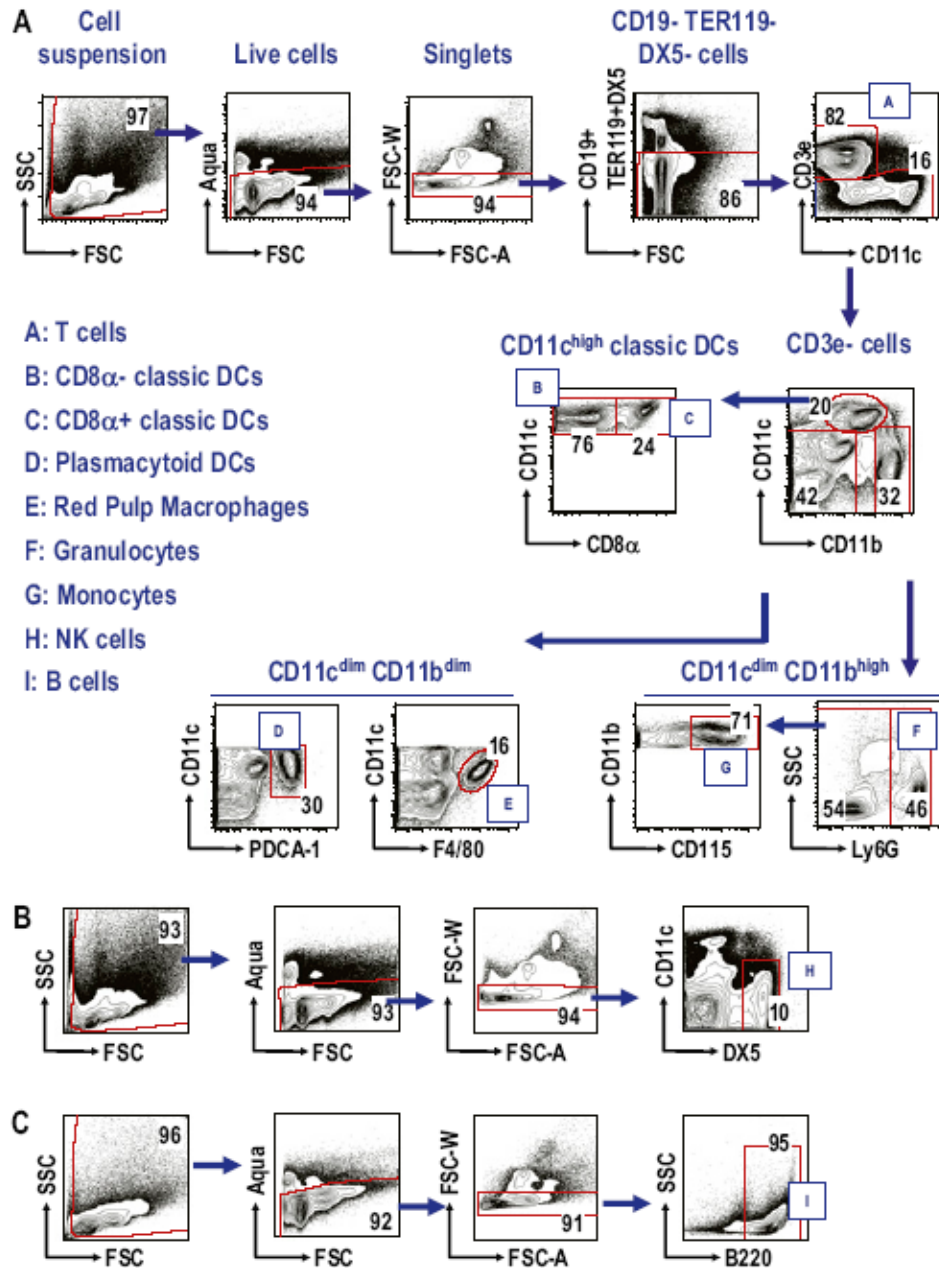
### **Targeting of Alexa 647-Labeled mAbs**

Anti-mDEC205-poly dA:dT mAb was labeled with Alexa 647 (Invitrogen Carlsbad, CA) per the manufacturer's instructions. C57BL/6 mice were injected i.p with 10  $\mu$ g labeled mAb. Uptake of labeled mAb by splenocytes was evaluated 3 hours after inoculation by multicolor flow cytometry (Idoyaga et al., 2009). The gating strategy is shown in figure 12.

**Figure 12:** Gating strategy for evaluating the targeting of injected anti-DEC205-poly dA:dT mAb to different subsets of DCs and other leukocytes in mouse spleen.

Splenocytes were incubated with anti-CD19 microbeads to remove B cells. Non-B cells (A and B) or positive selected B cells (C) were stained to detect distinctive leukocyte populations. A. Scheme to detect T cells, CD8<sup>-</sup> DCs, CD8<sup>+</sup> DCs, PDCs, red pulp macrophages, granulocytes, and monocytes. B. Scheme to detect NK cells. C. Scheme to detect B cells.<sup>81</sup>

Figure 12



## **DC Maturation**

Mice were injected i.p. with PBS or 15 ug anti-DEC205-poly dA:dT mAb. Spleens were collected 12 hours later and collagenase digested. Maturation was monitored by increased expression of MHC-II, CD86 and CD40 after gating on plasmacytoid, CD11c<sup>hi</sup> DEC<sup>-</sup>, or CD11c<sup>hi</sup> DEC<sup>+</sup> DCs (Idoyaga et al., 2009). The gating strategy to obtain these populations was the same as shown in figure 12.

## **Immunizations**

Mice were immunized twice i.p. at 4 week intervals with 5 µg of fusion HIV gag-p24 mAbs together with adjuvant (poly I:C or anti-DEC205-poly dA:dT mAb). One week after the second injection, splenocytes were restimulated with p24 (2 µM) or negative control peptide mix, along with 2 µg/mL of costimulatory αCD28 (clone 37.51) for 6 hours. Brefeldin A (10 ug/mL; Sigma-Aldrich, St. Louis, MO) was added for the last 5 hours. Cells were washed, incubated for 10 min at 4°C with 2.4G2 mAb to block Fcγ receptors, washed, and stained with Live/Dead Fixable Aqua viability dye, Pacific blue-conjugated anti-CD3, PerCP-conjugated anti-CD4, and Alexa 750-conjugated anti-CD8 mAbs for 20 min at 4°C. Cells were permeabilized (Cytofix/Cytoperm Plus; BD Biosciences, Bedford, MA) and stained with APC-anti-IFNγ, PE-anti-IL2, and PE-Cy7 anti-TNFα mAbs for 15 min at room temperature (BD Biosciences, Bedford, MA). Samples were acquired on BD LSRII flow cytometry and analyzed with FlowJo Software (Tree Star, Inc., San Carlos, CA).

### **RNA Preparation and RT-PCR**

Human MoDCs were collected 24 hours after electroporation with various siRNA. Cells were lysed with TRIzol (Invitrogen, Carlsbad, CA), followed by one round of chloroform extraction. Total RNA was precipitated with isopropanol and then washed with 75% ethanol. Reverse transcription was then performed on isolated RNA using High Capacity RNA-to- cDNA Kit (Applied Biosystems, Carlsbad, CA). RT-PCR conditions and primer sequences for RIG-I and MDA-5 were previously described (Sasai et al., 2006).

### **ELISA**

Human IFN- $\alpha$ , mouse IFN-  $\alpha$ , human IFN- $\beta$  (PBL Interferon Source), and human TNF (R&D systems) were assessed with commercial ELISA kits according to the manufacturer's instructions.

### **Electroporation with SiRNA**

All siRNA sequences were purchased from Sigma. The RIG-I siRNA sequence was 5'-AAGGCUGGUUCCGUGGCUUUUdTdT-3'. The MDA5 siRNA sequences were: 5'-GUUCAGGAGUUAUCGAACAdTdT-3', 5'-GUAACAUGUUAUCCGUUAdTdT-3', 5'-GGUGUAAGAGAGCUACUAAAdTdT-3'. The Control siRNA (targets luciferase) sequence was 5'-CUUACGCUGAGUACUUC GAdTdT-3'. For electroporation, human MoDCs were resuspended in opti-MEM without phenol red (Invitrogen) at a concentration of  $4 \times 10^7$  per milliliter.  $4 \times 10^6$  cells were electroporated with 1 nmol of

siRNA in a 4 mm cuvette and in a total volume of 200  $\mu$ l of opti-MEM. Cells were pulsed using the ECM830 Electro Square Porator<sup>TM</sup> (BTX Harvard Apparatus). The pulse conditions were a unique square wave pulse of 500 V and 0.5 ms. Immediately after electroporation, cells were transferred in complete medium [RPMI 2% human serum] supplemented with GM-CSF (20 ng/mL) and IL-4 (20 ng/mL).

## **Chapter 3**

### **Results**

#### **Production of anti-DEC205-poly dA:dT Antibodies**

At the onset of the project, two methods were employed to ligate molecules of interest to the DEC205 antibody: one method used the full-length mutant intein, Mxe GyrA, and the other used split inteins, either DNAe or NPU. The use of split inteins proved difficult. The anti-DEC205-DNAe intein was not produced in the media of transiently transfected HEK293T cells, and only a small amount (0.1-0.2 mg/L) of anti-DEC205-NPU intein was found in the media of transiently transfected HEK293T cells. Thus, only the full-length mutant intein method was utilized to produce the anti-DEC205-poly dA:dT antibodies.

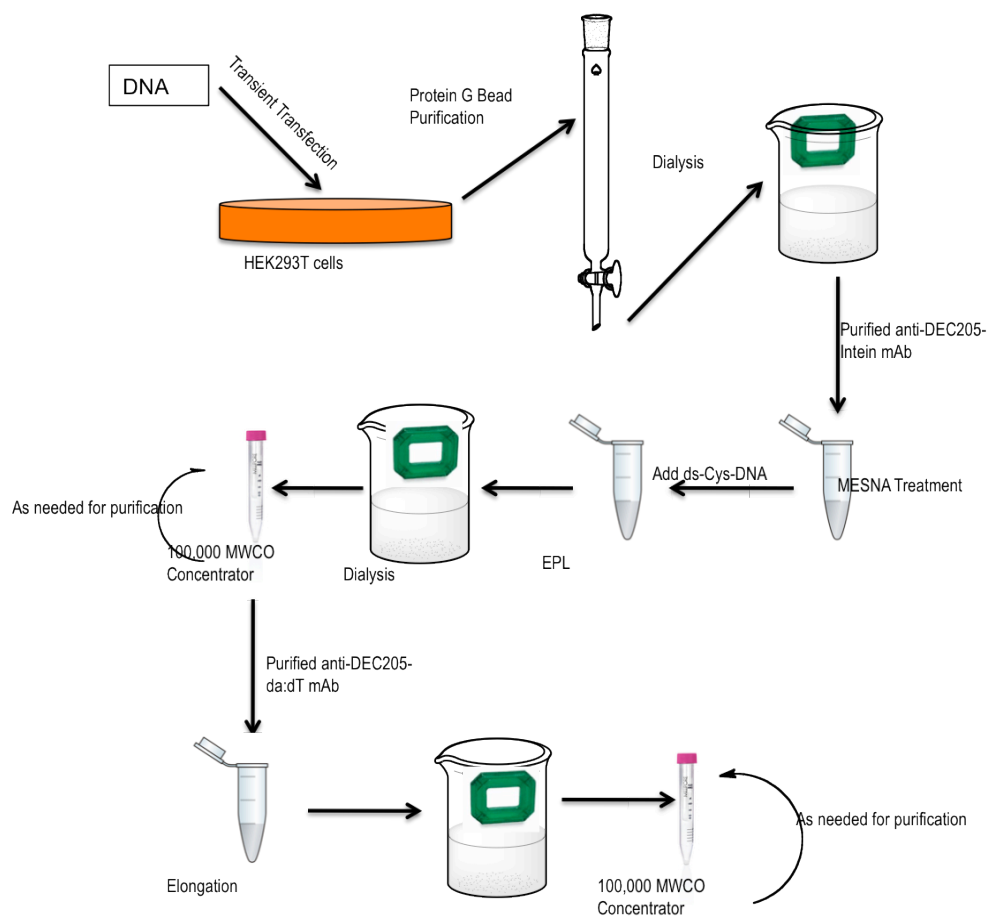
The overall scheme of production is shown in Figure 13. The anti-DEC205-Mxe gyrA intein antibodies were produced by transient transfection in HEK293T cells. After 3-5 days of expression, the secreted antibody in the media was loaded onto a Protein G column for purification. The antibody was then dialyzed against PBS to produce the purified anti-DEC205-intein antibodies.

The integrity of the purified proteins was confirmed by 10% SDS-Page gel stained with Coomassie blue (Figure 14A) and Western blot analysis (Figure 14B) using rat anti-

**Figure 13: Schematic of Production.** DNA encoding the DEC205 antibody with the Mxe gyrA intein attached to its C-terminus is transiently transfected into HEK293T cells. After 3-5 days of expression, the media is collected, and the antibody is purified using a column of Protein G beads. The antibody is dialyzed in PBS to produce the purified anti-DEC205-Mxe gyrA intein antibodies. This antibody is then incubated with 100 mM MESNA for 24 hours. The MESNA-treated antibody is combined with ds-Cys-DNA in order for expressed protein ligation to occur. The DNA-antibody conjugate is purified from unreacted Cys-DNA by dialysis and concentration. The DNA on the purified DNA-antibody conjugate is elongated using Klenow fragment. The anti-DEC205-poly dA:dT antibodies are purified using dialysis and concentration.



**Figure 13**



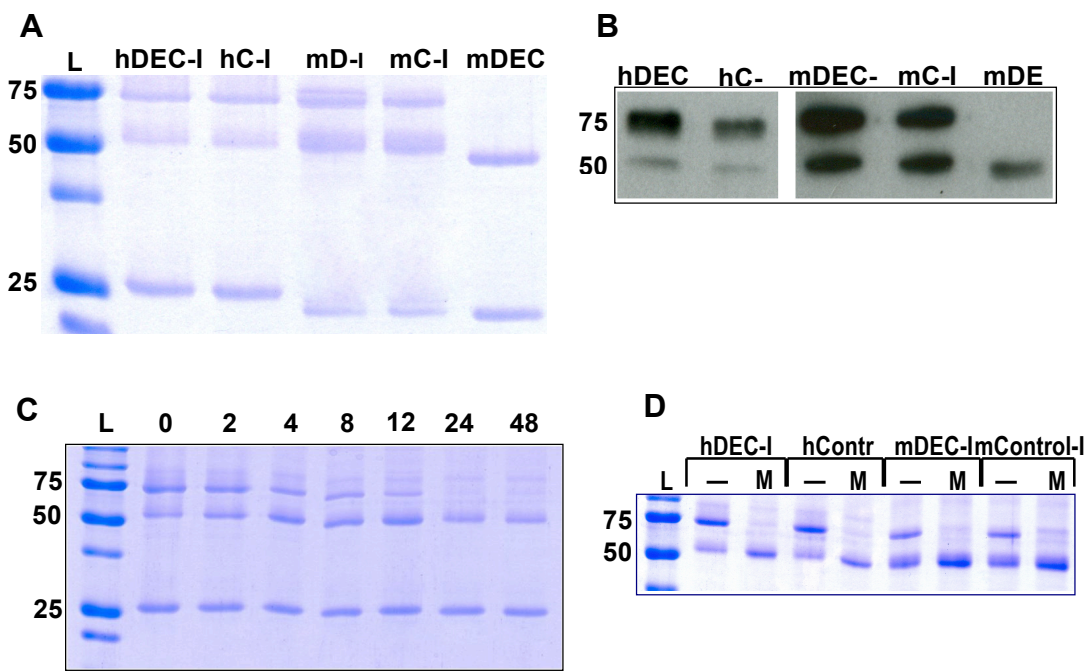
human (left panel) or goat anti-mouse (right panel) IgG Ab conjugated with HRP. The results indicated that the anti-DEC205-Mxe gyrA intein antibody was produced. The anti-hIgG (left) and mIgG (right) western blot showed two bands. The most prevalent band was at ~75 kD and represents that DEC205 antibody's heavy chain with the Mxe GyrA intein attached to the C-terminus. The band at ~50 kD can be interpreted as a non-specific IgG contaminant, or the DEC205 antibody without the intein attached. It is most likely the latter. The anti-DEC205-intein antibodies were produced in media supplemented with nutridoma. Nutridoma, unlike serum, does not have antibodies, and therefore, the media should not have IgG contaminants. The Coomassie blue stain showed three main bands. This includes the two bands described above, and a band at ~25 kD which is the right size for the light chain of the DEC205 antibody. Since there are only three bands on the Coomassie blue stain, the DEC205-Mxe GyrA intein antibody production is relatively pure. A negative control is shown in lane 5. This is expression of the DEC205 antibody without the Mxe GyrA intein attached to its C-terminus. As expected, only 2 bands appear in this lane: the 50 and 25 kD bands. This gives further support that the ~75 kD band is the DEC205-Mxe GyrA intein antibody band.

To evaluate the best method of transfection, different concentrations of the anti-DEC205-GyrA C79S C114S DNA were used to transfect the HEK293T cells. A total of five different concentrations were used: 5, 10, 15, 20, and 30 µg of DNA per plate. This was performed because the typical amount of DNA transfected, 30 µg, appeared to be killing the HEK293T cells as visualized by microscopy. The yield obtained from the plates transfected with 30 µg was approximately 0.75 mg/L. The best yield was obtained using

**Figure 14: Production of DEC205-Mxe GyrA intein antibodies. A & B.** The integrity of the anti-DEC205-intein antibodies were confirmed by 10% SDS-Page gel stained with **(A)** Coomassie blue and **(B)** Western blot analysis using (Left) anti-human (h) or (Right) anti-mouse (m) IgG Ab conjugated with HRP. Numbers are expressed in kDa. Anti-hIgG (hControl) and anti-mIgG (mControl) Mxe GyrA intein antibodies were also produced. **C.** Time course of MESNA treatment for anti-hDEC205-intein antibodies. Top numbers represent time in hours. Left numbers represent kDa. **D.** Activation of the DEC205 antibody. Anti-DEC-intein antibodies were treated with 100 mM MESNA for 18 hours. The removal of the intein from the heavy chain was confirmed by 10% SDS-Page gel stained with Coomassie blue. Numbers are expressed in kDa.

**Figure 14**

hDEC-I: anti-hDEC205-Mxe gyrA intein mAb  
hC-I: anti-hControl-Mxe gyrA intein mAb  
mD-I: anti-mDEC205-Mxe gyrA intein mAb  
mC-I: anti-hDEC205-Mxe gyrA intein mAb  
mDEC: anti-mDEC205 mAb  
L: Ladder  
M: MESNA treatment  
-: Untreated



15 µg of DNA per plate. The yield of antibody using this amount of DNA was approximately 1.5 mg/L of HEK293T cells as determined by total protein concentration.

To evaluate if the anti-DEC205-GyrA antibody was active towards thiolysis, the purified fusion was incubated with 100 mM sodium 2-mercaptoethanesulfonate (MESNA). Two µl aliquots of the mixture were taken at 0, 2, 4, 8, 12, 24, and 48 hours. These aliquots were loaded on a 12% SDS-page gel and stained with Coomassie blue to determine the extent of thiolysis at each time point. Thiolysis was measured by the extent of cleavage of the GyrA intein which leaves the antibody with a reactive  $\alpha$ -thioester on the heavy chain (Figure 6). Acceptable levels of thiolysis by MESNA was achieved after 24 hour incubation (Figure 14C). Since 48 hour incubation with MESNA did not improve the level of thiolysis, all subsequent experiments employed a 24 hour thiolysis step using 100 mM MESNA. All four antibodies tested with Mxe GyrA intein attached to its C-terminus were able to be thiolysed using this protocol (Figure 14D).

Next we designed an experiment to evaluate whether the DEC205 antibody thioester could undergo EPL. For this, we employed a short model peptide containing an N-terminal cysteine (for ligation) and a fluorescein label to provide a convenient readout of ligation. The DEC205 antibody thioester was incubated with 10 fold excess of short fluorescent peptide (Figure 15A). The sample was run on a 12% SDS-Page gel with varying concentrations of short fluorescent peptides, which were used as standards. The gel was then monitored for fluorescence. The fluorescent antibody band was then

compared to the intensity of short fluorescent peptide standards using ImageJ software (Figure 15B). The ligation efficiency was determined to be ~98%.

Commercially available amine-DNA (20-mer of As) was used to produce a DNA oligonucleotide with an N-terminal cysteine (Cys-DNA). The amine-DNA was reacted with N-hydroxysuccinimide activated Fmoc-Cys(StBu)-OH (Figure 10A). The product was purified by HPLC, and the protecting groups (Fmoc and StBu) were removed by addition of 30% ammonium hydroxide and 1 M DTT. The product was purified via HPLC and the identity of the compound was verified by MALDI-Mass Spectrometry (Figure 10B and 11). A complimentary oligonucleotide (20-mer of Ts) was then annealed to the Cys-DNA.

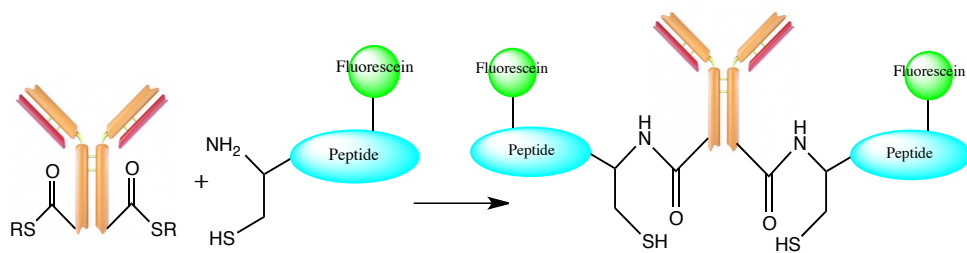
The double stranded (ds)-Cys-DNA was used for ligation to the MESNA-treated anti-DEC205 antibody. After an 18 hour reaction, the DNA-antibody conjugate was run on a 2-20% TBE native gel stained with ethidium bromide to confirm ligation (Figure 16A).<sup>79</sup> A band was visualized around 1000 base pairs, which disappeared upon proteinase K treatment, indicating it was a DNA-antibody conjugate. Ligation efficiency was indirectly approximated using a fluorescence assay.

First, the MESNA-treated antibody was incubated with ds-Cys-DNA or unmodified DNA. After overnight incubation, 10 fold excess of small fluorescent peptide was added to both reactions. The samples were run on a 12% SDS-PAGE gel after an 18 hour incubation, and the amount of antibody that was fluorescently labeled was determined

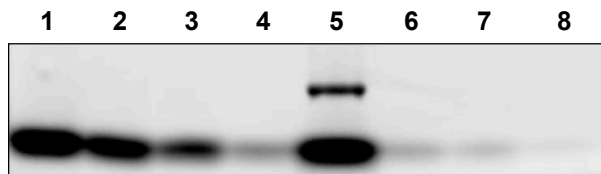
**Figure 15: Ligation with Short Fluorescent Peptide.** **A.** Schematic of the Reaction of the MESNA-treated antibody with the short fluorescent peptide. **B.** Fluorescence assay to determine ligation efficiency. 100 pmoles of MESNA-treated DEC205 antibody was reacted with 1000 pmoles of the short fluorescent peptide. The sample was run on a 10% SDS Page-gel and monitored for fluorescence. The short fluorescent peptide was at varying concentrations in other lanes to use as standards of fluorescence. ImageJ software was used to quantify the amount of fluorescent antibody.

**Figure 15**

**A**



**B**



Lane 1: 1000 pmoles Fluorescent peptide  
Lane 2: 500 pmoles Fluorescent peptide  
Lane 3: 250 pmoles Fluorescent peptide  
Lane 4: 100 pmoles Fluorescent peptide  
Lane 5: Anti-DEC205-peptide Reaction  
Lane 6: 50 pmoles Fluorescent peptide  
Lane 7: 25 pmoles Fluorescent peptide  
Lane 8: 10 pmoles Fluorescent peptide



using ImageJ software. There was no difference seen between pretreatment of antibody with unmodified DNA and antibody that had no pretreatment (data not shown). Thus, the antibody treated with unmodified DNA was used as a control to represent 100% reaction with fluorescent peptide. Anti-DEC205 antibody that was fluorescently labeled in the lane that was treated with Cys-DNA indicates the amount of antibody that did not react with the ds-Cys-DNA. Thus, the amount of cys-DNA ligation was determined by subtracting the amount of fluorescence in the Cys-DNA treated lane from the amount of fluorescence in the unmodified DNA treated lane. Ligation efficiencies for all the constructs ranged from 68-84%, with a typical ligation efficiency around 75% (Figure 16C).

This experiment assumes that there is no hydrolysis of the  $\alpha$ -thioester on the MESNA-treated DEC205 antibody during the initial reaction period. This assumption seems reasonable since there was no difference in antibody fluorescence when the antibody is pretreated with unmodified DNA compared to no pre-treatment. Furthermore, it is assumed that the same amount of thiolysis would occur during the initial reaction when comparing pretreatment with Cys-DNA and modified DNA as the only difference between the two reactions is the presence of the cysteine on the Cys-DNA.

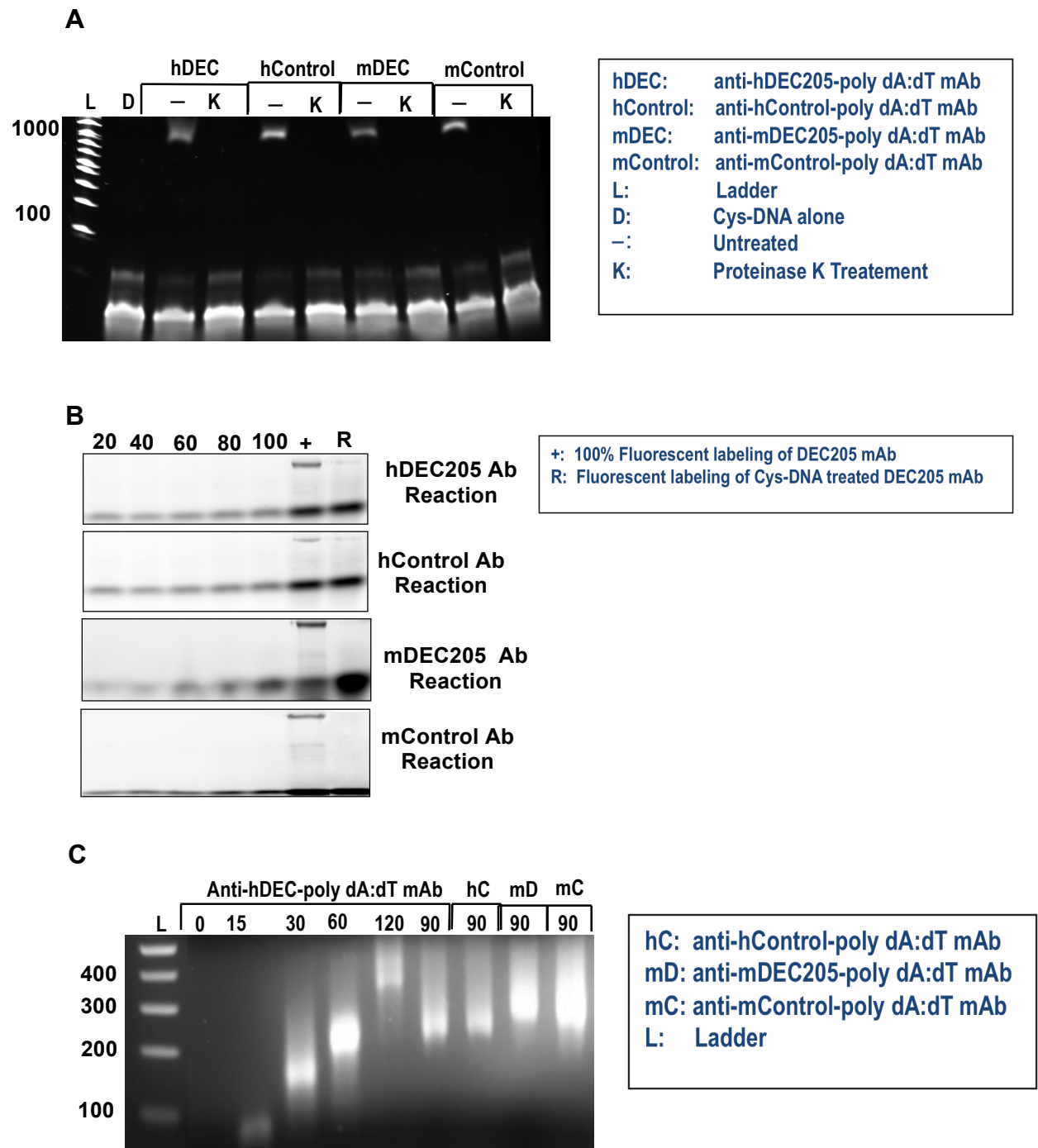
Only 20 base pairs of dA:dT were ligated to the anti-DEC antibody using EPL. Ligation of longer Cys-DNA to the antibody was less efficient (data not shown), and production of longer cys-DNA was cost ineffective. To introduce a larger dA:dT sequence into the antibody, since it has been shown that at least 40 base pairs of dA:dT are necessary for

immune activation of human MoDCs,<sup>28</sup> Klenow fragment in the presence of ATP and TTP was used to elongate the double stranded DNA on the fusion mAb.<sup>84</sup> A method was developed to elongate the DNA to approximately 250 base pairs, around the same length as commercially available poly dA:dT. The DNA-antibody conjugate was then treated with proteinase K and run on a 2% agarose gel stained with EtBr to check for elongation (Figure 16B). A length of 250 base pairs was seen after 90 minutes of Klenow reaction. Thus, this was the time point used to produce the anti-DEC205-poly dA:dT antibodies.

We next wanted to verify that the anti-DEC205-poly dA:dT antibodies were still active. With the MESNA treatment during the production, disulfide bonds could have been reduced, impairing the functionality of the antibody. Thus, a CHO stain was performed. Three different concentrations of the anti-DEC205-poly dA:dT mAb was incubated with CHO cells that expressed either mouse or human DEC205 receptor or CHO cells that did not have the receptor (CHO NEO). After incubation, antibodies bound to the CHO cells were labeled with either a mouse or human IgG-PE antibody. FACS analysis was then performed to determine the extent of binding. Results indicated that the antibodies bound specifically (to the CHO DEC205 cells, but not to the CHO NEO cells) and in a concentration dependent manner (Figure 17).

**Figure 16: DNA Ligation to DEC205 antibody.** **A.** MESNA-treated DEC205 or control mAb was added to Cys-DNA. Production of DNA-mAb conjugates was monitored on a 2-20% TBE native gel stained with EtBr. Numbers are expressed in base pairs. **B.** Indirect determination of anti-DEC205-dA:dT mAb ligation efficiency. After 18 hour ligation reaction between Cys-DNA and DEC mAb-thioester, 1000 pmoles (20 fold excess) of fluorescent peptide was added and allowed to react for a further 18 hours. The sample was run on a 10% SDS Page-gel and monitored for fluorescence. ImageJ software was used to determine the amount of DEC antibody that was fluorescently labeled. Numbers are in pmoles of fluorescent peptide. **C.** Elongation of DNA. Purified anti-DEC-dA:dT mAbs were reacted with Klenow fragment in the presence of dATP and dTTP. The mAbs were treated with proteinase K and run on a 2% agarose gel stained with EtBr. Numbers on left are expressed in base pairs. Numbers on top represent time in min.

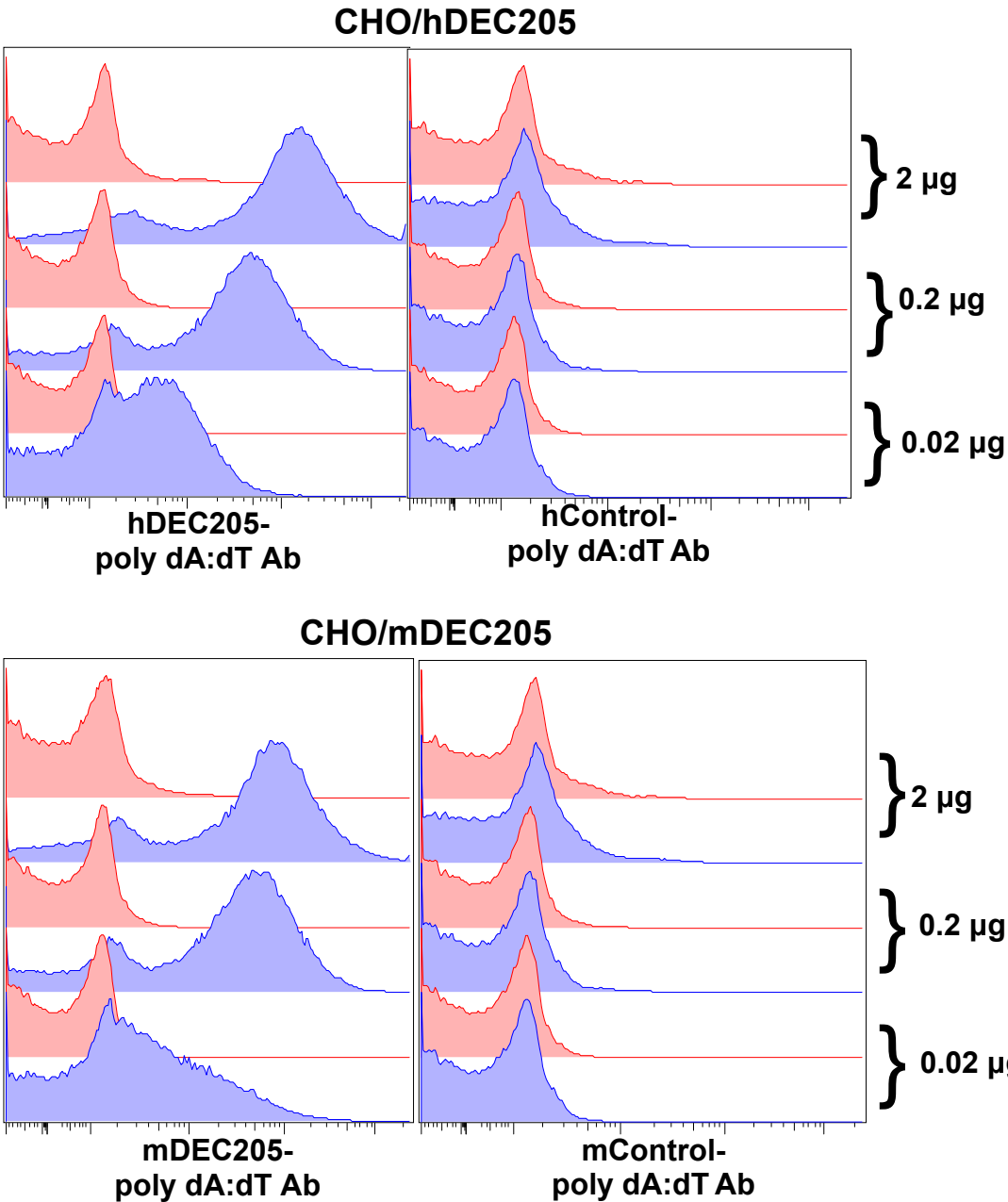
Figure 16



**Figure 17: CHO staining.** CHO cells with hDEC205 or mDEC205 receptors as well as CHO cells without DEC205 receptors (CHO NEO) were incubated with three different concentrations of anti-DEC205-poly dA:dT mAbs. The cells were then stained with either anti-hIgG or anti-mIgG-PE antibody. FACS analysis was performed to determine the extent of binding. Top panel is for hDEC205 CHO cells. Bottom panel is for mDEC205 CHO cells.

Figure 17

— CHO NEO  
— CHO DEC



### **Production of Cys-SiRNA**

We wanted to determine if siRNA could be linked to the DEC205 antibody. To test this, siRNA to programmed cell death ligand- 1 (PDL-1) was purchased from Sigma Aldrich (St. Louis, MO). PDL-1 is a molecule that binds to its receptor, PD-1, found on activated T cells, B cells, and myeloid cells, to inhibit their activation. Thus, by using siRNA to PDL-1, we wanted to determine if we could elicit a stronger immune response when an adjuvant and innate stimulus was given.

We were able to produce Cys-siRNA using the method described for the production of cys-DNA above. However, the yield was much lower, and not enough Cys-siRNA was made to attempt a ligation to the DEC205 antibody. It was determined that the treatment with 30% Ammonium hydroxide was destroying the integrity of the siRNA and was the reason why so little was produced.

The Cys-siRNA that was produced, however, was able to efficiently knockdown PDL-1 (data not shown). This indicates that the addition of the amino acid did not disrupt siRNA function.

This project was abandoned, however, due to reproducibility issues. The siRNA received from the company always consisted of varying purity. Extraneous peaks were always seen in the HPLC trace before any chemistry was performed.

### **Immune Response of anti-hDEC205-poly dA:dT mAb in MoDCs**

Human MoDCs were treated with anti-hDEC-poly dA:dT and control Ig-poly dA:dT mAb to test for innate immunity (Figure 18A). Anti-hDEC-poly dA:dT mAb caused production of IFN- $\alpha$  without the use of lipofectamine whereas anti-control-poly dA:dT mAb did not. As expected, only cells treated with lipofectamine produced IFN- $\alpha$  when unconjugated poly dA:dT was given (Figure 18A). The response was dependent on dose of anti-hDEC-poly dA:dT mAb (Figure 18A) and time of exposure (Figure 18B). To test the importance of hDEC receptor expression, MoDCs were pretreated with a high concentration (10  $\mu$ g/ml) of anti-MHC II, anti-CD11c, anti-DEC205, and anti-IgG for 2 hours. After 2 hours, the cells were treated with the anti-hDEC-poly dA:dT mAb. Only the pretreatment of anti-DEC205 mAb caused a significant decrease in the production of IFN- $\alpha$  by the MoDCs, indicating that the immune response was dependent on DEC205 receptor expression (Figure 18C).

To determine mechanism of immune activation, siRNA for RIG-I and MDA-5 was electroporated into the MoDCs on day 5 of their differentiation.<sup>85</sup> On day 6, RNA was collected from the cells and converted into cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems). RT-PCR was then performed using the conditions and primer sequences previously described (Sasai et al., 2006). Results of the RT-PCR indicated that there was sufficient knockdown with both the RIG-I and the MDA-5 siRNA. Next, the supernatant from the siRNA treated MoDCs was collected and an IFN- $\alpha$ , TNF- $\alpha$  and IFN- $\beta$  ELISA was performed. Production of cytokines by anti-mDEC poly dA:dT mAb was significantly reduced with RIG-I siRNA whereas the MDA-5 siRNA had no effect

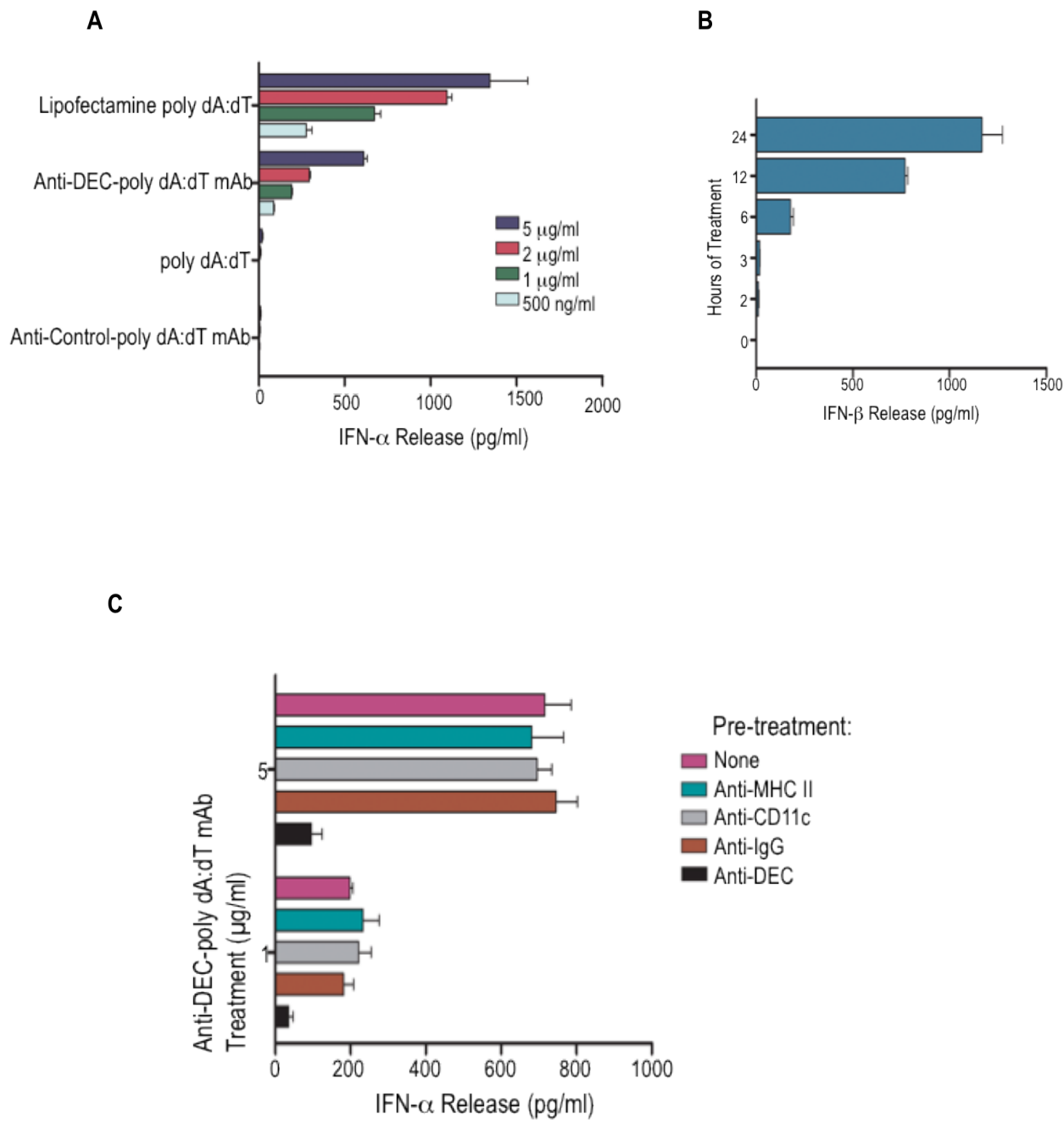


(Figure 19A, 19B, 19C). This result implies that DEC205 allows for cross-presentation, and the poly dA:dT gains cytoplasmic entry to activate RIG-I.

For comparison, other immune stimulants were given to the MoDCs. hDEC-poly dA:dT mAb caused the most production of IFN- $\alpha$  and IFN- $\beta$  when compared to poly IC (TLR 3 and MDA-5 agonist), resiquimod (TLR 7/8 agonist), CLO75 (TLR 7/8 agonist), and LPS (TLR 4 agonist) (Figure 19A, C). Anti-hDEC205-poly dA:dT mAb induced less TNF- $\alpha$  than resiquimod, CLO75, and LPS, but still a significant quantity (Figure 19B). Thus, high quantities of type I IFN are produced and transfection agents are not necessary for immune stimulation by anti-DEC-poly dA:dT antibody, and we proceed to test this approach to stimulate innate and adaptive immunity *in vivo* in mice.

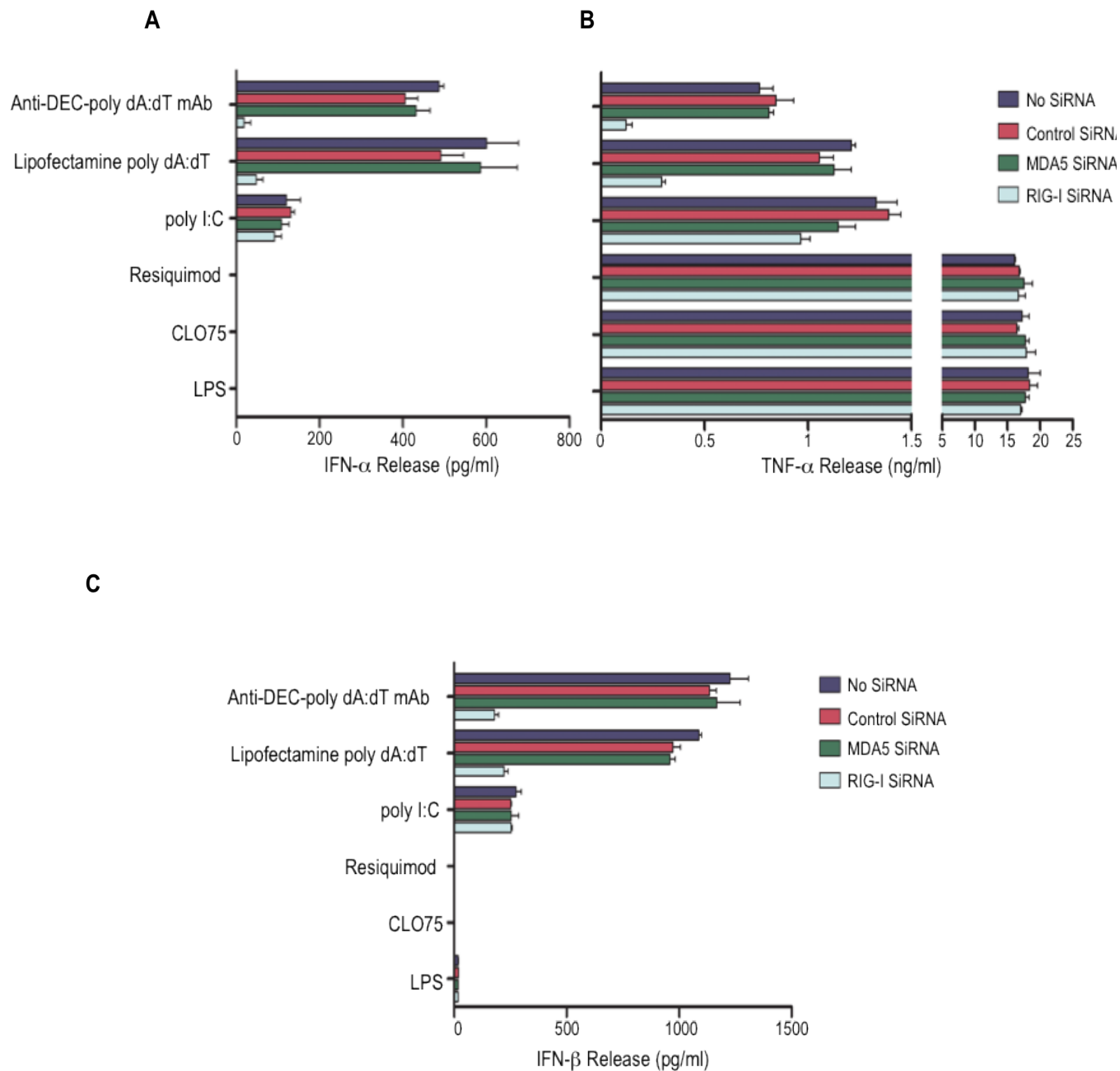
**Figure 18: Anti-DEC205-poly dA:dT mAb activates MoDCs.** **A.** ELISA for the production of IFN- $\alpha$  (pg/mL) by MoDCs treated with various doses (5 ug/mL to 500 ng/mL) of poly dA:dT, anti-hDEC-poly dA:dT mAb, and anti-hControl-poly dA:dT mAb. Graph is cumulative representation of 3 separate experiments. Error bars show standard error of mean (SEM). **B.** Time course of the production of IFN- $\beta$  (pg/mL) by MoDCs treated with anti-hDEC-poly dA:dT mAb. Y axis units are hours. Graph is cumulative representation of 2 separate experiments. Error bars show SEM. **C.** MoDCs were pretreated for 2 hours with 10  $\mu$ g of various mAbs before anti-DEC-poly dA:dT mAb was given. ELISA was performed to determine IFN- $\alpha$  (pg/mL) production. Y axis is amount of anti-DEC poly dA:dT mAb given ( $\mu$ g/mL) Graph is cumulative representation of 2 separate experiments. Error bars show SEM.

**Figure 18**



**Figure 19: Anti-DEC205-poly dA:dT mAb activates RIG-I. A, B, & C.** ELISA for the production of IFN- $\alpha$  (pg/mL) (A), TNF- $\alpha$  (ng/mL) (B), and IFN- $\beta$  (pg/mL) (C) by MoDCs transfected by electroporation with control, MDA-5, or RIG-I specific siRNA, then stimulated with various immune stimulants including anti-hDEC205-poly dA:dT mAb. Graphs are cumulative representation of 3 separate experiments. Error bars show SEM.

**Figure 19**



## Innate Immune Response in B6 Mice

To show targeting, the anti-mDEC205-poly dA:dT antibody was labeled with Alexa 647. After injection of the labeled protein in naïve B6 mice, flow cytometry was performed to determine targeting. The gating strategy is shown in figure 12. The results show that anti-mDEC205-poly dA:dT mAb mostly targets to the CD8<sup>+</sup> DCs. There is also targeting to B cells, granulocytes, NK cells, and T cells. There is very little targeting to CD8<sup>-</sup> DCs and red pulp macrophages, and no targeting to monocytes and plasmacytoid DCs (Figure 20). This is consistent with unconjugated anti-mDEC205 mAb.

To determine the innate response of anti-mDEC205-poly dA:dT antibody, an IFN- $\alpha$  ELISA was performed on the serum of injected naïve B6 mice (Figure 21A). Gratifyingly, anti-mDEC205-poly dA:dT mAb caused as much IFN- $\alpha$  release as poly I:C. The production of IFN- $\alpha$  by anti-mDEC205 poly dA:dT mAb was rapid in onset, occurring before three hours, and dose-dependent (Figure 21B). As expected, this response was ablated in DEC205 knockout (KO) mice, and anti-mControl-dA:dT mAb did not cause innate immunity.

To document the cellular source for IFN- $\alpha$  production, FACS was used to separate CD11c<sup>low</sup> plasmacytoid DC, CD8<sup>+</sup> DC, and CD8<sup>-</sup> DC populations in the spleens of anti-DEC-poly dA:dT mAb injected animals. Only the CD8<sup>+</sup> DCs produced IFN- $\alpha$  (Figure 21C). Although CD8<sup>+</sup> DCs had more upregulation of CD40, CD86 and MHCII, all types

of DCs showed these features of “phenotypic” maturation (Figure 22A), indicating that all types of DCs responded to the cytokine made by the CD8<sup>+</sup> DCs.

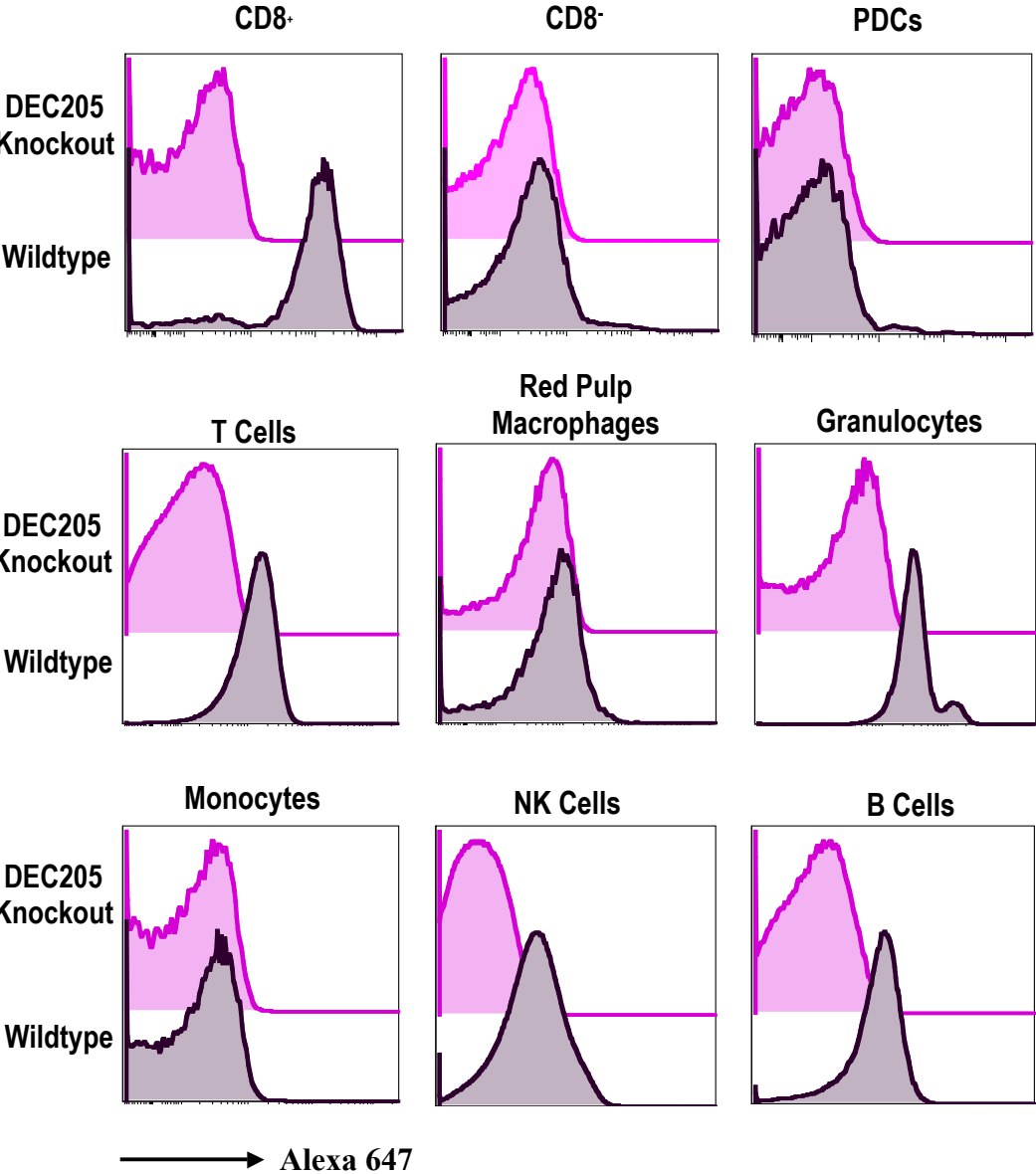
To determine whether stromal cells were also producing IFN- $\alpha$  in response to anti-mDEC205-poly dA:dT mAb, wild type or DEC KO mice were irradiated and then given bone marrow from a donor with the opposite genotype. After 2 weeks, the mice were injected with anti-mDEC205-poly dA:dT mAb, and the innate immune response was determined using an IFN- $\alpha$  ELISA (Figure 22B). Irradiated DEC205 KO mice with wild type bone marrow were able to produce significantly more IFN- $\alpha$  than irradiated wild type mice with DEC205 KO bone marrow, indicating that the majority of innate cytokine was produced from the bone marrow derived cells, namely the CD8<sup>+</sup> DCs.

To further demonstrate that the CD8<sup>+</sup> DCs were responsible for the production of IFN- $\alpha$ , CD11c-DTR mice were injected with diphtheria toxin (DT). This knocks out all CD11c<sup>+</sup> cells. The next day, anti-mDEC205-poly dA:dT mAb was injected into the animals. Mice that received the DT were unable to produce cytokine as evidenced by ELISA (Figure 22C).

**Figure 20. Targeting of anti-DEC205-poly dA:dT mAb.** Naïve B6 mice were injected with Alexa 647 labeled anti-mDEC205 poly dA:dT mAb. Uptake of labeled mAb by splenocytes was evaluated 3 hours after inoculation by multicolor flow cytometry.<sup>81</sup> The gating strategy is shown in figure 12.



**Figure 20**

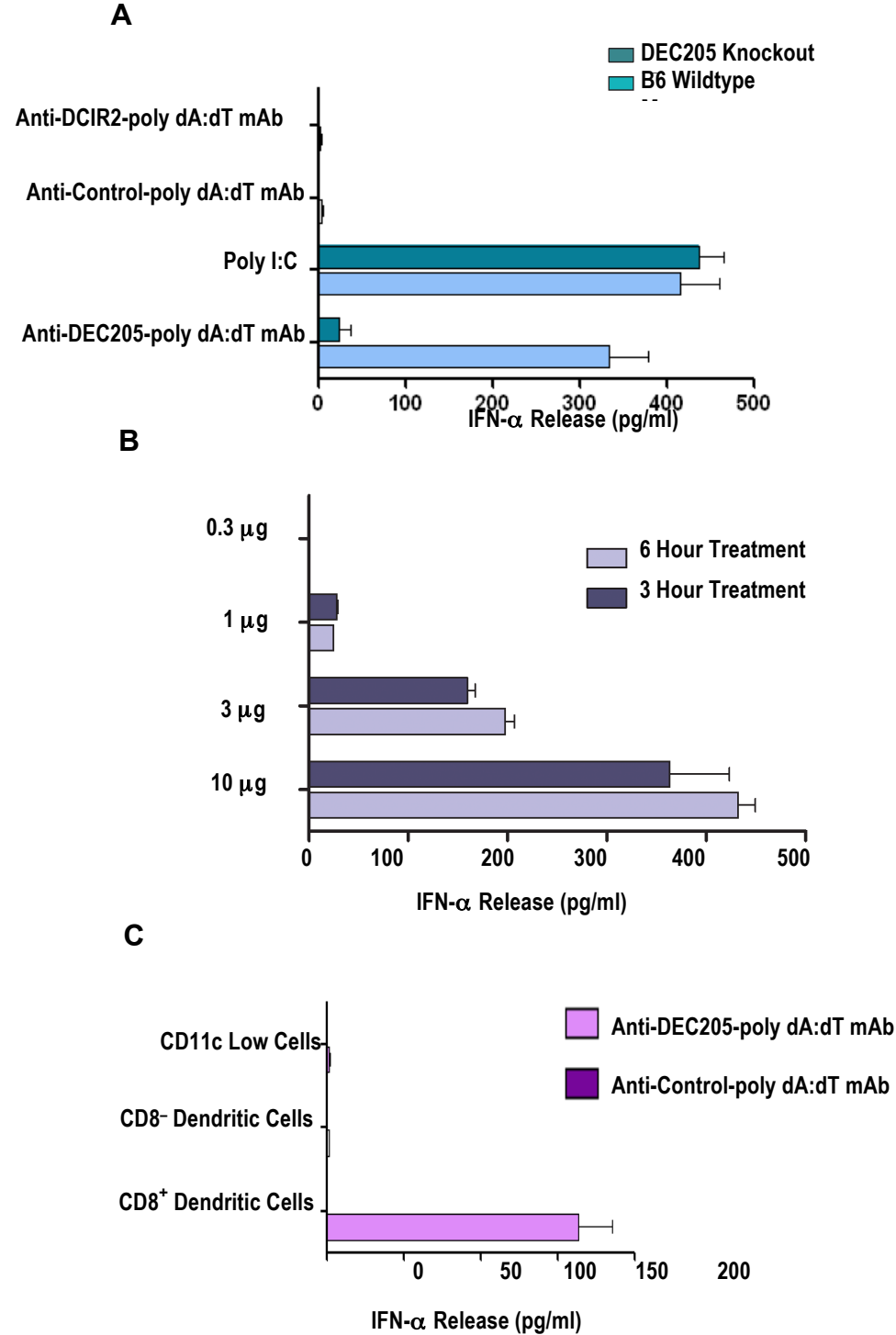


**Figure 21. Anti-DEC205-poly dA:dT mAb induces an innate immune response *in vivo* driven solely by DEC<sup>+</sup> CD8<sup>+</sup> DCs.** **A.** B6 or DEC205 KO mice were inoculated i.p. with 10 µg of anti-mDEC-poly dA:dT mAb, anti-mControl-poly dA:dT mAb, or poly I:C. After 6 hours, serum was collected and analyzed for IFN-α production using ELISA. Graph is cumulative representation of 5 separate experiments. Error bars show SEM.

**B.** Various doses of anti-mDEC-poly dA:dT mAb were inoculated i.p. in B6 mice. Serum was collected at 3 and 6 hours and then analyzed for IFN-α production using ELISA. Graph is cumulative representation of 2 experiments with 3 mice each. Error bars show SEM.

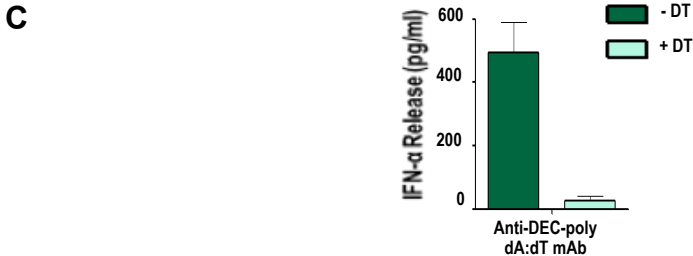
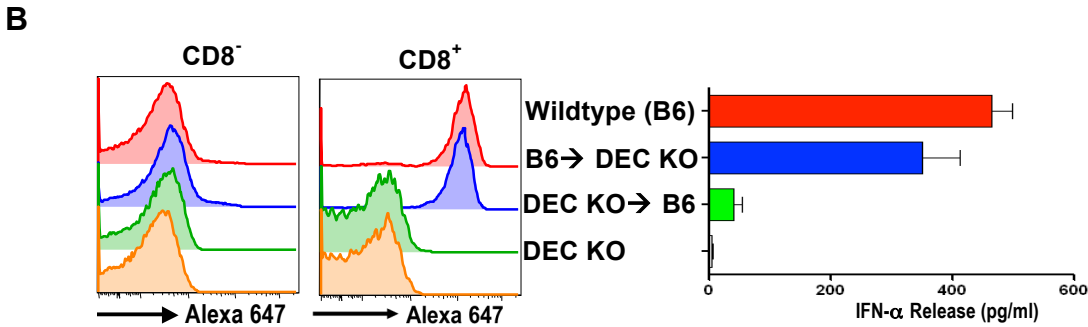
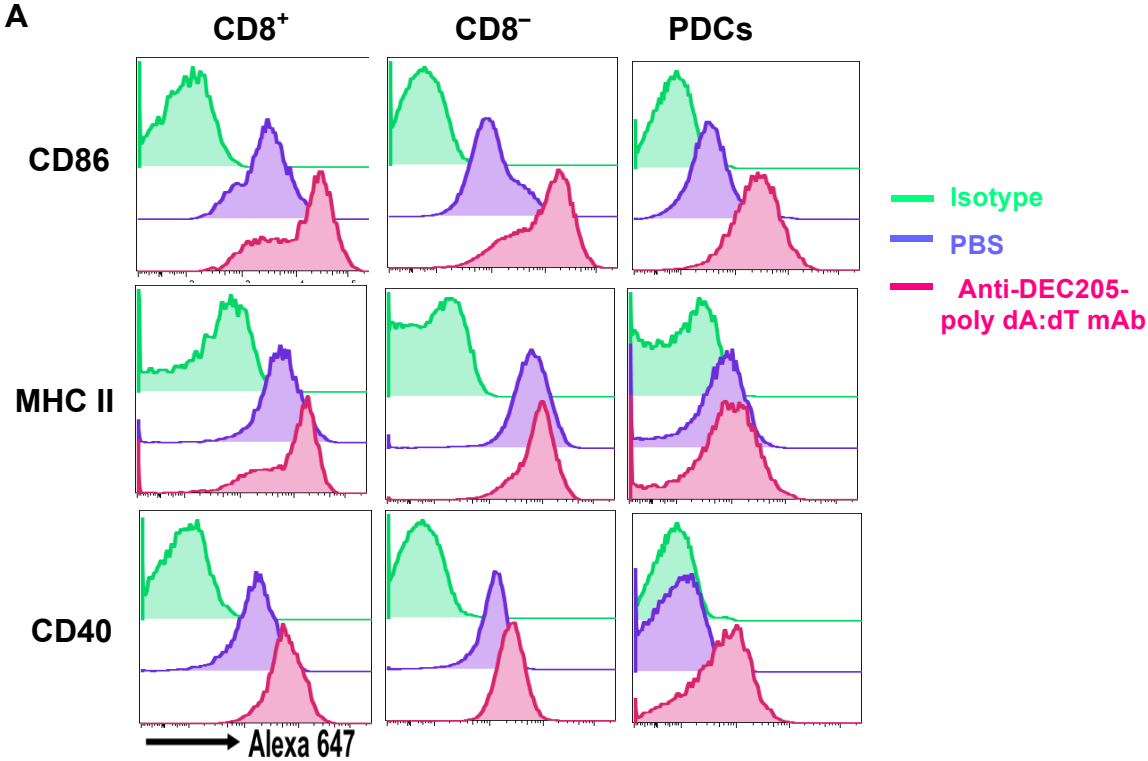
**C.** B6 mice were inoculated i.p. with 10 µg of anti-mDEC-poly dA:dT mAb. Splenocytes were harvested after 2 hours, and CD11c<sup>low</sup>, CD8<sup>+</sup> DC, and CD8<sup>-</sup> DC populations were sorted using FACS Aria with DiVa configuration. The cells were plated overnight in RPMI supplemented with 5% FCS, and the supernatant was collected for an IFN-α ELISA. Graph is cumulative representation for 1 experiment with 4 mice. Error bars show SEM.

Figure 21



**Figure 22. Maturation, Chimeras, and CD11c DTR mice.** **A.** B6 mice were stimulated with 10  $\mu$ g of anti-mDEC-poly dA:dT mAb or PBS. Maturation of CD8<sup>+</sup> DCs, CD8<sup>-</sup> DCs, and PDCA-1<sup>+</sup> PDCs was evaluated 12 hours after injection by multicolor flow cytometry. The gating strategy for these DC subsets is shown in figure 12. **B.** Wild type or DEC KO mice were irradiated and then given bone marrow from a donor with the opposite genotype (i.e. B6  $\rightarrow$  DEC KO is wild type bone marrow into irradiated DEC KO mouse). After 2 weeks, the mice were stimulated with 10  $\mu$ g of Alexa 647 labeled anti-mDEC-poly dA:dT mAb, and serum was collected 6 hours later for an IFN- $\alpha$  ELISA. Uptake of labeled mAb by splenocytes was also evaluated in these mice by multicolor flow cytometry in CD8<sup>+</sup> and CD8<sup>-</sup> DCs. Graph is cumulative representation for 2 experiments with 3 mice each. Error bars show SEM. **C.** CD11c DTR mice were injected with diphtheria toxin. The next day, anti-mDEC-poly dA:dT mAb was injected into the animals. Serum was harvested after 6 hours and an IFN-  $\alpha$  ELISA was performed. Graph is cumulative representation for 1 experiment with 3 mice. Error bars show SEM.

Figure 22



## **Adaptive Immune Response in B6 Mice**

To test its potential to induce adaptive immunity, anti-DEC205-poly dA:dT mAb was injected together with anti-DEC205-HIV Gag p24 mAb. The latter efficiently immunizes mice when an adjuvant acting systemically, such as poly I:C, is co-administered (Trumpfheller et al., 2006). After prime/boost immunization, splenocytes were restimulated with 15-mer overlapping peptide mixes spanning the sequence of both Gag p24 and control Gag p17 proteins. T-cells were then monitored for IFN- $\gamma$  production (Figure 23). The results indicated that a significantly higher amount of IFN- $\gamma$  was produced from immune T-cells when 15  $\mu$ g anti-mDEC205-poly dA:dT mAb was delivered compared to 15  $\mu$ g of poly I:C. Furthermore, since the poly dA:dT composes only half of the antibody-DNA conjugate, an even greater immune response was seen when anti-mDEC205-poly dA:dT mAb was compared to 7.5  $\mu$ g of poly I:C (Figure 23).

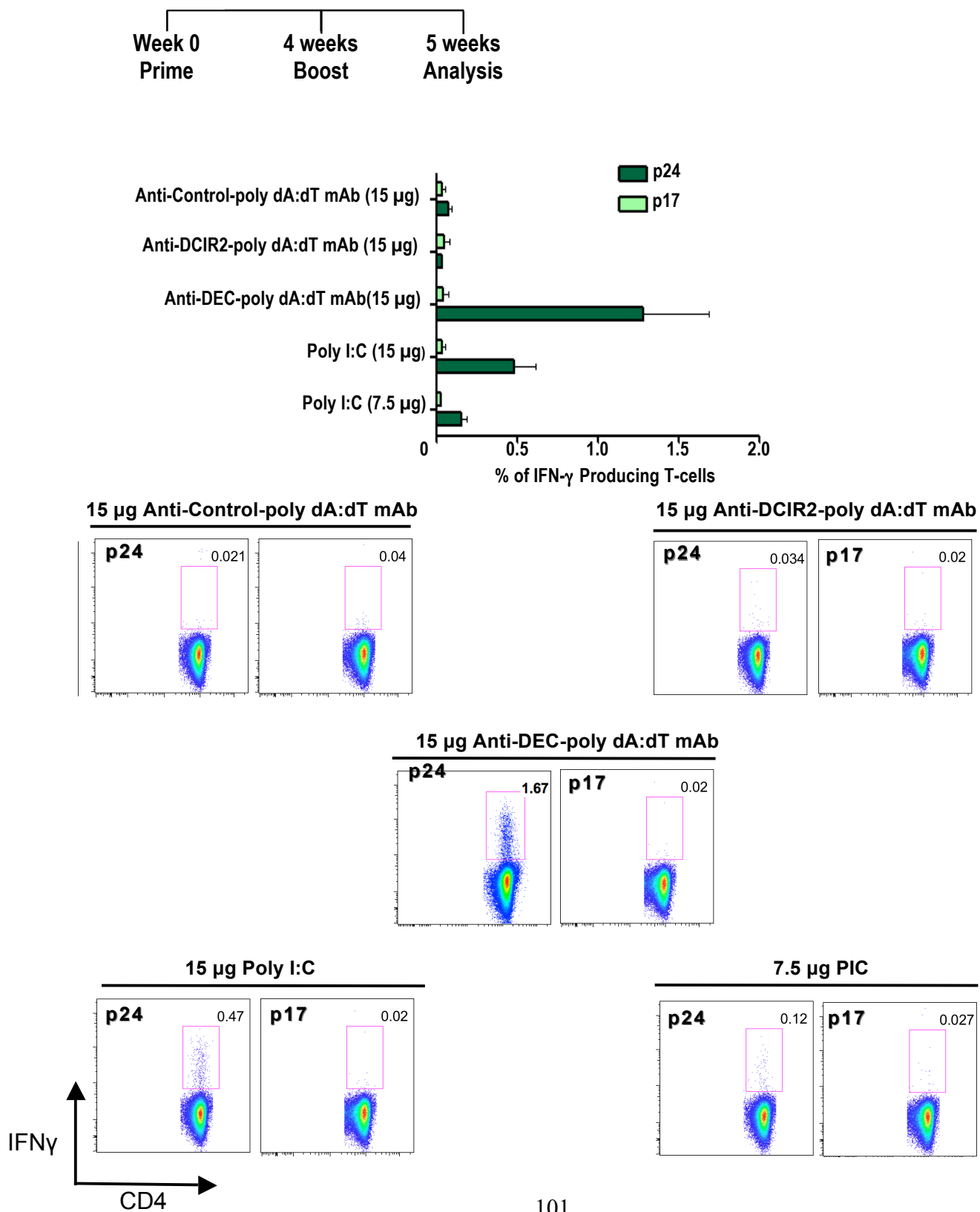
To test if anti-DEC targeted dA:dT could serve as an adjuvant for Gag targeted within another mAb to this DC subset, we used anti-Langerin-HIV Gag p24 mAb instead of anti-DEC205-Gag p24 mAb. Langerin, like DEC205, is a C-type lectin receptor. It has been shown that anti-Langerin mAb targets selectively to CD8<sup>+</sup> DCs and that adaptive immunity can be elicited when anti-Langerin mAb is used to target antigens. When the above experiment was performed, immunization did occur, but less than with anti-DEC205 targeted Gag p24 probably because Langerin is not abundant on cross presenting DCs in lymphoid organs of B6 mice (Figure 24).

We suspected that both the adjuvant and antigen would need to be targeted to the same DC subset for immunization to proceed. To test this hypothesis, HIV Gag p24 antigen was delivered specifically to CD8<sup>-</sup> DCs using an antibody to DCIR2 in conjunction with anti-DEC205-poly dA:dT mAb. Unexpectedly, a large number of IFN- $\gamma$  producing T-cells was seen, even more so than with anti-DEC205-HIV gag p24 mAb. Thus, as seen in Figure 22A, the cytokine production from the CD8<sup>+</sup> DCs is able to mature the CD8<sup>-</sup> DCs to allow for immunization to occur. A more robust response is seen probably because anti-DCIR2 mAb is better at inducing CD4<sup>+</sup> T cells as shown previously (Dudziak et al., 2007).

**Figure 23. Adaptive Immunity using anti-DEC205-Gag p24 mAb.** C57BL/6 mice were primed and boosted 4 wk apart with HIV gag p24 antigen conjugated to anti-DEC205 mAb. Anti-DEC-poly dA:dT mAb, poly I:C, anti-control-poly dA:dT mAb, or anti-DCIR2-poly dA:dT mAb was used as the adjuvant for the immunizations. IFN- $\gamma$  secretion in gated CD3<sup>+</sup>CD4<sup>+</sup> splenic T cells in response to HIV gag p24 peptides was measured 1 wk after boost. Top is schematic of procedure. Middle is histogram representing cumulative data of 3 experiments with 3 mice each. Error bars represent SEM. Bottom are representative FACS plots for each group.

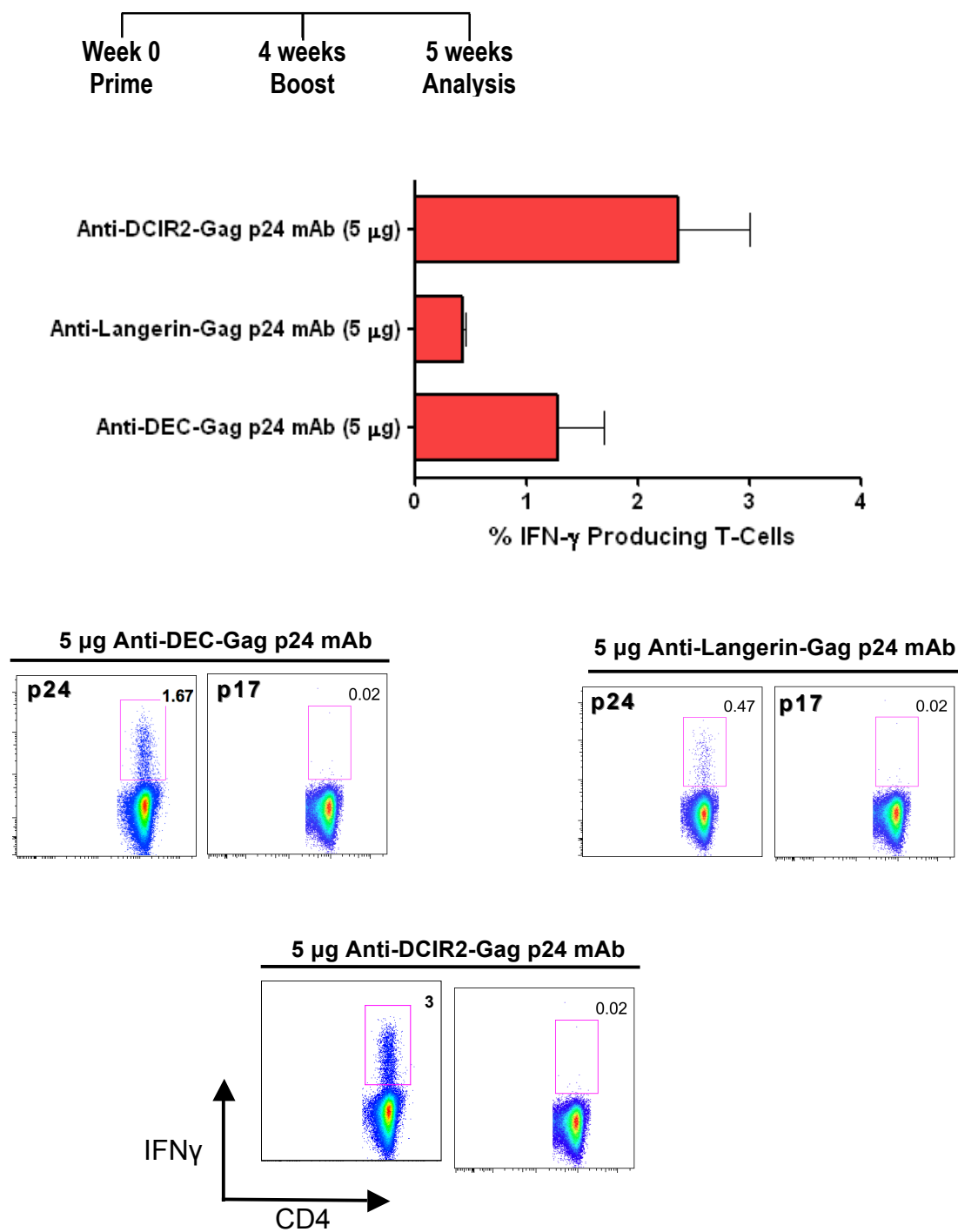


**Figure 23**



**Figure 24: Adaptive Immunity using anti-DEC205-poly dA:dT mAb.** a, C57BL/6 mice were primed and boosted 4 wk apart with HIV gag p24 antigen conjugated to anti-DEC205 mAb, anti-Langerin mAb, or Anti-DCIR2-mAb. Anti-DEC-poly dA:dT mAb was used as the adjuvant for the immunizations. IFN- $\gamma$  secretion in gated CD3<sup>+</sup>CD4<sup>+</sup> splenic T cells in response to HIV gag p24 peptides was measured 1 wk after boost. Middle is histogram representing cumulative data of 2 experiments with 3 mice each. Error bars represent SEM. Top is schematic of procedure. Bottom are representative FACS plots for each group.

**Figure 24**



### **Anti-DCIR2-poly dA:dT mAb**

DCIR2 is another C-type lectin receptor. It has been shown to target exclusively to CD8<sup>+</sup> DCs. To explore whether the targeting of poly dA:dT to CD8<sup>+</sup> DCs could also cause immunity, anti-DCIR2-poly dA:dT mAbs were produced (Figure 25). Although the fusion mAb specifically bound to CHO cells stably expressing the DCIR2 receptor, injection of anti-DCIR2-poly dA:dT mAb did not initiate innate immunity (Figure 21A). We also tested if adaptive immunity would develop if anti-DCIR2-poly dA:dT mAb was given as an adjuvant with anti-DCIR2, anti-DEC205 or anti-Langerin-HIV Gag p24 mAbs. An immune response was not seen in any case tested (Figure 23).

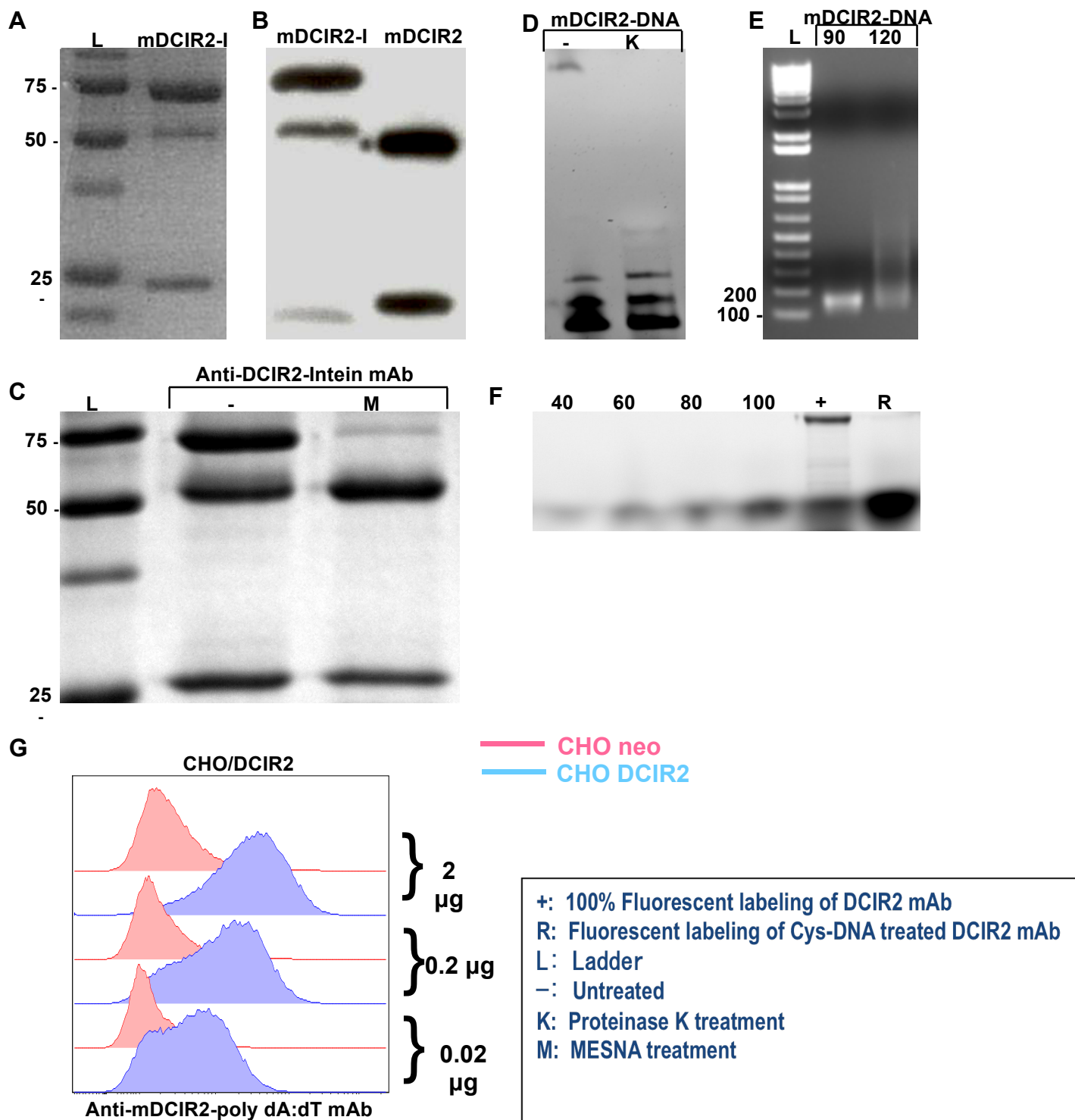
To investigate the cause of anti-DCIR2-poly dA:dT mAb ineffectiveness, FACs was used to sort plasmacytoid DCs (PDCs), CD8<sup>+</sup> DCs, and CD8<sup>+</sup> DCs from wildtype B6 mice. Poly dA:dT with lipofectamine, poly I:C with and without lipofectamine, anti-DEC205-poly dA:dT mAb, and anti-DCIR2-poly dA:dT mAb were then given to the sorted cells *in vitro* (Figure 26A). As published previously, only the CD8<sup>+</sup> DCs produced IFN- $\alpha$  when poly I:C was given without lipofectamine. All subsets were able to produce IFN- $\alpha$  when poly dA:dT or poly I:C was given in conjunction with lipofectamine. However, only the CD8<sup>+</sup> DCs were able to be stimulated by anti-mDEC205-poly dA:dT mAb, and none of the cells were able to respond to anti-DCIR2-poly dA:dT mAb (Figure 26A). Since CD8<sup>+</sup> DCs respond to poly dA:dT when given with lipofectamine, we conclude that poly dA:dT

is not delivered to the cytoplasm in the CD8<sup>-</sup> DCs and therefore does not cause immune activation.

To further test this hypothesis, human DEC205 CD11c mice were used. These mice express human DEC205 on both CD8<sup>-</sup> and CD8<sup>+</sup> DCs. When these mice were treated with anti-hDEC205-poly dA:dT mAb, only the CD8<sup>+</sup> DCs were able to elicit an immune response (Figure 26B). This further supports that the CD8<sup>+</sup> cross-presenting DCs are needed to allow cytoplasmic entry of the poly dA:dT.

**Figure 25. Production of anti-DCIR2-poly dA:dT mAbs.** **A,B,** The integrity of the anti-DCIR2-intein mAb was confirmed by 10% SDS-Page gel stained with (A) Coomassie blue and (B) Western blot analysis using goat anti-mouse IgG Ab conjugated with HRP. Numbers are expressed in kDa. **C.** Thiolytic cleavage of the DCIR2 mAb. Anti-DCIR2-intein mAbs were treated with 100 mM MESNA for 18 hours. The removal of the intein from the heavy chain was confirmed by 10% SDS-Page gel stained with Coomassie blue. Numbers are expressed in kDa. **D.** DNA ligation to antibody. MESNA-treated DCIR2 mAb was added to Cys-DNA. Production of DNA-mAb conjugates was monitored on a 2-20% TBE native gel stained with EtBr. **E.** Elongation of DNA. Purified anti-DCIR2-dA:dT mAbs were reacted with Klenow fragment in the presence of dATP and dTTP. The mAbs were treated with proteinase K and run on a 2% agarose gel stained with EtBr. Numbers on left are expressed in base pairs. Numbers on top represent time in min. **F.** Indirect determination of anti-DCIR2-dA:dT mAb ligation efficiency. After 18 hour ligation reaction between cys-DNA and activated DCIR2 mAb, 1000 pmoles (20 fold excess) of fluorescent peptide was added and allowed to react for a further 18 hours. The sample was run on a 10% SDS Page-gel and monitored for fluorescence. ImageJ software was used to determine the amount of DCIR2 antibody that was fluorescently labeled. Numbers are in pmoles of fluorescent peptide. **G.** CHO cells with DCIR2 receptor as well as CHO NEO cells were incubated with three different concentrations of anti-DCIR2-poly dA:dT mAbs. The cells were then stained with anti-mIgG-PE antibody. FACS analysis was performed to determine the extent of binding.

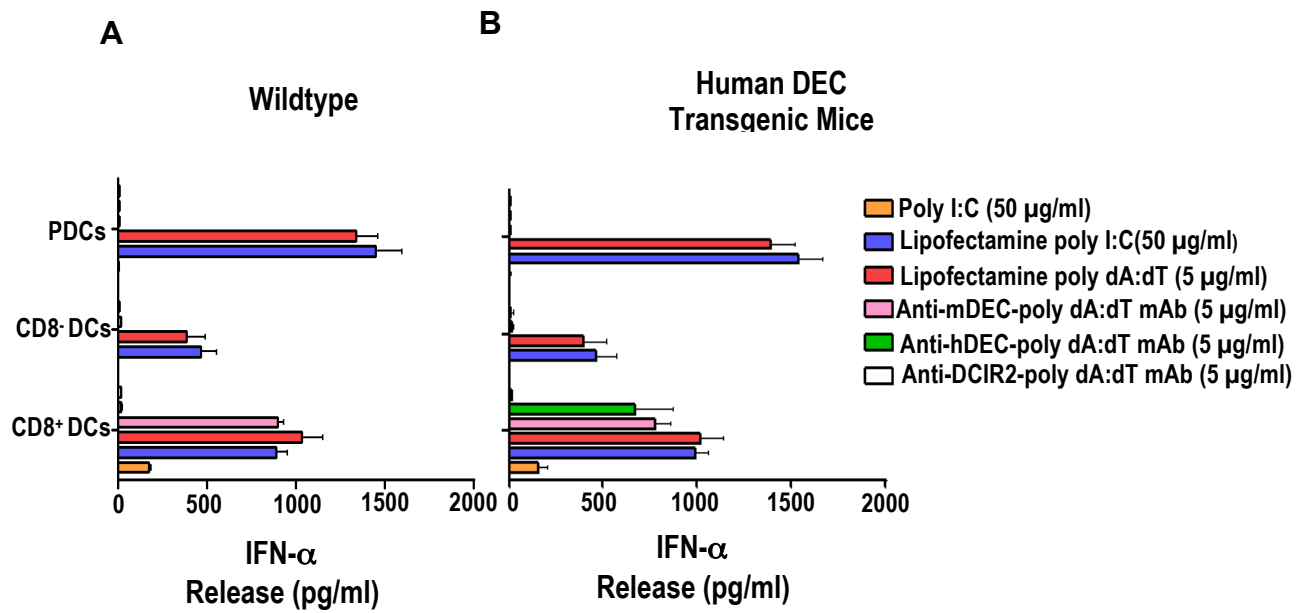
**Figure 25**



**Figure 26. CD8<sup>+</sup> DCs are needed for cytoplasmic entry of poly dA:dT.** A,B. CD8<sup>+</sup>, CD8<sup>-</sup>, and PDCs were sorted from either wildtype (A) or human DEC CD11c mice (B). Cells were plated at 300,000 cells/mL and then stimulated with various immune stimulants with or without the use of lipofectamine. An IFN- $\alpha$  ELISA was performed on the cell's supernatant the following day.



**Figure 26**



## **Chapter 4**

### **Discussion**

#### **Introduction**

Ralph M. Steinman and Zanvil A. Cohn first described dendritic cells in 1973, and the new cells were characterized by their morphology and excellence at inducing immune reactions (Steinman et al., 1978). It took many years and compelling evidence to convince the scientific community that DCs were distinct from macrophages. The main function of DCs is to present antigen to T cells, controlling both tolerance and immunity. Antigens have been successfully delivered to DCs using uptake receptors, like DEC205, to induce both tolerance and immunity (Hawiger, et al., 2001). In terms of immunity, it has been shown that direct delivery of an antigen to DCs produces an immune response 100 to 1000 times greater than untargeted antigen (Trumpfheller et al., 2006). To mature DCs, however, an adjuvant is necessary in these experiments.

Although antigens have been targeted specifically to DCs using a variety of uptake receptors, adjuvants have always been given systemically. In fact, it was shown that poly I:C, the most commonly used adjuvant to induce CD4<sup>+</sup> T cell immunity, acts mostly on non-hematopoietic cells (Longhi et al., 2009). Thus, it was believed that a systemic immune response was required to induce immunity, and DCs were not capable of inducing adaptive immunity alone.

To test this hypothesis, we were able to ligate poly dA:dT, a type I interferon immune stimulant, to an antibody to the DEC205 receptor. We show that the anti-DEC205-poly dA:dT mAbs target to CD8<sup>+</sup> DCs, and these cells are able to produce IFN- $\alpha$  in response. The response was shown to be RIG-I dependent in human MoDCs, indicating that the poly dA:dT is entering the cytoplasm, probably through the same mechanism as cross-presentation. We also demonstrate that DCs are sufficient to induce adaptive immunity when both the adjuvant (poly dA:dT) and antigen (Gag p24) are delivered exclusively to them.

There are three major findings discussed in this thesis. First, the method to ligate DNA to the DEC205 antibody is novel. Furthermore, we were able to elongate the DNA to produce poly dA:dT. Second, we illustrate that CD8<sup>+</sup> DCs are sufficient to induce innate and adaptive immunity *in vivo* when antigen and adjuvant are targeted selectively to them. Finally, the thesis provides insight to the mechanism of cross-presentation.

## **Implication of Anti-DEC205-poly dA:dT mAb Production**

DNA-antibody conjugates have been created since 1992 where the first immuno-PCR was described (Sano et al., 1992). In these experiments, the antibody is used to detect some antigen, and the DNA moiety is used to increase detection by PCR amplification (Fredriksson et al., 2002, Hendrickson et al., 1995, and Mckie et al., 2002). However, immuno-PCR has not gained wide use, mainly because of the antibody's molecular heterogeneity and lack of precise chemical control at the site of DNA attachment.

EPL has been used in the production of DNA-protein conjugates for immuno-PCR since 2005 (Burbulis et al., 2005). Termed “tadpoles,” these DNA-protein conjugates are homogenous and the DNA molecules are added to the protein of interest in a site-specific manner. However, due to ease of production, only proteins of interest that could be expressed in bacteria, such as streptavidin (Sydor et al., 2002) and single-chain antibodies, (Ruelen et al., 2009) were used to produce tadpoles.

The use of antibodies as the protein of interest for EPL was not described until 2011 (Mohlmann et al., 2011). In this work, EPL was used to link an extra domain of fibronectin (ED-B) antibody to biotin. ED-B containing fibronectin is associated with angiogenesis and tissue remodeling (Menrad et al., 2005), and high levels of ED-B have been detected in solid tumors (Borsi et al., 2002 and Santimaria et al., 2003). The researchers of this article want to eventually use EPL to link cytotoxic agents to ED-B antibody as an antibody-based cancer treatment. However, in these experiments, EPL was not used to link the antibody of interest to DNA.

The approach described in this thesis offers a novel way to produce DNA-antibody conjugates. EPL has never been used to create DNA-full length antibody conjugates before. Thus, this method provides a new way to prepare DNA-antibody conjugates where there is defined stoichiometry and regioselectivity at the coupling site. This is ideal for immuno-PCR. With this method, full-length antibodies (not just single-chain antibodies) can be utilized, making it possible to target a wider variety of proteins of interest in the assay.

There are other applications for the production of DNA-antibody conjugates using this method. One idea is to use the method to target siRNA. One of the hardest obstacles to overcome when using siRNA is cell delivery. Transfection agents are needed to allow for cell entry. Furthermore, specific cells cannot be targeted by siRNAs; siRNA will knockdown the gene of interest in all cells it enters. By linking the siRNA to an antigen uptake receptor antibody, the siRNA will have ease of entry and will target specific cells. Of note, this method will probably only work for cells that undergo cross-presentation to allow the siRNA to enter the cytoplasm of the targeted cell.

One of the disadvantages to the method described above was that only short ds-DNA was linked to the antibody. Longer Cys-DNA had lower ligation efficiency to the DEC205 antibody. We overcome this obstacle by elongating the DNA that was attached to the antibody using Klenow Fragment. This is the first description of elongating DNA that is attached to an antibody. The sequence of elongation, however, is not specific. Since we

were ligating poly dA:dT, this was not an issue in our case, but the method is limited for researchers who wish to add longer, sequence specific DNA to antibodies.

## **CD8<sup>+</sup> DCs are Sufficient to Induce Innate and Adaptive Immunity**

DC activation is not a linear process, and the nature, intensity, and duration of the activation signal are important in determining effector function of DCs (Kapsenberg et al., 2003). As such, it was hypothesized that inflammatory cytokines could represent the actual mediators of DC activation *in vivo* and may serve to trigger adaptive immune reactions in response to pathogens that fail to engage DC-expressed pattern-recognition receptors (Reis e Sousa et al., 2005).

However, researchers reported that indirect activation by inflammatory mediators generated DCs that supported CD4<sup>+</sup> T cell clonal expansion but failed to direct T helper cell differentiation (Reis e Sousa et al., 2005 and Nolte et al., 2007). Only direct exposure of pathogen components, such as CpG-containing DNA or LPS, resulted in fully activated DCs that promoted T helper responses (Reis e Sousa et al., 2005). Moreover, cytokines originating from nonhematopoietic cells were neither sufficient nor required for DC activation *in vivo* (Nolte et al., 2007).

When mice are injected with poly I:C, a synthetic double stranded RNA agonist for TLR3 and MDA-5, type I interferon was rapidly produced as an innate response, and this acts on DCs as a component of adjuvant action for adaptive immunity.

Nonhematopoietic cells produce the bulk of the innate type I interferon, and both DCs and nonDCs are required for immunization to proceed (Longhi et al., 2009). However, innate stimuli have not been selectively delivered to DCs to determine if these cells alone are capable of integrating innate and adaptive immunity in the intact animal.

The results of this thesis indicate that CD8<sup>+</sup> DCs are sufficient to induce innate immunity. First, it is shown that the anti-DEC205-poly dA:dT mAb's main target is the CD8<sup>+</sup> DCs (Figure 20). Further proof of selective targeting is that only the CD8<sup>+</sup> DCs produce type I interferon when stimulated with anti-DEC205-poly dA:dT mAb *in vitro* (Figure 25) and *ex vivo* (Figure 21C). The mouse chimera experiment indicates that type I interferon production from nonhematopoietic cells, i.e. stromal cells, is minimal (Figure 22B). Thus, this is the first proof that selective targeting of CD8<sup>+</sup> DCs is sufficient to induce innate immunity (Figure 21A).

Using anti-DEC205-poly dA:dT mAb as the adjuvant and anti-DEC205-Gag p24 mAb or anti-Langerin-Gag p24 mAb as the antigen, we selectively targeted CD8<sup>+</sup> DCs. The results indicated that selective targeting to CD8<sup>+</sup> DCs was sufficient in inducing an adaptive immune response as evidenced by the number of CD4<sup>+</sup> IFN- $\gamma$  producing T-cells (Figure 23 and 24). This result was specific as anti-control-poly dA:dT mAb did not induce immunity, and the immunity induced was stronger than when poly I:C was given at a similar dose (Figure 23). Poly I:C did work better when a larger dose was used as an adjuvant, however. This is probably due to the fact that there are more stromal cells than dendritic cells in the intact animal. Thus, if a larger dose of poly I:C is given, more IFN- $\alpha$  can be produced than when the immune stimulant is targeted only to DCs.

One surprising experiment is that when the adjuvant was targeted to CD8<sup>+</sup> DCs and the antigen was targeted to CD8<sup>-</sup> DCs using anti-DCIR2-Gag p24 mAb, adaptive immunity



was elicited (Figure 24). Previous results indicated that cytokines alone are not sufficient to induce immunity and that the immune cell must have direct exposure to the adjuvant. However, our results indicated that the cytokine produced from the CD8<sup>+</sup> DCs was sufficient to induce the phenotypic changes used to depict DC maturation in both PDCs and CD8<sup>-</sup> DCs (Figure 22A). Furthermore, the *in vitro* results using the sorted mouse DCs indicated that poly I:C has difficulty entering the cells; a much greater immune response was seen when poly I:C was given with lipofectamine than when it was given without (Figure 26). Previous work has also shown that the majority of cytokine is produced from the nonhematopoietic cells when poly I:C is injected into mice. Thus, it is likely that for CD4<sup>+</sup> T cell immunity, the adjuvant is needed to induce type I interferon, but is not required to directly stimulate the DCs.

## Implications for Cross-Presentation

Although it is known that the DEC receptor is capable of cross-presentation, the mechanism is not well understood ((Dudziak et al., 2007). Two theories have been proposed. The first theory hypothesizes that cross-presenting DCs have specialized phagosomes that contain MHC class I machinery. Hence, the endocytosed antigen does not have to compete with loading of endogenous peptides in the ER, but instead would be brought to these phagosomes for MHC class I peptide presentation (Guermonprez et al., 2003). The second theory proposes that cross-presenting DCs possess the machinery for antigen escape from endosome into the cytoplasm to gain access to the proteasome and TAP transporters (Dudziak et al., 2007).

Our results support the latter theory. The production of type I interferon is reduced with the use of RIG-I siRNA in human monocyte derived DCs (Figure 19). Thus, the poly dA:dT must be delivered to the cytoplasm of the DC after endocytosis in order to cause immune stimulation.

The use of anti-DCIR2-poly dA:dT mAb also provides insight to the mechanism of cross-presentation. Anti-DCIR2-poly dA:dT mAb was unable to induce innate or adaptive immunity, even though the antibody was shown to be functional by CHO staining (Figure 25G). We thought that the reason for this was that CD8<sup>+</sup> DCs were unable to respond to poly dA:dT. This was not the case, however, as shown in the *in vitro* experiment where CD8<sup>+</sup> DCs responded to poly dA:dT given with lipofectamine (Figure 26A). This lead us

to the hypothesis that the poly dA:dT was not being delivered to the cytoplasm because the CD8<sup>-</sup> DCs cannot cross-present.

To test this hypothesis, human DEC205 CD11c transgenic mice were used. Human DEC205 is expressed on both CD8<sup>+</sup> and CD8<sup>-</sup> DCs in these animals. When given anti-hDEC205-poly dA:dT mAb, only the CD8<sup>+</sup> DCs were able to produce type I interferon (Figure 26B). Thus, cross-presentation is not receptor dependent, but cell type dependent. The CD8<sup>+</sup> DCs must express some proteins that allow its endocytosed material into the cytoplasm. The names of these proteins still remain to be identified.

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