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Receptor-Mediated Delivery of Antigens to Dendritic Cells: Anticancer Applications

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Abstract: Recently, there has been a surge of interest in the use of *ex vivo* antigen-pulsed dendritic cells (DCs) in the immunotherapy for cancer. DCs are powerful adjuvants with the ability to prime naive $CD4^+$ and $CD8^+$ T cells. As antigen sources, various preparations, including peptides, proteins, crude tumor lysates, and DCs transfected or transformed with various viruses, have been used. These procedures that involve the isolation of patient DCs and reintroduction after *in vitro* manipulation are time-consuming and expensive. The DC populations used frequently in *ex vivo* clinical studies are IL-4 and GM-CSF cultured DCs that may not represent the *in vivo* DC populations. An attractive method of targeting *in vivo* DCs is to utilize various ligands or antibodies that bind discrete populations of DCs. These cell surface receptors will direct the antigen to different antigen processing pathways depending on the targeted receptor to induce cytotoxic T cell or T helper responses. This review will discuss the various approaches and receptors that have been used for antigen targeting that may eventually be translated to alternative DC-based immunotherapies.

Keywords: Dendritic cells; antigen presentation; receptor; C-type lectins; scavenger receptor; TLR; FcR; bacterial toxins; chemokine receptors; DC binding peptides

Introduction

While vaccination has ultimately proved to be an effective measure against the spread of some viruses, many remain uncontrollable by vaccination, as do most bacterial, protozoan, and "worm" (helminth and cestode) infectious agents. The most successful vaccines used to combat infectious disease are the live or live-attenuated organisms as used in polio and smallpox vaccines. With purified proteins or peptides, in most cases powerful adjuvants or suitable danger signals are needed to prime naive T cell responses. It is possible that targeting antigen directly to antigen-presenting cells (APCs) may provide a new level of potency above that afforded by co-administration of antigen with adjuvant.

The past decade has seen a dramatic increase in the number of studies aimed at "vaccinating" against existing disease in patients, particularly cancer. Numerous tumor-associated antigens have been identified and expressed as recombinant proteins. Further, with the help of T cell epitope prediction algorithms and experimental observations, cytotoxic T cell and helper T cell epitopes have been identified for tumor antigens (Table 1).[1,2] These recombinant proteins and synthetic peptides have been utilized with different adjuvants and delivery systems in preclinical studies and some in clinical studies. While numerous adjuvants have been utilized in clinical trials, Alum remains the only adjuvant licensed for use in human vaccines. Despite concerted efforts, the efficacy of clinical trials using vaccination to treat existing

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Table 1. A Selection of Recent Tumor Antigens and Their T Cell Epitopes[a]

antigen	tumor	HLA	epitope	ref
		(i) Unique Antigens		
CASP-5	colon, gastric endometrial	A2	FLIIWQNTM	97
COA-1	colorectal	DR4/DR13	TLYQDDTLTLQAAG	98
OGT	colorectal	A2	SLYKFSPFP	99
NFYC	lung (squamous)	B52	QQITKTEV	100
		(ii) Tumor Specific Antigens		
KK-LC-1	melanoma	B15	RQKRILVNL	101
KM-HN-1	melanoma	A24	NYNNFYRFL	102
		A24	EYSKECLKEF	102
		A24	EYLSLSDKI	102
SAGE-1	melanoma	A24	LYATVIHDI	103
Sp17	melanoma	A1	ILDSSEEDK	104
SSX-2	melanoma	A2	KASEKIFYV	105
		(iii) Differentiation Antigens		
CEA	colon, gut	A2/3/24/DR4/11/13*		
		DR3	AYVCGIQNSVSANRS	106
		DR7	TYYRPGVNLSLSC	106
		DR7	EIIYPNASLLIQN	106
		DR14	EIIYPNASLLIQN	106
		DR14	NSIVKSITVSASG	106
kallikrein-4	prostate	DP4	SVSESDTIRSISIAS	107
		DR4	LLANGRMPTVLQCVN	107
		DR7	RMPTVLQCVNVSVVS	107
mammaglobin-A	breast	A3	PLLENVISK	108
		(iv) Overexpressed Antigens		
adipophilin	adipocytes	A2	SVASTITGV	109
Ep-CAM	epithelial cells	A24	RYQLDPKFI	110
FGF5	brain, kidney	A3	NTYASPRFK	111
mdm-2	brain, lung, muscle	A2	VLFYLGQY	112
MMP-2	ubiquitous	A2	GLPPDVQRV	113
PBF	ovary, pancreas spleen, liver	B55	CTACRWKKACQR	114
RAGE-1	retina	A2	LKLSGVVRL	115
			PLPPARNGGL	115
		B7	SPSSNRIRNT	115
STEAP1	prostate	A2	MIAVFLPIV	116

[a] Complete peptide database and T cell-defined tumor antigens can be found at http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm. Numerous melanoma antigens have been identified for groups i, iii, and iv and are not included in this table.

cancers has at best been modest. As with the more insidious or complex pathogens that have thus far eluded development of successful preventative vaccines, targeting tumor antigens directly to APCs may represent the next step forward in the development of more potent anticancer treatments.

An ideal vaccine should prime naive $CD8^+$ as well as $CD4^+$ T cells. The CD4 T helper response is important in initiating and maintaining long-term immune responses.[3,4] By utilizing intact recombinant proteins, it is more likely that there are peptide sequences that bind MHC class II molecules and initiate CD4 responses and also present peptides that bind to more than one MHC class I or II haplotype. This is a distinct advantage of using intact recombinant tumor antigens. However, to overcome the limitations of using single cytotoxic T cell epitopes, polytope vaccines have been designed by producing recombinant proteins consisting of a combination of T helper and/or cytotoxic T cell epitopes.[5]

Dendritic cells (DCs) are key players in the immune system in that they link the innate immune response to the adaptive immune response.[6] DCs act as sentinels by sampling

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the environment for foreign antigens which are then processed and bound to surface MHC class I or II molecules for presentation to T cells in the lymph node. Peripheral DCs are immature, and in this state, they actively phagocytose antigens; once they mature, they express costimulatory markers, are less phagocytic, and readily migrate to the draining lymph nodes and present antigen in the context of MHC to T cells. These DCs can interact with naive T cells to initiate primary immune responses leading to activated effector $CD4^+$ helper and $CD8^+$ cytotoxic T cells. It is therefore crucial for antigens to bind or gain access to the intracellular pathways in DCs before they mature. In the conventional antigen presenting pathway, antigens endogenously synthesized or viral proteins from virus-infected cells or antigens that have gained access to the cytoplasm are presented by the class I antigen presentation pathway. Antigens that gain access to the cell by endocytosis or phagocytosis are directed to the lysosomes where they are processed and then presented by the class II antigen presentation pathway. DCs have an alternative pathway for class I presentation denoted the "cross-presentation" pathway, where cell-associated or soluble antigens access and are presented by MHC class I molecules to $CD8^+$ T cells.

DC-Targeted Antigen Delivery

Current dendritic cell-based vaccines use autologous DCs that are harvested from patients and then loaded *ex vivo* with antigen prior to re-administration to patients. While various strategies have been utilized to load antigen into DCs (Table 2), by necessity all have been highly laborious. Targeting DCs *in vivo* via specific surface receptors represents a more direct and less laborious strategy and has been the subject of considerable recent investigation. A multitude of receptors have been investigated, including mannose receptor (MR), DC-SIGN, scavenger receptor (SR), DEC-205, and Toll-like receptors.

C-Type Lectins. (1) Mannose Receptor. The mannose receptor (MR) is primarily present on DCs and macrophages, which recognize carbohydrates (mannose, fucose, glucose, GlcNAc, and maltose) on the cell walls of infectious agents (bacteria and yeast). Once binding occurs, aggregation and receptor-mediated endocytosis and phagocytosis take place. The MR is part of the multilectin receptor family and provides a link between innate and adaptive immunity.[7]

The high level of expression of MR on DCs and macrophages indicates that it is a key molecule in antigen recognition.[8,9] The MR on macrophages and immature DCs is involved in endocytosis and phagocytosis[10] and facilitates an important pathway for antigen uptake and delivery to MHC class II molecules.[11,12] Further, it has been demonstrated that the MR is also involved in antigen uptake and delivery to MHC class I molecules[8] particularly with mannose modified by oxidation.[13]

The MR is found in endosomes which transit to the cell surface, where it binds ligands and recycles from the cell

Table 2. Methods and Antigens Used for Antigen Loading of Dendritic Cells

method	antigen (peptide/protein)	ref
	Peptides/Proteins	
peptide pulse	PSCA and PSA	117
	MUC1	118
	hAFP	119
	PSCA, PAP, PSMA, PSA	120
	PSA, PSMA, survivin, prostein, trp-p8	121
protein pulse	CEA	122
	PAP	123
penetratin	MUC1 (peptide and protein)	124
	RNA	
mRNA	autologous tumor	125
	allogeneic cells	126
	cell lines	127
tumor RNA		128
RNA	*in vitro* transcribed	129
	Tumor	
tumor lysate	allogeneic medullary thyroid carcinoma	130
	melanoma	131
	CT-26 cells	132
	MCF-7, MDA-MB-231	133
apoptotic tumor cells	NH-1 (B-ALL)	134
	Complexes	
HSP−Ag complex	hepatocellular carcinoma cells	135
mannan-Ag	MUC1	136
	Virus	
adenovirus	HCV NS3	137
	αFP	138
	CEA	139
lentivirus	Sca-2, GP38, RABP1	140
	Fusion	
tumor DC fusion	allogeneic COLM-6 colon cancer cells	141
	multiple-myeloma cells	142

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surface through the endosomal pathway. It then dissociates due to the lower pH found in endosomes.[14] Endocytosis by the DC via the MR takes place in small coated vesicles, and shortly thereafter, the MR and its ligand appear in larger vesicles, followed by colocalization with MHC class II molecules in lysosomes.[12] Mannosylated peptides and proteins are able to stimulate MHC class II restricted peptide specific T cells with 200−10000-fold higher efficiency than are peptides or proteins which have not been mannosylated.[12] Furthermore, uptake via the MR by DCs results in 100-fold enhanced presentation of soluble antigens to T cells as compared to antigens internalized via the fluid phase.[11] After internalization, the MR transports antigens to MHC class II-containing compartments in immature DCs for antigen processing and presentation to T cells via MHC class II.[15]

Various reports demonstrate that CD1 proteins are capable of antigen presentation.[16,17] Human CD1b can present non-peptide components of mycobacterial antigens to T cells, including lipid mycolic acid and lipoarabinomannan. The antigen presentation pathway for lipoarabinomannan has been characterized, and the macrophage MR is clearly responsible for uptake.[18] The MR is abundant in early endosomes and during MHC class II loading, Lipoarabinomannan is taken into early endosomes via the MR and from late endosomes is loaded onto CD1b molecules for T cell presentation.[18] Their study linked the MR to presentation of glycolipids via CD1 and suggested that the MR plays a critical role in processing of carbohydrate antigens.

A fusion protein containing the cysteine-rich domain of the murine MR and the Fc portion of human IgG1 was able to bind cells which were positive for MHC class II, sialoadhesin, and CD11c but negative for markers such as F4/80, FDC-M2, CD11b, B220, and CD4; these cells were found to localize to B cell follicles and initiate humoral immune responses and activation of T cells.[19]

The use of mannan to aid induction of T1-mediated immune responses and cytotoxic T cells (CTL) has also been investigated. When cationic liposomes with HIV-1 DNA incorporated were coated with mannan before administration, HIV specific CTL responses, T1-type cytokine IFN-γ, and delayed-type hypersensitivity responses were all enhanced, as were IgG2a and IgA antibody responses.[20] Furthermore, HER2 protein complexed to cholesteryl group-bearing mannan or pullulan polysaccharides can evidently induce $CD8^+$ CTL cells which reject $HER2^+$ tumors.[21]

It has been demonstrated that mannan conjugated to the tumor-associated antigen MUC1 can induce strong T1 or T2-type immune responses, depending on the mode of conjugation.[22] MUC1 conjugated to mannan under reducing conditions (where no aldehydes or Schiff bases are present) induces strong T2-type immune responses with a high level of IgG1 antibodies, IL-4 cytokine production, a low CTL precursor frequency, and no protection in mice against a tumor challenge.[23−28] However, conjugation of MUC1 to

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mannan under oxidizing conditions (where aldehydes and Schiff bases are present) yields an immunogen that generates a T1-type response, as indicated by $CD8^+$ CTLs, a low level of IgG2a antibodies, and IL-12 and IFNγ cytokine production.[23−28] Both conjugate formulations evidently bind equally to the MR and are taken up into early endosomes.[8] However, the reduced mannan−MUC1 fusion protein is preferentially presented by the MHC class II pathway, whereas the oxidized mannan−MUC1 fusion protein is preferentially presented by the MHC class I pathway. The aldehydes in the oxidized mannan−MUC1 fusion protein are crucial for endosomal escape of antigen into the cytoplasm.[13] These observations provided the first demonstration that the MR aids the introduction of antigens into the MHC class I pathway. Furthermore, *ex vivo* targeting of the macrophage or DC MR followed by transfer of these cells into mice, induces strong CTL responses, and protects mice against MUC1 tumor challenge.[8,29] In contrast to mouse studies, oxidized mannan MUC1 fusion protein induced predominantly humoral immune responses in human clinical trials (see below). This was due to the presence of pre-existing natural galα(1,3)gal antibodies in humans (absent in mice) which diverted the immune response.[30]

Rather than utilizing a carbohydrate-linked antigen to target the MR, a recombinant human anti-MR/tumor antigen has been used.[31] The pmel17 melanoma-associated antigen was fused to the heavy chain to produce a fully human protein, B11-pmel17. DCs treated with B11-pmel17 presented pmel17 in the context of class I and class II molecules. CTL generated via B11-pmel17-pulsed DCs has multiple restricting elements but nevertheless only lysed HLA-matched targets. Chemical modification of the N-terminus of the ectodomain L2 region of the ErbB2 receptor to introduce a single mannose group has been undertaken.[32] This mannosylated protein was more potent than the nonmannosylated *Escherichia coli* protein at maturing DCs. Furthermore, the mannosylated antigen was internalized by DCs more efficiently and also enhanced stimulation in an autologous MLR. To retain the characteristics of mannose rich carbohydrates and the target mannose receptor on DCs, antigens have been expressed in yeast. Several recombinant ovalbumin (OVA) proteins were generated in *Pichia pastoris* that contained no glycosylation, only O-linked glycosylation and N- and O-glycosylation.[33] In all cases, mannosylated OVA generated more potent antigen specific proliferation than the nonmannosylated OVA. When MR knockout and wild-type mouse bone marrow-derived DCs (BMDC) were compared in their ability to present the mannosylated OVA to OT-II T cells, no differences were seen, indicating that other mannose binding receptors also play a part in mannosylated antigen processing and presentation.

(2) DC-SIGN. The most recently identified member of the MR family, the C-type lectin DC-SIGN, is expressed in large amounts on immature DCs, endothelial vascular cells, and lymphatic vessels in tonsils. DC-SIGN was identified through its high-affinity interaction with ICAM-3 which facilitates interactions of DCs with T cells and contributes to the regulation of primary immune responses.[34,35] In addition to positive immunomodulatory functions, it is now evident that DC-SIGN contributes to HIV pathogenesis. HIV-1 binds to DC-SIGN, is transported by DCs into lymphoid tissues, and consequently facilitates HIV-1 infection of target $CD4^+$ T cells.[34,35]

In an effort to elucidate the molecular basis of internalization of antigen by DC-SIGN, the putative internalization motif within the cytoplasmic tail was modified, resulting in reduced internalization after exposure to antigen.[36] The carbohydrate specificity of murine DC-SIGN (mSIGNR1) is similar to that of human DC-SIGN. Both bind to mannose-containing ligands and interact with Le(x/y) and Le(a/b) antigens; however, mSIGNR1 also interacts with sialylated Lex, a ligand for selectins.[37] The humanized antibody

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hD1V1G2/G4 (hD1), directed against DC-SIGN, was recently cross-linked to KLH.[38] This chimeric antibody−protein complex (hD1−KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment of DCs. These DCs induced proliferation of patient PBMC at a concentration 100-fold lower than that of KLH-alone pulsed DCs. In addition, hD1−KLH complex-targeted DCs induced proliferation of naive T cells which recognized KLH T cell epitopes presented by MHC class I and II.[38]

In a recent study primarily aimed at generating L-SIGN specific recombinant antibodies, Dakappagari and colleagues generated an antibody that cross-reacted with DC-SIGN and investigated its potential for functional targeting of antigen to DCs.[39] A sequence encoding a known Th epitope from tetanus toxoid (TT) was incorporated into the antibody sequence, and the resulting antibody (E10-632DR) was pulsed with DCs and tested for ability to stimulate recall proliferative responses using T cells from previously TT-immunized donors. Despite prior immunization, only T cells from one of four donors proliferated in response to Th helper epitope alone, in positive control assays. Notably, however, in that donor, autologous DCs pulsed with E10-632DR induced significant proliferation above that of the control antibody, and furthermore, the magnitude of these proliferative responses correlated directly with the amount of E10-632DR used to pulse DCs.

While recombinant antibodies represent an obvious candidate vehicle for targeting of antigen to DC-SIGN, other less obvious vehicles have been investigated. Steeghs et al.[40] have reported an intriguing study in which a novel form of LPS (lgtB), derived from a mutant *Neisseria meningitidis* strain was found to have high affinity and be readily internalized by human DC-SIGN. Rather than generating composites of lgtB with recombinant peptides or pathogen-derived proteins, the group assessed the differential effects of the mutant bacterial strain on DCs as compared to those of the wild-type strain. Internalization of mutant strains was markedly enhanced, and further, subsequent cellular responses induced by mutant-pulsed DCs were skewed toward a T1 response, rather than the T2 responses induced by wild-type strains.

(3) DEC-205. DEC-205 is an integral membrane protein homologous to the macrophage mannose receptor, which binds carbohydrates and mediates endocytosis.[41] Following ligand binding, DEC-205 is rapidly internalized by means of coated pits and vesicles and is delivered to multivesicular endosomal compartments that resemble the MHC class II-containing vesicles implicated in antigen presentation. Extensive investigations into the molecular mechanisms of DEC-205-mediated antigen uptake and processing have now been conducted, which collectively suggest that this molecule represents a promising target for facilitation of delivery of antigens to APCs.

The cytosolic tail of DEC-205 was fused to the external domain of the CD16 Fcγ receptor and was studied in stable L cell transfectants.[15] The DEC-205 tail recycled CD16 through MHC II-positive late endosomal/lysosomal vacuoles and also mediated a 100-fold increase in the level of presentation of antigen to $CD4^+$ T cells.

An anti-DEC-205 monoclonal antibody chemically coupled to ovalbumin (OVA) protein stimulated OVA specific $CD4^+$ and $CD8^+$ T cells by $CD11c^+$ lymph node DCs, but not by $CD11c^-$ DCs.[42] Receptor-mediated presentation by MHC class I was 400 times more efficient than that induced by OVA alone and was TAP-dependent. Following subcutaneous injection of an anti-DEC-205−antibody−OVA conjugate, uptake by draining lymph node DCs was evident within 48 h, and OVA derivatives were presented to TCR-transgenic $CD8^+$ cells 400 times more effectively than when OVA alone was utilized. Additionally, simultaneous delivery of anti-DEC-205−antibody−OVA conjugates with anti-CD40 antibody (a DC maturation stimulus) further enhanced immune responses (IL-2, IFN-γ production, CTL, and tumor protection) *in vivo*.[42] A subsequent study showed that anti-DEC-205 monoclonal antibody conjugated to the melanoma antigen tyrosinase-related protein TRP-2 protected mice against B16 tumor cell growth and slowed growth of established B16 tumors.[43] Moreover, 70% of mice were cured of existing tumors via injection of anti-DEC-205 monoclonal antibody conjugated to two different melanoma antigens (TRP-2 and gp100); both CD4 and CD8 responses were induced. Thus, targeting DCs using DEC-205-directed antibody−antigen conjugates represents a novel method of inducing long-lasting antitumor immunity.

Despite preliminary data suggesting that DNA vaccines might provide a robust and cheap means of vaccination

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against a multitude of diseases, more than two decades after their initial development no human DNA vaccine has yet proved to be sufficiently effective to warrant commercialization. Strategies aimed at increasing the "general" immunogenicity of DNA vaccines such as incorporation of cytokine DNA have led to greater immunogenicity in experimental contexts but have not achieved the ultimate aim of protective immunity. Recently, successful DEC-205 targeting via a DNA vaccine has provided evidence that targeting to relevant APCs may substantially enhance their immunogenicity and could represent the next avenue of investigation toward achieving protective immunity against disease via administration of DNA constructs in humans. Moreover, DNA encoding a single-chain antibody fragment (scFv) from NLDC-145, a monoclonal antibody against murine DEC-205, fused with a model protein stimulated both humoral and cellular responses *in vivo*.[44]

(4) Dendritic Cell-Associated C-Type Lectin-1 (Dectin-1) and Lectin-2 (Dectin-2). Dectin-1 and -2 are C-type lectin receptors expressed on DCs, macrophages, neutrophils, and monocytes and are receptors for β-glucan-recognizing β1,3- and β1,6-linked glucans on yeast cell walls.[45,46] Dectin-1 can initiate inflammatory responses by the presence of an ITAM in its cytoplasmic tail. Dectins are expressed on $CD8\alpha^{-}CD4^{-}$ DCs and dermal DCs. The ability of Dectin-1 and Dectin-2 to present antigen was studied using OVA conjugated to an anti-Dectin monoclonal antibody.[47,48] Using adoptive transfer of $CD8^{+}$ T cells from OT-I mice to B6CD45.1 mice, Carter et al. demonstrated that when mice were immunized with a 1 μg dose of free or conjugated antigens, only conjugated antigens generated significant expansion of T cells in the draining lymph nodes of these mice. Furthermore, only the conjugated antigens stimulated antigen specific IFNγ as detected by ELISpot analysis.

Scavenger Receptor. The scavenger receptor (SR) is primarily present on macrophages and can internalize endotoxins, oxidized low-density lipoproteins, and other negatively charged proteins. Maleylated ovalbumin evidently binds to the SR, enhancing its presentation to ovalbumin specific MHC class I-restricted CTLs by macrophages and B cells.[49] Maleylated diphtheria toxoid has also been demonstrated to be more immunogenic than nonmaleylated diphtheria toxoid, yielding enhanced antibody and T cell proliferative responses.[50] In chickens, immunization with maleylated bovine serum albumin (BSA) resulted in specific binding to SRs and modulated the Th1 immune response via antibodies. In addition, high levels of IFNγ mRNA were detected in splenocytes, while nonmaleylated BSA induced Th2 immune responses.[51]

When the alcohol metabolites malondialdehyde and acetaldehyde are combined with hen egg lysozyme protein, stable adducts (oxidative products) are formed. Strong antibody responses and T cell proliferation are induced after immunization in animal models. Studies have suggested that such immune responses may be mediated by SRs that recognize malondialdehyde and acetaldehyde-adducted proteins.[52] In an elegant study into the potential of injectable nanoparticles to selectively deliver antigen to a target cell type, Broz et al.[53] successfully targeted the SR *in vitro*, using ~200 nm triblock copolymer "nanocontainers". Biotin was incorporated into these particles at the production stage, allowing targeting molecules to be coupled via streptavidin. The oligonucleotide polyguanylic acid was used as the SR-targeting agent, and particles were loaded with fluorescent molecules (as model "antigen") to allow visualization of binding of the nanoparticle to and uptake of the nanoparticle by target cells, and intracellular antigen processing. Strong receptor specific binding of nanoparticles was observed within 30 min of the addition of nanoparticles to SR^{+} cells, peaking at ~3 h. No binding to SR^{-} cells was observed. Using confocal microscopy, Broz et al. demonstrated that localization of the particles was to intracellular vesicles and surmized processing through the endocytotic pathway.[53] Interestingly, there was a lack of nonspecific binding of the nanoparticles to cells devoid of SR. One of the main

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hindrances suffered by conventional antigen-targeting approaches (nonspecific uptake by phagocytes) can thus be circumvented using receptor specific nanoparticles that exhibit very weak polymer−protein interaction.

Heat shock proteins (HSPs) are molecular chaperones that control folding of biosynthetic proteins. Previous studies have demonstrated that HSPs from tumor cells are capable of generating tumor specific CTL responses *in vivo*.[54] In addition, antigens covalently linked to HSPs or fused as a recombinant construct initiate MHC class I-mediated immune responses.[55] The receptor for HSP on DCs has been identified as LOX-1, a member of the scavenger receptor family.[56,57] HSP did not bind other scavenger receptors, CD36, SRA-1, MARCO or CLA-1. Mice immunized with OVA conjugated to an anti-LOX-1 antibody were protected from a lethal OVA^+ tumor challenge, and the therapeutic response was dependent on the presence of $CD8^+$ T cells.[57]

Toll-like Receptors. Using specific Toll-like receptor (TLR) ligands which target both DCs and B cells, it has proven to be possible to induce both cellular and humoral immune responses following immunization. It has been demonstrated that TLR7 agonists directly activate plasmacytoid DCs and TLR8 agonists directly activate myeloid DCs and monocyte-derived DCs. TLR7 agonists are more effective than TLR8 agonists at inducing IFNγ, IFN-inducible protein, and IFN-inducible T cell α chemoattractant production by human PBMC; in contrast, TLR8 agonists are more effective than TLR7 agonists at inducing proinflammatory cytokines and chemokines, TNFα, IL-12, and MIP-1α.[58] In one study, when a TLR7 agonist (R-848) was injected into mice, enhanced IL-12p70 and IFNγ expression and enhanced expression of CD80, CD86, and CD40 on DCs were observed. However, $CD8^+$ T cell responses were poor when the agonist was administered with HIV-1 Gag protein.[59] In contrast, when a TLR7/8 agonist was conjugated to HIV-1 Gag protein, substantial Th1/$CD8^+$ T cell responses were induced. Thus, targeting both TLR7 and TLR8 is effective in eliciting a spectrum of immune responses *in vivo*.[59]

TLR9 knockout mouse DCs are unable to induce secretion of IL-12 and type 1 IFN when CpG or viral plasmids are used. However, DNA vaccination experiments showed that TLR9 knockout mice are able to induce Th1 antibody and IFNγ responses; thus, TLR9 signaling is not an absolute requirement for eliciting T and B cell responses to DNA-encoded antigens. However, TLR9 signaling tended to enhance plasmid adjuvant effects on antigen specific immune responses.[60]

To investigate the role of TLR4, the *in vivo* antitumor effect of intratumoral administration of DCs after chemotherapy using an oral fluoropyrimidine anticancer drug TS-1 followed by immunotherapeutic agent OK-432 was investigated in two syngeneic tumor-bearing mouse models. In both Meth-A fibrosarcoma-bearing BALB/c mice and SCCVII-bearing C3H/HeN mice, 1 week of oral administration of TS-1 effected partial eradication of established tumors.[61] Intratumoral injection of DCs and OK-432 caused only slight inhibition of the tumor growth. However, administration of TS-1 followed by DCs and OK-432 resulted in a marked inhibition in tumor growth. The same therapy was also used to treat SCCVII-bearing C3H/HeJ mice in which TLR4 is deficient and no immunotherapeutic effect was observed.[61] Thus, TLR4 signaling is important in the success of this therapy.

A totally synthetic vaccine has been developed which targets TLR2 and consists of a single T cell epitope (a target epitope from influenza virus, *Listeria monocytogenes*, or ovalbumin) that is recognized by either $CD8^+$ T cells or B cells, and a TLR2 lipid moiety *S*-[2,3-bis(palmitoyloxy)-propyl]cysteine, situated between the peptide epitopes to form a branched configuration.[62] Each of the vaccines was capable of inducing $CD8^+$ T cell responses and antibodies. The lipidated vaccines, but not the nonlipidated vaccines, were able to mediate protection against viral or bacterial infection and mediate prophylactic and therapeutic anticancer activity. Thus, a totally synthetic, epitope-based vaccine which targets TLR2 may represent a simple method of inducing cellular and humoral immune responses.

There has been much emphasis recently on enhancing the potency of vaccines by incorporating adjuvants that target

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TLRs on DCs, in animal models. TLR2,[62] TLR4,[61] TLR7,[63] TLR8,[64] and TLR9[65] have all been targeted with adjuvants, and all have demonstrated induction of enhanced immune responses above that of control vaccinations with the TLR agonist absent. In addition to the TLR7/8-targeted study described above,[59] another group[66] has generated actual TLR−antigen composite proteins in an effort to "directly" target antigen to TLRs. Hueleatt et al. generated proteins containing both a TLR 5 ligand (flagellin) and either of the potentially protective OVA antigens or a novel polypetide sequence containing two immunoprotective epitopes derived from the *L. monocytogenes* antigens p60 and listeriolysin. Both vaccines resulted in potent antigen specific T and B cell responses, including rapid induction of antigen specific IgG1 and IgG2a antibody and the development of protective $CD8^+$ T cell responses.

Substantial TLR-targeting vaccine trials have not yet been performed in humans. It remains to be determined whether the "TLR-targeted adjuvant" approach will yield significant benefits in humans or whether the more "direct targeting" approach adopted by Wille-Reece et al.[59] and Huleatt et al.[66] will be required; this is likely to depend on the TLR subtype that is targeted, the antigens that are utilized, and the disease in question.

FIRE and CIRE. F4/80-like receptor FIRE is expressed specifically on $CD8^-CD4^+$ and $CD8^-CD4^-$ DCs and weakly on monocytes and macrophages.[67] CIRE is a C-type lectin receptor expressed on $CD8^-CD4^+$ and $CD8^-CD4^-$ DCs with no expression on macrophages or monocytes.[68] Since rat antibodies are immunogenic in mice, 5 μg of rat anti-FIRE (6F12) and rat anti-CIRE (5H10) antibodies were injected into mice and boosted after 6 weeks and anti-rat Ig titers measured and compared to a control rat antibody.[69] The IgG response (mainly IgG1) to anti-FIRE and anti-CIRE was 100-fold greater than that of the nontargeted control rat antibody. In contrast to a rat anti-DEC-205 antibody (NLDC-145), the magnitude of the response was not increased by the addition of CpG oligodeoxynucleotide. It was apparent that the enhanced antibody responses could be attained without a danger signal as there was no elevation of the level of CD80 or CD86 expression on DCs *in vivo* after administration of anti-FIRE or anti-CIRE antibodies.

Fc Receptors. Fc receptors (FcR) for immunoglobulins link the humoral immune response and the cellular immune response.[70] They also link the innate immune response to the adaptive immune response by binding to pathogens and immune complexes internalized by APCs for presenting T cell epitopes to activate T cells. There is a different FcR for each class of immunoglobulin: FcαR (IgA), FcϵR (IgE), FcγR (IgG), and Fcα/μR (IgA and IgM). In mice, there are four types of FcγRs: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV. Previous studies have shown that immune complexes present antigen more efficiently than antigen alone via the FcγR. The availability of various FcR deficient mice has enabled the importance of the subtypes of FcRs in antigen presentation to be studied. Immune complexes consisting of OVA and polyclonal rabbit anti-OVA antibody were presented 10 times more effectively to T cells than noncomplexed OVA *in vivo*.[71] *In vivo* T cell proliferation in mice treated with OVA immune complexes was no different in γ-chain$^{-/-}$ mice which lack FcγRI, FcγRIII, and FcγRIV compared to the wild type. The extent of $CD8^+$ T cell proliferation was strongly reduced in FcγRI/II/III$^{-/-}$ mice compared to that in the wild type; however, there was no change in T cell proliferation in FcγRII$^{-/-}$ mice. Therefore, all receptors other than FcγRIV are capable of uptake of immune complexes and presentation to $CD8^+$ T cells.

(1) FcγRIII. The human M-$DC8^+$ DC subset represents ~1% of PBMC, larger than the DC1 and DC2 populations, expresses high levels of FcγRIII, and lacks expression of FcγRI.[72,73] Delivery of a tetanus toxoid peptide (amino acids 830−843) or a hepatitis C virus peptide (amino acids 1248−

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1261) by conjugation to an anti-CD16 antibody (3G8) was 500 times more effective in activating specific $CD4^+$ T cell clones than the free antigen.[74] Using an MHC class I-restricted T cell epitope (amino acids 369−377), it was demonstrated that the M-$DC8^+$ cells were incapable of cross-presenting antigens.

(2) FcαRI. FcαRI is expressed on cells of the myeloid lineage. Interstitial-type DCs, $CD34^+$ progenitor-derived DCs, and monocyte-derived DCs express FcαRI.[75] Expression of FcαRI is upregulated after activation, and cross-linking of FcαRI induces internalization of the receptor and activation of DCs.[76] Mice do not express FcαRI, and to study the presentation of antigens targeted via this receptor, IIa1.6 cells transfected with the receptor and bone marrow-derived DCs (BMDCs) from FcαRI transgenic mice have been used.[77] BMDCs cultured with GM-CSF alone or GM-CSF and IL4 which had an immature phenotype expressed low levels of FcαRI which was upregulated by TNFα. Human monocyte-derived DCs (MoDCs) had an FcαRI-positive and -negative population, probably reflecting immature and activated DCs. Only 40% of donors expressed a low level of FcαRI on MoDC which increased to 75−80% of donors when TNFα was used. When the presentation of OVA antigen by MoDCs was studied via delivery of OVA as an IgA immune complex to OVA specific T cells, T cell activation was minimal despite culturing with TNFα. Therefore, it is unlikely that targeting antigen to human FcαRI will result in the generation of stronger immune responses.

Chemokine Receptors. Chemokines can be inflammatory, and their secretion can be induced by inflammatory stimuli or homeostatic where their production is important in normal trafficking of lymphoid cells. Chemokine receptors are expressed on leukocytes, and there expression profile changes with cell differentiation. To target chemokine receptors on immature DCs, Biragyn et al. fused two chemokines, interferon inducible protein 10 (IP-10), monocyte chemotactic protein 3 (MCP-3), and β-defensin 2 and 3 to the variable region of a single-chain antibody based on the idiotype of a murine B cell lymphoma.[78,79] In these studies based on the therapeutic effect on a syngeneic lymphoma, it was suggested that only antigen targeting with inflammatory cytokines and not homeostatic chemokines leads to therapeutic immunity. Using specific antibodies (κ light chains) to chemokine receptors and a cloned human DR4-restricted CD4 T cell specific for mouse Ck,[40−48] the delivery of antigen via several chemokine receptors (CCR1, CCR2, CXCR4, CCR5, CCR6, and CXCR1) was investigated.[80] $CD14^+$ monocytes expressed high levels of CCR1, CCR2, and CXCR4, whereas CCR5, CCR6, and CXCR1 were expressed at intermediate levels. Monocyte DC expressed low levels of all six chemokine receptors. When monocytes were used, an APC antibody specific to CCR1, CCR2, and CXCR4 induced 1000−10000-fold more efficient proliferation than the isotype-matched control antibody. Antibodies specific for CCR5 were 100-fold more efficient, while antibodies to CCR6 and CXCR1 were only 10-fold more potent than the control antibody.[78,79] When immature DCs were used, antigen delivery via CCR1 and CXCR4 was only 10−100-fold more efficient, while the others were even less efficient than controls. In contrast to Biragyn's *in vivo* studies, antigen targeted to a homeostatic chemokine receptor, CXCR4, also stimulated efficient proliferation of T cells.

Bacterial Toxins. Bacterially derived toxins evidently represent attractive candidates for use as vehicles for the delivery of antigen to APCs. The level of genetic manipulation now possible can eliminate their toxicity without compromising their ligand binding or enzymatic qualities; this allows for targeted delivery of experimentally coupled antigens to APCs and subsequent intracellular processing culminating in the presentation of the antigen to the wider immune system. This avenue of research has primarily focused on induction of $CD8^+$ (cytotoxic) T cell responses via APC targeting of $CD8^+$ epitopes that are subsequently processed in the cytoplasm by proteosomes and presented on MHC class I molecules, which have proven to be protective in animal models. Successful vaccination (or treatment regimes) against some human diseases will probably require induction of $CD4^+$ as well as $CD8^+$ T cell responses. Recently, the bacterial toxins *Bordetella pertussis* adenylate cyclase[81] and modified anthrax toxin lethal factor[82] have led to simultaneous facilitation of both $CD4^+$ and $CD8^+$ T cell responses against artificially coupled secondary antigens.

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One of the earliest demonstrations that bacterial toxin-mediated delivery of a secondary antigen could promote effective $CD8^+$ T cell responses against that antigen was provided more than 10 years ago.[83] A $CD8^+$ T cell epitope derived from *choriomeningitis* virus was inserted into the *B. pertussis*-derived adenylate cyclase toxin (CyaA) without interrupting the enzymatic properties of the toxin that facilitated its entry into host cells. The construct was used to sensitize cells to *in vitro* killing by peptide specific $CD8^+$ T cells. Subsequently, $CD8^+$ T cell responses were induced *in vivo*[84,85] and ultimately protection of the host against viral challenge.[86] Furthermore, $CD8^+$ T cell-mediated protection against *L. monocytogenes* was demonstrated using a bacterially derived toxin utilizing components of anthrax toxin which facilitated entry into the cell and delivery to the cytosol. Following these early investigations, many reports revealing that various bacterial toxins are amenable to fusion with model antigens and subsequent induction of $CD8^+$ T cell responses *in vivo* have emerged, including reports on *Pseudomanas* exotoxin,[87] the B subunit derived from *E. coli* heat labile enterotoxin,[88] and *Diphtheria* toxin.[89] These and other bacterial-toxin-vehicle-based studies have historically focused on achieving antigen presentation via MHC class I molecules and subsequent $CD8^+$ T cell responses. While a potent $CD8^+$ T cell response has proven to be protective against some pathogens in experimental animal models, these observations may not hold true for humans, and for at least some pathogens, co-induction of both $CD8^+$ and $CD4^+$ responses will be required for induction of long-term protective immunity via vaccination, or effective treatment of existing disease. Recently, a number of groups have investigated simultaneous targeting of antigens to MHC class I and II molecules via bacterial toxin derivatives, to elicit both $CD8^+$ and $CD4^+$ T cell responses in vaccine recipients.

In a preliminary study, a $CD4^+$ T cell epitope derived from *E. coli* maltose binding protein (MalE) was inserted into *B. pertussis* CyaA, at sites previously determined not to disrupt binding and invasiveness into target cells.[90] Genetic modification was employed to abolish the ability of the constructs to convert ATP to cyclic AMP (and thus, their toxicity). *In vitro* presentation assays suggested that depending on the insertion site of the MalE epitope, constructs facilitated its delivery into cells for endosomal processing and ultimately MHC class II presentation with varying degrees of success. Constructs with the MalE epitope inserted within the first third of the molecule proved to be the most efficient, and two of these were tested *in vivo*. Mice were immunized with the constructs and then sacrificed 1 week later and their splenocytes harvested. Proliferation assays using a range of concentrations of the MalE peptide to stimulate potential responses revealed substantial proliferative responses had occurred within 6 h of stimulation with peptide, and these responses titrated consistently with the concentration of peptide added. Thus, without adjuvant, genetically modified CyaA was able to deliver a $CD4^+$ epitope efficiently, resulting in endosomal processing and MHC II presentation, both *in vitro* and *in vivo*.[90] In addition, the group then endeavored to simultaneously elicit $CD8^+$ and $CD4^+$ responses using the CyaA epitope delivery system.[81] An OVA-derived $CD8^+$ T cell epitope ($OVA_{257-264}$) and a $CD4^+$ T cell epitope derived from *E. coli* maltose binding protein ($MalE_{100-114}$) were incorporated into the same CyaA molecule (CyaA-MalE-OVA) and tested both *in vitro* and *in vivo* for simultaneous $CD8^+$ and $CD4^+$ T cell response induction. *In vitro*, both MHC class I and MHC class II presentation and responses were observed. Using various inhibitors and TAP-deficient DCs to elaborate on this finding, it was demonstrated that after interaction of the construct with CD11b, the adenyl cyclase domain can either be translocated into the DC cytosol, leading to processing via the conventional MHC class I pathway, or be degraded via the endocytic pathway, culminating in MHC class II presentation. Congruent with the *in vitro* results, *in vivo* administration of CyaA-MalE-OVA simultaneously induced CTL responses to the OVA-derived $CD8^+$ epitope and $CD4^+$ responses to the MalE-derived $CD4^+$ epitope.[81]

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Furthermore, modified anthrax toxin lethal factor (LFn) fusion proteins have been shown to stimulate $CD4^+$ T cell responses against HIV proteins in humans.[82] While the mechanisms of APC entry are yet to be determined, endosomal processing of HIV-derived GAG and NEF proteins (collectively termed "Fn-HIV") and subsequent MHC class II presentation was clearly demonstrated *in vitro*.[82]

While a vigorous $CD8^+$ T cell response is evidently sufficient to prime protection against some pathogens, it has proven to be ineffective against more persistent or "insidious" pathogens such as HIV and most cancers. Induction of simultaneous $CD4^+$ and $CD8^+$ responses represents a significant move forward in the quest for preventative vaccines, and "vaccines" designed to treat existing conditions. Recombinant bacterially derived toxins are emerging as cheap and adaptable candidates toward achieving this aim.

DC-Binding Peptides. To obtain peptides that specifically bind DCs, a 12-mer phage library was panned successively on human monocytes, T cells, and B cells. Langerhans-like DCs and unbound phage were used to pan on myeloid DCs.[91] Three peptides (FYPSYHSTPQRP, AYYKTASLAPAE, and SLSLLTMPGNAS) which specifically bound DCs were selected. These peptides bound distinctly different proteins on the surface of immature DCs and were internalized. DCs pulsed with a recombinant fusion protein between the peptide and the hepatitis C virus nonstructural protein 3 (NS3) activated autologous $CD4^+$ and $CD8^+$ cells from HCV-infected individuals. The fusion protein primed naive $CD4^+$ and $CD8^+$ T cells in a NOD-SCID mouse xenotransplanted with PBLs and DCs from HCV subjects. Mice immunized with BMDCs pulsed with the fusion protein generated antigen specific cytokines (IFNα and TNFα) and proliferative responses. In another study in which a phage library containing 20-mer peptides was utilized to pan Langerhans cells, the peptide GPEDTSRAPENQQKTFHRRW was isolated with high frequency.[92] A tetrameric peptide formulated as a multiple-antigen peptide specifically targeted the Langerhans cells and internalized a 40 nm quantum dot nanoparticle. Phage incorporating the specific peptide primed antibody responses to the phage, while phage incorporating control nonbinding peptides were not immunogenic.

Clinical Studies

Despite preliminary attempts to target antigen to DCs *in vitro* and a number of preclinical efficacy studies, success in clinical trials has been limited. In contrast, there have been many clinical trials that have utilized DCs pulsed *ex vivo* with DC-targeted antigen. However, a number of Phase I clinical trials targeting the MR have been completed. In one trial, 25 patients with advanced metastatic adenocarcinoma were injected with increasing doses of the mannan−MUC1 fusion protein.[93] High titers of IgG1 anti-MUC1 antibodies were produced in 13 of 25 patients (ranging from 1/320 to 1/20480 as assessed by ELISA). In addition, T cell proliferation was found in 4 of 15 patients, and CTL responses in 2 of 10 patients.[93,94] Using flow cytometry, intracellular IL-2, IL-4, IFNγ, and TNFα were produced by $CD4^+CD69^+$- and $CD8^+CD69^+$-activated T cells from PBMCs of immunized patients, after MUC1 antigen stimulation.[95] In a randomized double-blind study, 31 postmenopausal women with estrogen-positive stage II breast cancer with no more than four ipsilateral nodes involved and no evidence of distal disease were treated with a placebo (15 patients) or the mannan−MUC1 fusion protein (16 patients).[96] All patients received tamoxifen. Four of the 15 patients treated with a placebo had a relapse of disease, while none of the patients treated with the mannan−MUC1 fusion

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protein relapsed (currently 9 years). Nine of 13 patients treated with the mannan−MUC1 fusion protein had anti-MUC1 antibodies, and 40% displayed IFNγ as detected by ELISpot analysis. These studies suggest that targeting the MR using mannan−antigen conjugates represents a promising immunization strategy that stimulates both humoral and

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cellular responses in patients. Collective considerations herein strongly support the concept of conducting vaccine trials with early stage patients or patients with low tumor burdens.

Conclusion

The potent adjuvant effect of DCs capable of priming naive $CD4^+$ and $CD8^+$ T cell responses has triggered a large number of clinical trials using *ex vivo* DC pulsed with a variety of antigens, taking advantage of this property of DCs. While it has shown promise in some instances and is a valuable research tool, harvesting DCs from potential vaccinees, pulsing them with antigen *ex vivo*, and then reintroducing them into those same recipients does not represent a practical method of preventative vaccination "en masse". Commercial application of this strategy, should it prove to be effective in the long run, will conceivably be confined to those that already have a potentially terminal disease (such as cancer or HIV) and can afford the (necessarily very expensive) treatment regime. Furthermore, there is still debate regarding the type of human DC to use as well as the dose and route of immunization for optimal therapeutic effects. Because of the variation in mouse DC populations that are used in mouse experiments and human DCs, there is no clear extrapolation between mouse and human studies. Targeting DC populations *in vivo* with various DC specific antigens may meaningfully expand on these discrepancies. The studies reported above foreshadow approaches that may give rise to clinically relevant antigen specific $CD4^+$ and/or $CD8^+$ T cell responses in humans, that could be utilized as inexpensive alternatives to *ex vivo* pulsing of DCs within large populations.

Even with the various improved antigen delivery strategies, it will still be necessary to accommodate features in the novel vaccines that can overcome tumor heterogeneity, downregulate class I molecules and/or tumor antigen, prime CD4 and CD8 responses, cater for multiple HLA types, and provide

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a maturation stimulus. Some of these can be rectified by utilizing whole protein antigens, multiple-protein antigens, or polytope fusion proteins for immunization or potent adjuvants. However, it may also be necessary to conduct the clinical trials in patients with minimal residual disease before immune evasion strategies of the tumor are triggered.

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