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*J Immunol* 2004; 172:7425-7431; doi: 10.4049/jimmunol.172.12.7425

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Peptides Identified through Phage Display Direct Immunogenic Antigen to Dendritic Cells

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Dendritic cells (DC) play a critical role in adaptive immunity by presenting Ag, thereby priming naive T cells. Specific DC-binding peptides were identified using a phage display peptide library. DC-peptides were fused to hepatitis C virus nonstructural protein 3 (NS3) while preserving DC targeting selectivity and Ag immunogenicity. The NS3-DC-peptide fusion protein was efficiently presented to CD4⁺ and CD8⁺ T cells derived from hepatitis C virus-positive blood cells, inducing their activation and proliferation. This immunogenic fusion protein was significantly more potent than NS3 control fusion protein or NS3 alone. In chimeric NOD-SCID mice transplanted with human cells, DC-targeted NS3 primed naive CD4⁺ and CD8⁺ T cells for potent NS3-specific proliferation and cytokine secretion. The capacity of peptides to specifically target immunogenic Ags to DC may establish a novel strategy for vaccine development. The Journal of Immunology, 2004, 172: 7425–7431.

Dendritic cells (DC)³ are a complex, heterogeneous group of multifunctional APCs that play critical roles in combating viral infection, and other pathogenic conditions, by inducing humoral and cellular immunity (1, 2). Recent studies (1–7) demonstrate that DC differentiate into at least three subsets including Langerhans cells, myeloid DC (MDC), and plasmacytoid DC. Bone marrow-derived DC progenitors migrate through the bloodstream and home to peripheral tissues to sample for foreign Ags (1). After Ag capture, DC migrate to locally draining lymph nodes to prime Ag-specific CD4⁺ T cells that in turn regulate multiple effector cells, including CD8⁺ CTLs, NK cells, and B lymphocytes (1). Recent studies demonstrate the utility of DC as effective vaccines in human clinical trials (8–10).

We undertook this study to test the hypothesis that DC-targeting 12-mer peptides derived from a phage display library would increase Ag immunogenicity and would enhance naive T cell priming. We used hepatitis C virus (HCV) nonstructural protein 3 (NS3), an Ag proposed for HCV vaccines (11–14), as our model immunogen.

Materials and Methods

Mice and reagents

Female BALB/c mice and NOD.CB17-SCID mice were used (6- to 8-wk old; The Jackson Laboratory, Bar Harbor, ME) and housed in the Tulane vivarium. The study was approved by the Tulane Institutional Animal Care Committee. Murine mAbs were: CD14, HLA-DR, CD3, CD4, CD8 (BD Biosciences, Franklin Hills, NJ); CD62L (Caltag Laboratories, Burlingame, CA); CD86, CD83, and anti-human IFN-γ, IL-2, IL-10, TNF-α (BD PharmMingen, San Diego, CA); CD40, HLA-ABC (R&D Systems, Minneapolis, MN); CD1a (DAKO, Carpinteria, CA); CD80, CD83, CD45RA, CD45RO, CD69 (Coulter/Immunotech, Fullerton, CA); streptavidin-PE (SA-PE); and anti-M13 Ab (Amersham Pharmacia Biotech, Piscataway, NJ). Other reagents were: LPS, murine GM-CSF, murine IL-4 (Sigma-Aldrich, St. Louis, MO); recombinant human GM-CSF (Immunex, Seattle, WA); and recombinant human IL-4 (R&D Systems, Minneapolis, MN). Complete medium is RPMI 1640, heat-inactivated 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 50 µM 2-ME, 1% sodium pyruvate, and 1% essential amino acids (all from Life Technologies, Gaithersburg, MD). The Ph.D.-12 phage display library was purchased from New England Biolabs (Beverly, MA).

Synthesis of peptides

Peptides were synthesized using 9-fluorenylemethoxycarbonyl chemistry, purified using high pressure liquid chromatography to 90% purity, and biotinylated by the Auxiliary Biochemistry Core Laboratories, Louisiana State University (New Orleans, LA).

Human cells

PBMC were isolated from the blood of healthy or HCV⁺ individuals by Ficoll gradient centrifugation. HCV RNA was detected in the blood of HCV-infected patients by RT-PCR. PBMC (10⁵/well) were seeded in six-well plates for 2 h at 37°C. Subsequently, nonadherent cells were removed by several washes using PBS plus 2% FCS and frozen for autologous MLR experiments. Adherent monocytes were cultured with GM-CSF (100 ng/ml) and IL-4 (10 ng/ml) in complete RPMI for 6 days (6). CD1a⁺ DC were phenotyped by FACS before use. Rhesus macaque or chimpanzee DC were prepared in a parallel manner by using human GM-CSF (100 ng/ml) and human IL-4 (50 ng/ml) for 6 days (15). Mouse DC were prepared from bone marrow of BALB/c mice as described (16). On day 6, CD1c⁺ I-A⁺ DC were harvested, phenotyped, and used for DC-peptide staining.

DC-binding peptides

We panned the Ph.D.-12 phage display library (17). Phages (1.5 × 10¹¹) were incubated serially with monocytes, T cells, and B cells, then with Langerhans-like DC (7), then MDC, using unbound phage for each subsequent step. MDC-binding phage were eluted with glycine-HCl (0.2 M, pH 2.2), neutralized with Tris base (1 M, pH 9.1), and amplified in Escherichia coli. A total of four rounds of selection were conducted. EMBL Nucleotide Sequence Database Accession Numbers for peptides 3, 12, and 18 were AJ544526, AJ544527, and AJ544528, respectively.
Fusion of DC-peptides to HCV NS3

The NS3 coding sequence was amplified from an HCV proviral cDNA using PCR (gift from Dr. S. Dash, Tulane University, New Orleans, LA). After restriction digestion, the PCR product was inserted into compatible sites in plasmid pET24d (Novagen, Madison, WI). The forward primer encoded NS4A_{11-32} peptide and thus was fused at the 5’ end of the NS3 coding region to enhance the stability of the protein (18). To generate the final construct, the NS4A_{11-32}-NS3 coding region was ligated to DNA encoding peptide 3 or control peptide, each of which was followed by a vector-encoded C-terminal histidine 6-coding sequence. Both genetic fusions were verified by sequencing using an ABI-377 Automated Sequencer (Applied Biosystems, Foster City, CA). Recombinant NS3 fusion proteins (FP) were expressed in E. coli BL21(DE3) after 4 h of induction using 1 mM isopropyl-β-D-thiogalactopyranoside. Cell-free extracts were generated by sonication, and the recombinant fusion proteins were purified using a Ni-NTA Superflow Column (Qiagen, Valencia, CA). Bacterial endotoxin was subsequently removed using the END-X B15 Endotoxin Removal Affinity Resin (Seikagaku America, Falmouth, MA). Protein quantification and purity were assessed by PAGE.

Immunoblotting

Immunoblotting of purified recombinant NS3-FP and NS3-control FP (cFP) was performed as described previously (7). Gels were transferred onto nitrocellulose membranes. The transferred proteins were detected using anti-NS3 Ab (Novocasta, Newcastle, U.K.) and visualized with the ECL Western blotting detection system (Amersham Pharmacia Biotech).

T cell proliferation

Purified CD4⁺ T cells were obtained by depletion of CD8⁺, CD19⁺, CD56⁺, CD1a⁺, and CD14⁺ cells using specific bead-conjugated Abs (Miltenyi Biotec, Auburn, CA). CD8⁺ T cells were purified by depleting CD4⁺ cells in an analogous manner. Monocyte-derived DC were incubated with immunogen (1 µg/ml) or control for 12 h at 37°C. DC were cocultured with T cells (10⁵/well of a 96-well plate) for 4 days in complete RPMI 1640 + substituting 10% human AB⁺ serum (Gemini Bio-Products, Woodland, CA) for FCS. Cells were pulsed for the last 16 h with 0.5 µCi of [³H]Thymidine per well (New England Nuclear, Brattleboro, VT). [³H]Thymidine incorporation was measured using a beta counter. Supernatants of cocultures were assayed for cytokines by ELISA (R&D Systems) on day 4.

NOD-SCID mouse model

Female NOD.CB17-SCID mice were maintained as we previously described (19). PBMC were obtained from the blood of healthy, HCV⁻ naive donors. DC (5 x 10⁶/mouse), T cells, and B cells (3 x 10⁶ in 300 µl of PBS/mouse) were combined with NS3-FP (5 µg/mouse), NS3-cFP (5 µg/mouse), or nothing and administered i.p. three times at 5-day intervals. Five days after the last immunization, mice were sacrificed, and human cells were recovered from the peritoneum and separated by Ficoll density gradient centrifugation (20). CD3⁺ T cells were bead-enriched (Miltenyi Biotec) and analyzed by FACS for intracellular cytokines, as we described, by gating on human CD3⁺ cells (19, 21).

In addition to the NOD-SCID mouse model, groups of BALB/c mice were immunized with bone marrow-derived CD11c⁺ DC (3 x 10⁶/mouse) plus NS3-FP (1 µg), NS3-cFP (1 µg), or no supplement and subsequently injected both i.p. and into the footpads of the BALB/c mice. This immunization was repeated four times at 5-day intervals. Five days after the last immunization, mice were sacrificed, and spleen and lymph nodes were subsequently isolated. Spleens and lymph nodes of immunized mice were minced, and cell suspensions were prepared. Subsequently, splenic CD4⁺ and CD8⁺ T cells were purified using magnetic beads. Bone marrow-derived DC were then pulsed with NS3 (500 ng/ml) or HIV gp160 (500 ng/ml) for 12 h at 37°C. Pulsed DC were extensively washed and cocultured with autologous CD4⁺ (5 x 10⁵/well of a 96-well plate) and DC-peptides, or control peptide, conjugated with SA-PE, and analyzed by FACS. E. Rhesus macaque monocyte-derived DC were stained with biotinylated DC-peptides or control peptides and analyzed by FACS. F. FACS of bone marrow-derived DC stained with biotinylated DC-peptides or control conjugated with SA-PE. All experiments were repeated at least three times.
MDC (5 × 10^4) were incubated with biotinylated peptides (10 μg/ml each) for 1 h at 4°C. Subsequently, cells were washed, incubated with SA-PE (1:100) for 1 h, fixed with 0.1% paraformaldehyde, and analyzed on a FACS Caliber using standard CellQuest software (BD Biosciences, San Jose, CA). T cells were analyzed as described previously (19). At least 10^4 gated events/condition were acquired for all analyses.

Flow cytometry

DC were cultured in a tissue chamber (coverglass-chambered eight-well slide; Nunc, Roskilde, Denmark) for 12 h at 37°C. DC were incubated with either biotinylated peptide 3 or control peptide for 30 min at 37°C. Cells were fixed with 2% paraformaldehyde for 15 min and subsequently permeabilized with 1% Triton X-100 in PBS-fish skin gelatin (Sigma-Aldrich) for 15 min. Biotinylated peptides were detected with 0.1% streptavidin labeled with Alexa 488 (Molecular Probes, Eugene, OR). The nuclei were stained with ToPro-3 (Molecular Probes). Cells were visualized using a Leica (Leica Microsystems, Exton, PA) TCS SP2-confocal microscope (19).

Statistical analysis

All values were determined as means ± SE. Statistical analysis was conducted by the Student t test or χ² test as appropriate. Significance was defined as p < 0.05.

Results

Generation and characterization of DC-binding peptides

The Ph.D. 12-mer peptide phage display library was screened for peptide ligands that specifically bound to human DC. After 4 rounds of screening, 20 candidate phages were identified. DC were incubated with individual phages and analyzed by FACS. Phages 3, 12, and 18 significantly bound human DC (not shown). Peptide sequences of these three phages were deduced from nucleic acid sequences, and were designated as peptides 3 (FYPSYHSTPQRP), 12 (AYYKTASLAPAE), and 18 (SLSLLTMPGNAS). A 12-mer peptide ligands that specifically bound to DC were first treated with unlabeled peptides (biotin free), stained with biotinylated peptides, conjugated with SA-PE, and analyzed by FACS. x-axis, Mean fluorescence intensity (MFI) of gated events. DC were incubated with various concentrations of DC-binding peptides or control peptide, conjugated with SA-PE, fixed, and analyzed by FACS. Data represent the mean of three experiments. E, DC were activated with E. coli LPS (100 ng/ml) for 48 h, stained with biotinylated DC-peptides or control peptide, and analyzed by FACS. All experiments were performed at least three times.

CD8⁺ T cells (50 × 10^4)/well of a 96-well plate) for 5 days. On day 5, supernatants of the cocultures were collected for cytokine analysis. [3H]Thymidine incorporation was measured using a beta counter.

FIGURE 2. Peptides (pep) bind to distinct and saturable sites on DC. A, DC were stained with biotinylated DC-peptides (thin lines), or control (Ctrl) peptide (thick lines), or after pretreatment with the same peptide not biotinylated (dotted line) and were analyzed by FACS. B, Incubation with a given DC-binding peptide did not inhibit binding of other DC-binding peptides. C, DC were incubated with various concentrations of DC-binding peptides or control peptide, conjugated with SA-PE, analyzed by FACS. x-axis, Mean fluorescence intensity (MFI) of gated events. D, MDC were treated with biotinylated DC-peptides for 0, 2, 4, or 20 h and analyzed by FACS. Data represent the mean of three experiments. E, DC were activated with E. coli LPS (100 ng/ml) for 48 h, stained with biotinylated DC-peptides or control peptide, and analyzed by FACS. All experiments were performed at least three times.

Peptides bind to distinct and saturable sites on DC

The cognate ligands for these peptides are unknown. Thus, we next tested whether these peptides bound distinct epitopes. DC were incubated with biotinylated and nonbiotinylated peptide no longer bound the corresponding biotinylated peptide. In contrast, binding of one specific peptide did not inhibit binding of the other two peptides. Thus, the three DC-binding peptides bound distinct, saturable DC surface epitopes. The half-maximal relative mean fluorescence of binding for each peptide was reached at a peptide concentration of <2 μg/ml (Fig. 2C), suggesting dissociation constants in the nanomolar range.

Biotinylated peptide 3 bound immature DC within 1 h at 4°C (Fig. 2D). Staining was decreased during the next 20 h at 37°C (Fig. 2D) consistent with Ag uptake by DC (15, 22). Importantly, DC remained viable during this period. Peptides bound immature (Fig. 2E, upper panels) and mature MDC (Fig. 2E, lower panels) comparably, although LPS-mediated maturation appeared to increase peptide binding slightly by inducing a new, minor population of high peptide-binding DC.
FIGURE 3. DC-binding peptides did not change the phenotype or functions of DC. A, The phenotype of DC treated with DC-peptides was analyzed by FACS. Thick line, Untreated DC; dotted line; DC treated with peptides; dashed line, isotype control. DC treated simultaneously with DC-peptides did not inhibit the proliferation of allogeneic CD4+ T cells (B) or T cell activation (C). All experiments were repeated at least three times.

FIGURE 4. Fusion of DC-binding peptide with HCV NS3. A, Schematic depicting of the NS3-FP or NS3-control FP construct. Cntl and Ctrl, Control; h6, histidine 6 tag. B, SDS-PAGE of the Ni2+-affinity-purified products (left) and Western blot (right). The transferred proteins were detected using an anti-NS3 Ab and visualized by ECL Western blotting detection system. MW, Molecular mass marker. C, Detection of NS3-FP using a monoclonal anti-NS3 Ab conjugated with a goat anti-mouse IgG FITC by FACS; thick line, NS3-FP; dotted line, NS3-cFP; thin line, isotype control.
Peptides do not alter DC costimulatory function

To show whether the binding of DC-peptides can modulate the phenotype and the accessory functions of DC, human DC were treated with synthetic DC-peptides alone and cultured at 37°C. Data show that DC treated with the peptides did not undergo phenotypic or functional changes to induce allogeneic CD4⁺ T cell proliferation and activation (Fig. 3).

Genetic fusion of DC peptides to NS3 facilitates DC capture and presentation

We next tested the functionality of DC-binding peptides when NS3 was genetically fused to peptide 3 or control peptide. The coding region of NS3 was fused in frame to DC peptide 3 or control peptide, with a vector-encoded C-terminal histidine tag using pET24d (Fig. 4A). After SDS-PAGE and Western blotting, 85-kDa full length recombinant FP were detected, along with predominant N-terminally truncated 72-kDa forms (Fig. 4B). As shown in Fig. 4C, the binding of the recombinant NS3-DC peptide 3 FP (NS3-FP) to DC was demonstrated by FACS and was significantly better than the binding of NS3-cFP.

To demonstrate NS3-FP function, DC were pulsed with NS3-FP, NS3-cFP, NS3 alone, or nothing and used to activate autologous CD4⁺ or CD8⁺ T cells from HCV-infected individuals. CD4⁺ and CD8⁺ T cell proliferation was increased ~3-fold (p < 0.05) by incubation of DC with NS3-FP compared with NS3-cFP, or NS3 alone (Fig. 5, A and C). Furthermore, CD4⁺ or CD8⁺ T cells cocultured with NS3-FP-loaded DC became activated and up-regulated CD69, CD45RO, and CCR6 (not shown). IFN-γ and TNF-α production induced by DC treated with NS3-FP was significantly higher (p < 0.05) than that stimulated by DC pulsed with NS3-cFP or NS3 alone (Fig. 5, B and D). DC loaded with NS3-FP up-regulated CD83, and CD40 and IL-12 p70 higher than NS3-cFP (Fig. 5, E and F), suggesting that conjugates do not suppress normal DC function (23).

Studies in vivo

To study efficacy of DC-peptides that were genetically fused to NS3 in vivo; we used our NOD-SCID mouse model (19). NOD-SCID mice were xenotransplanted with peripheral blood cells. DCs from HCV⁻ subjects, and the immunogens (NS3-FP or NS3-cFP) were administered directly into mice to prime naive T cells in vivo. Vaccinated mice with autologous DC charged with NS3-FP induced significantly more NS3-specific T cell priming as evidenced by NS3-specific cytokine secretion (IFN-γ and TNF-α), CD69 expression, and CD4⁺ T cell proliferation compared with DC pulsed with NS3-cFP alone (Fig. 6, A–D).

Moreover, significant priming of NS3-specific CD8⁺ T cells was observed in NS3-FP-vaccinated mice compared with NS3-cFP vaccinated mice (Fig. 6, E and F). T cells from mice immunized with NS3-FP or NS3-cFP did not respond to the control immunogen HIV gp160, suggesting that T cell activation was NS3 specific (Fig. 6, C and E).

To further show the efficacy of NS3-FP in vivo, BALB/c mice were immunized with bone marrow-derived DCs pulsed with immunogenic fusions. Data showed that bone marrow-derived DC combined with NS3-FP significantly enhanced the expansion of murine CD4⁺ and CD8⁺ T cells (Fig. 7, A and B) and their subsequent activation as evident by IFN-γ and TNF-α production (Fig. 7, C and D).

Discussion

DC as a critical vector for vaccine strategy induce potent Ag-specific immunity in various settings including human clinical trials (8–10). Optimal means of delivering immunogenic Ag to DC remain undefined, but current immunotherapy relies largely on ex vivo methods. Although useful to demonstrate proof-of-concept, ex vivo growth of DC is logistically difficult (24). Although DC-based immunotherapy highlights its critical role in inducing Ag-specific immunity, however, at present, DC-targeting strategies are hampered by lack of DC-specific target molecules. Nonetheless, we and others (17, 25) reported previously that peptide libraries...
could be used to derive 12-mer peptides that bind specifically to matrix or cell surface molecules. Thus, to target immunogenic Ag specifically to DC via targeted molecules expressed solely by these cells, we used a defined phage display peptide library that does not require a priori knowledge of the DC molecule targeted.

In this paper, we show that small peptides can be derived from a phage display peptide library that specifically bind to their ligands expressed on DC without modulating the phenotype of these cells. Additionally, FACS analyses indicate that DC exhibit bimodal peptide binding capacity, with the majority of these cells binding peptide uniformly, and a small (~5%) subset exhibiting extremely high level binding. This high level binding was demonstrated with all three DC-binding peptides, despite the fact that they bind distinct DC epitopes. Thus, we speculate that this capacity may be an intrinsic property of these 12-mer peptides. Alternatively, the high binding subset may represent a unique DC subset. Further work is required to distinguish these possibilities.

**FIGURE 7.** Activation of CD4⁺ and CD8⁺ T cells using bone marrow-derived DC pulsed with NS3-FP in vivo. Groups of BALB/c mice were immunized with bone marrow DC, NS3-FP, NS3-cFP, or no supplement. This immunization was repeated four times at 5-day intervals. After the fourth immunization, spleens and lymph nodes of immunized mice were isolated. Cell suspensions were prepared, and T cells were isolated. Purified splenic murine CD4⁺ T cells and CD8⁺ T cells were then cocultured with pulsed bone marrow-derived DC at graded doses, with NS3 alone or HIV gp160. T cell proliferation was measured by [³H]thymidine uptake, and T cell activation was analyzed by ELISA.
Furthermore, we present a novel strategy for targeting immunogenic Ag to DC using 12-mer binding peptides. Our results clearly show that Ag-DC-peptide fusions retained their immunogenicity, are captured, and are presented to T cells, thereby inducing potent Ag-specific T cell activation. We also demonstrate Ag-specific priming of naive human and murine T cells in vivo in a novel manner in which DC will not need to be pulsed in ex vivo and then administered into mice. Thus, our approach may be complementary to the current methods which involve viral vectors (26). Ag receptor-DEC205 (23), or peptide-binding to MHC (8, 9, 27).

Recently, it was shown in an elegant approach that using an anti-DEC205 Ab to target DC induced tolerance rather than active immunity, unless a DC activation signal was first used (23). We show here that T cells activated or primed through DC-targeted Ag demonstrated increased cytokine production as a population and also on an individual cell basis. For example, the mean fluorescence intensity of IFN-γ, IL-2, and TNF-α expression was higher in NS3-FP-activated T cells than in NS3-cFP-activated T cells. Using NOD-SCID mice, survival of T cells was also promoted by the NS3-FP vaccine compared with NS3-cFP as judged by higher proportions of CD4+ and CD8+ T cells in animals vaccinated with NS3-FP. Moreover, data clearly show that by immunizing BALB/c mice with bone marrow-derived DC pulsed with immunogenic fusions, this stimulated murine T cells in vivo. The mechanisms underlying these phenomena remain undefined but likely involve more than the immaturity of DC or simply delivering more Ags to DC. DC loaded with the immunogenic fusions were able to prime both CD4+ and CD8+ T cells in vivo with no detectable evidence for tolerance or for IL-10 production. In fact, the interaction between the DC-peptides and their cognate surface ligands may prevent signals leading to tolerance. Moreover, DC-targeted fusion proteins captured by DC do not appear to impair accessory immunological responses. Further in vivo studies may shed light on the molecular mechanisms by which these peptides induce active immunity.

Together, DC-directed Ag stimulated potent Th1 priming of naive T cells, suggesting that this approach may be useful for preventive vaccinations. Additionally, this approach potently induced memory T cell responses, suggesting that this strategy may also be useful in mediating therapeutic immunity. Finally, in light of concerns regarding the safety, purity, and manufacture of various vaccines, our strategy may provide an alternative approach.

Acknowledgments
We thank Dr. Rafi Ahmed, Dr. S. Dash, and Karol Sestak for support and fruitful discussions. We thank Julia Tuckler and Jessica B. Hebert for technical assistance.

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