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Induction of Vigorous Helper and Cytotoxic T Cell as well as B Cell Responses by Dendritic Cells Expressing a Modified Antigen Targeting Receptor-Mediated Internalization Pathway

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Efficient Ag presentation is essential to induce effective cellular and humoral immune responses. Thus, one central goal of current immunotherapy and vaccine development is to enhance Ag presentation to induce potent and broad immune responses. Here, a novel Ag presentation strategy is developed by transducing dendritic cells (DCs) to produce and secrete a model hepatitis B virus Ag fused with a cell-binding domain and to process the fusion Ag as an exogenous Ag to efficiently induce both humoral and cellular immunity. The principle of this strategy is illustrated by genetically modifying DCs to secrete a model hepatitis B virus Ag fused with a cell-binding domain and to process the fusion Ag as an exogenous Ag after receptor-mediated internalization for MHC class I and II presentation. Vigorous Ag-specific CD4+ helper and CD8+ cytotoxic T cell, as well as B cell, responses were induced by the transduced DCs in mouse models. Thus, this novel strategy uses a receptor-mediated internalization process to efficiently induce all arms of the adaptive immunity and may provide a powerful means to develop potent vaccines and immunotherapies. *The Journal of Immunology, 2000, 165: 4581–4592.

The adaptive immunity is maintained by cellular and humoral immune responses that are initiated and modulated by professional APCs such as dendritic cells (DCs) (1–4). T cells consisting of CD4+ Th cells and CD8+ CTLs only recognize antigenic peptides associated with either MHC class I or II (MHC-I, MHC-II) (1–4). CD8+ CTLs recognize and destroy MHC-I-restricted Ags, which are mainly degraded in the cytosol, translocated into the lumen of the endoplasmic reticulum, and presented to the MHC-I. In contrast, CD4+ T cells recognize MHC-II-restricted Ags, which are mainly internalized by professional APCs and processed in the endosomal pathway, where the Ags are degraded into peptides that are presented to the MHC-II. A crucial step for inducing immune responses is the presentation of Ags by MHC-II molecules on APCs to CD4+ T cells, because activated CD4+ T cells are responsible for regulation of essentially all other functions of the immune system, including CTLs, B cells, and macrophages (1–4). Thus, one central goal of current immunotherapy and vaccine development is to enhance Ag presentation to both MHC-I and -II for inducing potent and broad immune responses (1–4).

An Ag presentation strategy, which is able to induce CD4+ Th cell, CD8+ CTL, and B cell responses, will lead to the development of potent immunotherapy or vaccines against pathogens and tumors. In this study, we design a novel Ag presentation strategy by transducing DCs to produce and secrete a fusion protein consisting of a hepatitis B virus (HBV) nucleocapsid protein HBeAg/ HBeAg fused with a cell-binding domain such as the Fc fragment of IgG. The secreted fusion proteins, in addition to inducing Ab responses, are transported back to DCs via receptor-mediated internalization. It has been demonstrated that Ag presentation by receptor-mediated internalization of DCs can be enhanced up to 1000-fold, compared with fluid phase Ag pinocytosis (5–9). As a result, the fusion Ags are processed in the endosomal pathway and presented by DCs as exogenous for MHC-II presentation to induce CD4+ Th cells. The internalized Ags can also be presented to MHC-I (cross-priming) by DCs to directly activate CTLs (10–16). Thus, this strategy uses a unifying mechanism to activate all arms of the adaptive immunity. In this study, we demonstrate that this receptor-mediated Ag presentation strategy is able to induce vigorous Th cell, CTL, and B cell responses against the model HBV nucleocapsid protein in mouse models.

Materials and Methods

Construction of expression vectors

A plasmid encoding the full-length HBV (adw subtype) genome was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The HBV precore/core gene was found to contain a single base pair deletion, which causes a frame shift at codon 79, resulting in two consecutive stop codons at 84 and 85. This gene was repaired by inserting the deleted base using PCR mutagenesis as described previously (17) and was confirmed by DNA sequencing. The full-length HBeAg gene was generated by PCR amplification of the repaired HBV genome with a pair of primers (5’-primer (P-A): 5’TAAAGCTTAATCAGGACTTTCCTC-3’, corresponding to the nucleotide sequence 1904–2020 of the HBV genome with an additional HindIII restriction site), and 3’-primer (P-B): 5’TGTCTCTGATGTTTACGAGATGCCTCTCT-3’, corresponding to the nucleotide sequence 2437–2457 of the HBV genome with an additional XhoI and C1a I sites). The truncated HBeAg gene with the...
deletion of the arginine-rich, C-terminus sequence of HBV genome (aa 150–185) that was cleaved during viral infection was generated by PCR amplification with a pair of primers (5′-primer: P-A, 3′-primer: 5′-GTCGACGGCC GCTACGATGAGTGTGATTCCTGGAACTG-3′ corresponding to the nucleotide sequence 2324–2350 of the HBV genome with an additional Nol restriction site). The full-length HBcAg gene was generated by PCR amplification with a pair of primers (5′-primer: 5′-TAAAGCTTAAATTTAGCAGTATTTCTTGAAGTGTCGAAC-3′, corresponding to the nucleotide sequence 1901–1932 of the HBV genome with an additional HindIII restriction site) and the primer P-B. The human IgG Fc fragment was generated by PCR amplification with the plasmid pEF6/ CLL-1 containing human IgG heavy chain cDNA (18) as a template. The pair of primers for the PCR are 5′-primer: 5′-ATAAGGCGGCCGCTA AACCTTACATGCGCA-3′, corresponding to the nucleotide sequence 785–802 of the heavy chain with an additional Nol site and 3′-primer: 5′-TATCTTATGCACTAATTTATCACTCCCAGGAGACGG-3′ (P-C), corresponding to the nucleotide sequence 1447–1468 of the heavy chain with a ClaI site.

A murine retroviral vector, pLNCX-ΔNGFR (nerve growth factor receptor) vector that contains the ΔNGFR marker, was constructed as described previously (19) and used for this study. The retroviral vector HBc-Fc, which expresses the secretory HBc-Fc fusion protein consisting of the truncated HBcAg leader in frame to the IgG Fc, was constructed by a three-piece ligation of the truncated HBc fragment, IgG Fc, and HindIII/ ClaI-cut pLNCX-ΔNGFR (19). The retroviral vector HBcAg, which expresses a secretory HBcAg protein, was constructed by inserting the HBcAg gene into the HindIII/ClaI cut pLNCX-ΔNGFR. The retroviral vector HBcAg, which expresses a cytosolic HBcAg protein, was constructed by inserting the HBcAg gene into the HindIII/ClaI cut pLNCX-ΔNGFR. To construct the vector, the human IgG Fc cDNA fragment was linked with a mouse V, signal leader sequence by two PCR. In the first PCR, the IgG Fc cDNA was used as a template for the amplification with a pair of primers (5′- primer: 5′-GCAGCTTCCAGATGGTCTGCTGCAAAACTCACAATGCCCCACTGTGCCCAGAC-3′, corresponding to the nucleotide sequence 785–815 of the heavy chain and a partial V, leader sequence, and the 3′-primer P-C). The second PCR using the product of the first PCR as a template was conducted with a pair of primers (5′-primer: 5′-TAAAGCTTAAATTTAGCAGTATTTCTTGAAGTGTCGAAC-3′, corresponding to the nucleotide sequence 1901–1932 of the HBV genome with an additional HindIII and Nol restriction site, and the 3′-primer P-C). The Fc cDNA with a leader sequence was cloned into the HindIII/ClaI cut pLNCX-ΔNGFR. These resultant vectors were identified by restriction enzyme analysis and confirmed by DNA sequencing.

Production of retroviral vectors and DC transduction

To produce retroviral vectors, packaging cells (PA317; ATCC) were cultured in 100-mm culture dishes with DMEM containing 10% heat-inactivated FBS, 60 ng mGM-CSF/ml, and 100 U mIL-4/ml for 4 days. For generating DCs, BM cells were cultured in RPMI 1640 supplemented with 6% FBS, 60 ng mGM-CSF/ml, and 100 U mIL-4/ml for 4 days. BM-derived DCs were then cultured in medium containing a mixture of the recombinant HBcAg (100 μg/ml) and HBeAg (100 μg/ml) (American Research Products, Belmont, MA) for an additional 4 days.

CTL assays

For CTL assays, pooled splenocytes from immunized mice were restimulated in vitro in RPMI 1640 containing synthetic peptide HBcAg13–27 (1 μM) for 4–6 days. EL-4 (H-2b) and p815 (H-2b) target cells were incubated with a synthetic peptide, HBeAg13–27 (Chiron), at a concentration of 10 μg/ml overnight and labeled with 150 μCi of sodium 35Cr chromate solution (Amersham International, Arlington Heights, IL) for 90 min. Different numbers of effector cells were incubated with a constant number of target cells (1 × 105) in 96-well V-bottom plates (200 μl/well) for 3 h at 37°C. The supernatants (10 μl) from triplicate cultures were collected. In some experiments, the restimulated effector cell populations were incubated with the anti-CD4 or anti-CD8 Abs (30 μl/well, Pharmingen) for 30 min to deplete CD4+ or CD8+ T cells before CTL assays. The percent lysis was calculated as (experimental release − spontaneous release)/spontaneous release) × 100. Maximum release was determined in parallel by 1% Triton X-100. Spontaneous release was always <5% of the maximum release in the assays.

ELISA

Anti-HBc/Ag Abs in the sera of immunized mice were determined by ELISA. Briefly, microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with a mixture of recombinant HBcAg and HBcAg proteins (each 50 ng/well) were incubated with serially diluted sera in a blocking buffer (KPL, Gaithersburg, MD) at room temperature for 2 h. Bound Ab was detected after incubation with peroxidase or alkaline phosphatase-conjugated Abs against mouse IgG (Sigma, St. Louis, MO), IgG1, IgG2a, or IgG2b (Pharmingen) diluted in the blocking buffer. A polyclonal anti-HBc/Ag Ab (Dako, Carpinteria, CA) was used as a negative control, and non-immunized mouse sera as a negative control. The Ab titer was defined as the highest dilution with OD440 < 0.2. The background OD440 for normal mouse sera was <0.1.

Tumor challenge studies

The tumor cell line EL4 (C57BL/6, H-2b thymoma; ATCC) was transfected with the plasmid pCMV-HBcAg using lipofectin (Life Technologies) and then selected in the presence of 1 mg/ml G418 (Life Technologies) (19). The G418-resistant clones were subcloned and then screened for HBcAg expression by immunoprecipitation and PCR. The EL4-HBcAg cells expressing HBcAg were maintained at 37°C in 5% CO2 in DMEM containing 10% FBS and 1 mg/ml G418. In the tumor protection experiments, C57BL/6 mice were immunized i.p. with 1 × 105 transduced DCs on days 0 and 3, and then intradermally challenged with 3 × 108 exponentially growing EL4-HBcAg cells 1
wk after the first immunization. Tumor sizes were measured every 2–3 days, with tumor volumes calculated as follows: (longest diameter) × (shortest diameter)² (22, 23).

**Western blot analysis**

Murine BM cells were transduced with various recombinant retroviral vectors and differentiated into DCs in vitro as described above. After 4 days of culture with mGM-CSF and mIL-4, a total of 1 × 10⁶ DCs transduced with each construct and their culture media were harvested. The transduced DCs were then lysed with a buffer (10 mM Tris, 150 mM NaCl, pH 7.4, 1% Triton X-100 (Sigma), 0.5 mM PMSF, and protease inhibitor mixture tablets (Boehringer Mannheim, Indianapolis, IN) on ice for 10 min. Cell lysates and culture media were then precipitated with mouse Abs against HBeAg (Chemicon International, Temecula, CA) or Abs against anti-human IgG Fc (Sigma), followed by incubation with protein G-Sepharose (Sigma). The precipitates were then resuspended in 20 μl loading buffer and subjected to Western blot analysis (24). Briefly, protein samples (20 μl) were loaded onto a 10% SDS-PAGE gel and transferred to a Hybond P (Amersham Pharmacia Biotech, Piscataway, NJ), which was blocked by overnight incubation in PBS (pH 7.5) containing 5% nonfat dried milk (Carnation) and 0.1% (v/v) Tween 20 (Fisher Scientific, Pittsburgh, PA) at 4°C. After washing with a buffer (PBS containing 0.1% (v/v) Tween 20), the membrane was incubated with rabbit anti-HBeAg Ab (Dako) or goat anti-human Fc Ab (Sigma) diluted in a PBS buffer containing 2.5% nonfat milk and 0.1% Tween 20 (1:1000) at room temperature for 1 h. After washing, the membrane was incubated with an HRP-labeled anti-rabbit (Amersham Pharmacia Biotech) or anti-goat (Sigma) Ab in the buffer (1:10,000) at room temperature for 1 h. After a final wash, the membrane was visualized with an ECL-Plus chemiluminescent detection kit (Amersham Pharmacia Biotech) and exposed on a Kodak (Rochester, NY) film. Protein band intensity of the Western blot on the film was determined and analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with Image-Quant software 1.2 version.

**Statistical analyses**

All data are presented as the mean and SE. ANOVA was used to determine the levels of differences between groups. Different groups were compared by Student-Newman-Keuls test with SigmaStat 2.03 software (SPSS, Chicago, IL). Values of p were considered significant at 0.05.

**Results**

**Construction and expression of fusion proteins**

DCs, the most potent APCs, express Fc receptors in vivo, which is critical for MHC-II- as well as -I-restricted Ag presentation route for efficient MHC-II- as well as -I-restricted Ag presentation (10–16). Therefore, an IgG Fc fragment was fused with the model HBV nucleocapsid gene to illustrate this strategy (Fig. 1A). Although both HBeAg and HBeAg proteins are encoded by the HBV pre-core/core gene, the secretory HBeAg protein is initiated at a start codon 29 residues upstream of the start codon for HBeAg (25–27). In this study, the arginine-rich amino acid residues (aa 150–180) at the HBeAg C terminus that are cleaved during HBV infection (25–27) were deleted. The truncated HBeAg was fused in frame with the human IgG Fc cDNA gene and then cloned into a retrovector under the CMV promoter control (HBe-Fc) (Fig. 1A) (19). Several control retrovector vectors expressing HBeAg (secretory), HBeAg (cytosolic), or the Fc fragment with a signal sequence (secretory) were constructed and are schematically represented in Fig. 1A. By using radiolabeling and immunoprecipitation/SDS-PAGE (19), it was found that the HBeAg-Fc proteins (HBe-Fc) were efficiently produced and secreted from transfected cells. Both intracellular and secreted HBe-Fc were directly precipitated by protein A beads, indicating that the fusion protein retains its binding ability to protein A (data not shown).

To assess the expression of the HBe-Fc, HBeAg, and HBeAg proteins in DCs, we transduced mouse BM cells in medium supplemented with mSCF and IL-6, using recombinant murine retroviral vectors (LNCX-ΔNGFR) (19) that express HBe-Fc, HBeAg, HBeAg, or Fc (Fig. 1A). The transduced cells were then cultured in medium containing murine IL-4 and GM-CSF for their differentiation into DCs (21, 23, 28–31). After several days of culture, a significant fraction of the cells showed distinct DC morphology. Approximately 32% of the BM-derived cells were consistently transduced with each of these vectors, as determined by flow cytometric assay with an Ab against the NGFR marker (19) (Fig. 1B). The HBe-Fc, HBeAg, HBeAg, or Fc gene in the transduced DCs was transcribed as demonstrated by RT-PCR assays (data not shown). Finally, Western blotting analysis demonstrated that comparable levels of HBe-Fc, HBeAg, and HBeAg proteins were expressed in the transduced DCs, and both HBe-Fc and HBeAg were efficiently secreted (Fig. 1C).

**Priming of naive CD4⁰ T cells by fusion construct in vitro**

First we evaluated whether this receptor-mediated Ag presentation strategy is capable of priming naive murine CD4⁰ T cells in vitro. It was demonstrated that the human IgG can efficiently bind to the Fc receptors on murine APCs (32, 33). Interaction of Fc with FcγRs on DCs triggers cell activation, causing the up-regulation of cell surface molecules involved in Ag presentation (21, 23, 28–31). To evaluate whether the expression of HBe-Fc in the transduced DCs could induce CD activation, we examined surface markers of DCs transduced with HBe-Fc, HBeAg, or HBeAg by flow cytometric assays. As shown in Fig. 2A, higher levels of MHC-II, CD40, and CD86 were expressed on DCs transduced with HBe-Fc and on untransduced DCs in the presence of anti-CD40 than on DCs transduced with HBeAg or HBeAg. This result suggests that the secretion and subsequent interaction of the fusion protein Fc with FcγRs activate DCs.

The transduced DCs were then cocultured with naive CD4⁰ T cells to monitor T cell proliferation and cytokine production. When CD4⁰ T cells were cocultured with the DCs transduced with either HBeAg, HBeAg, or Fc, only low or background levels of GM-CSF and IFN-γ were detected in the culture medium by ELISA, and no apparent T cell proliferation was observed during 2 wk of coculture (Fig. 2B). In contrast, after CD4⁰ T cells were cocultured with the DCs transduced with HBe-Fc for only 5 days, T cells actively proliferated, and high levels of GM-CSF and IFN-γ in the culture medium were detected (Fig. 2B). The primed CD4⁰ T cells responded to HBeAg-pulsed DCs, but not to irrelevant HBsAg-pulsed DCs (data not shown). Repeated experiments showed a similar result. This result suggests that HBe-Fc was more efficiently processed and presented to MHC-II by DCs to prime naive CD4⁰ T cells than HBeAg and HBeAg.

**Role of MHC-II and FcγRs for CD4⁰ T cell priming**

To determine the MHC-II-restricted presentation of HBe-Fc and the role of Fc receptors in this strategy, BM cells from MHC-II KO or FcγRs KO C57BL/6 mice that are deficient in the FcγRI, II, and III genes, or wild-type (WT) C57BL/6 mice were transduced with the retrovector HBe-Fc. The derived DCs were cocultured with naive CD4⁰ T cells from WT mice. High levels of GM-CSF and IFN-γ were detected in the media of the coculture with the transduced WT DCs, but not in the coculture with the transduced MHC-II KO DCs in repeated experiments (Fig. 2C). Similarly, significantly lower levels of IFN-γ were produced in the coculture with the transduced FcγRs KO DCs, as compared with those in the coculture with the transduced WT DCs (Fig. 2D). Collectively, these results suggest that the priming of CD4⁰ Th cells by HBe-Fc DCs is MHC-II dependent and greatly assisted by FcγRs. No apparent CD8⁰ T cell activation was detected in the in vitro coculture with DCs transduced with all these constructs, although CD8⁰ T cells were efficiently induced in the mice immunized with the DCs transduced with HBe-Fc (see below). The failure to prime naive CD8⁰ T cells in this culture condition may...
be because CD4\(^+\) T cells are required for efficient activation of CD8\(^+\) T cells by DCs (15, 34, 35).

Induction of strong Th, CTL, and B cell responses in vivo

Next we evaluated the potency of this strategy to induce immune responses in vivo. C57BL/6 mice were divided into four groups, and each mouse was immunized with one i.p. injection of half a million DCs transduced with HBcAg, HBeAg, Fc, or HBe-Fc. At different times after DC injection, the mice were sacrificed and peripheral blood, spleens, and other tissue samples were collected. Lymph nodes in the vicinity of the injection sites were significantly enlarged in the mice administered with the HBe-Fc DCs, but not in the mice administered with other transduced DCs.

To determine whether Th cell responses are induced, CD4\(^+\) T cells from the immunized mice were cocultured with naive mouse DCs that were pulsed with recombinant HBeAg and HBcAg proteins (American Research Products, Boston, MA). During 2 wk of coculture with different ratios of T cells vs DCs, CD4\(^+\) T cells from the mice immunized with HBeAg-, HBeAg- or Fc-DC did not actively proliferate, and only low levels of IL-2, GM-CSF, and IFN-\(\gamma\) were detected in the coculture media (Fig. 3A). In contrast, in the cocultures with the CD4\(^+\) T cells from the mice immunized with HBe-Fc-DCs, CD4\(^+\) T cells actively proliferated after only 48-h coculture even at a 1:1000 (DCs:T cells) ratio. Furthermore, levels of IL-2, IFN-\(\gamma\), and GM-CSF in the coculture media were significantly higher than those in the cocultures with the CD4\(^+\) T cells from the mice administered HBeAg- or HBeAg-DCs (Fig. 3A). Anti-CD4, but not anti-CD8 Abs, dramatically blocked the production of these cytokines by the cocultured cells (Fig. 3B).

Repeated experiments showed similar results. In addition, an irrelevant Ag, the recombinant HBsAg protein (American Research Products), was used to pulse DCs in parallel with HBe/cAg. The

FIGURE 1. Construction and expression of HBe-Fc fusion proteins. A. Schematic representation of recombinant retroviral vectors. The HBe-Fc fusion gene was cloned into a retroviral vector with a truncated NGFR marker (LNC-\(\Delta\)NGFR) (19). The control HBcAg (cytosolic) gene, HBeAg (secretory) gene, or Fc cDNA fragment with a signal sequence (secretory) was cloned into the retroviral vector under the CMV promoter control. The black square represents the signal sequence. B. Transduction of BM-derived DCs. Murine BM cells in the presence of mSCF and mIL-6 were transduced with different recombinant retrovirus vectors and differentiated into DCs in the presence of mGM-CSF, and mIL-4. On day 8 of in vitro culture, transduced and untransduced BM-derived DCs were stained with an anti-NGFR Ab (Boehringer Mannheim), followed by incubation with a FITC-conjugated anti-mouse IgG (Sigma) on ice for 30–60 min. The cells were then analyzed on a FACScan (Becton Dickinson, Mountain View, CA) and the percentages of the cells positive for the NGFR marker are indicated. C. Expression of different constructs in DCs. Murine BM stem cells were transduced by recombinant retroviruses containing HBe-Fc, HBeAg, HBeAg, or Fc, and differentiated into DCs in vitro. After 4 days of culture with mGM-CSF and mIL-4, a total of 1 \(\times\) 10\(^8\) DCs transduced with each construct and their culture medium were harvested. Cell lysates (C) and culture media (M) were precipitated with mouse Abs against HBcAg/HBeAg (Chemicon) or Abs against anti-human IgG Fc (Sigma), followed by incubation with protein G-sepharose (Sigma). A. Twenty microliters of each precipitate was then used for Western blot analysis and chemiluminescent detection (ECL-Plus; Amersham). HBe-Fc-DC, HBcAg-DC, and HBeAg-DC samples were stained with a rabbit anti-HBc/eAg (Dako), and Fc-DC samples were stained with a goat anti-human Fc Ab. The m.w. of secreted HBeAg was smaller than that of intracellular HBeAg due to the cleavage of the C-terminal amino acid sequence of HBeAg precursor (25–27). B. Protein band intensity of the Western blot was determined and analyzed by a PhosphorImager (Molecular Dynamics) with an Image-Quant software.
FIGURE 2. In vitro priming of naive T cells by HBe-Fc-transduced DCs. A, Surface markers of transduced DCs. BM-derived DCs transduced with each construct were stained for MHC-II (M5/114.15.2), CD40 (HM40-3), and CD86/B7.2 (GL1) (PharMingen) on day 6 of DC culture, and analyzed by FACScan. Nontransduced BM-derived DCs on day 5 of DC culture were incubated with or without an anti-CD40 (2.5 μg/ml; PharMingen) for 24 h and then subjected to flow cytometric assay. Data were prepared with CellQuest software (Becton Dickinson). The transduced DCs were directly stained with a second Ab conjugate as a negative control. B, In vitro priming of naive CD4+ T cells by HBe-Fc-transduced DCs. DCs transduced with HBe-Fc, HBeAg, HBcAg, or Fc were cocultured with naive CD4+ T cells isolated from C57BL/6 mice at a ratio of 1:100. The concentrations of GM-CSF and IFN-γ in the media on day 6 of coculture are shown. Data represent the mean ± SE of triplicate samples from one of four independent experiments. p < 0.05, HBe-Fc compared with HBeAg, HBcAg, or Fc. C, MHC-II-dependent CD4+ T cell priming. BM cells from five MHC-II-KO or WT C57BL/6 mice were transduced with the recombinant retrovirus vector HBe-Fc, and the transduced BM-derived DCs were cocultured with naive CD4+ T cells isolated from WT mice at a ratio of 1:100. Kinetics of GM-CSF and IFN-γ concentrations in the coculture media is shown. Each curve represents the mean ± SE of triplicate samples from one of two independent experiments, p < 0.05. D, FcγRs in priming of naive CD4+ T cells. BM cells from five FcγRs KO or WT C57BL/6 mice were transduced with the recombinant retroviruses HBe-Fc. DCs derived from the transduced BM cells were cocultured with naive CD4+ T cells isolated from WT mice at a ratio of 1:100 in triplicate. The levels of IFN-γ in the media on day 5 of coculture were determined, and data represent the mean ± SE of two independent experiments, p < 0.05.
FIGURE 3. A–C. In vivo induction of CD4^{+} T cell responses. CD4^{+} T cells were isolated from pooled splenocytes of five to six mice (each group) 4 mo after immunization by using a column (R&D Systems). The cells were then cocultured in duplicate with HBe/cAg-pulsed DCs at a ratio of 1000:1 (T:DC), and the concentrations of IL-2, IFN-\( \gamma \), GM-CSF, IL-4, and IL-5 in the media were determined by ELISA after 2 days of coculture (A), \( p < 0.05 \), HBe-Fc compared with HBeAg, HBcAg, or Fc. The CD4^{+} T cells isolated from the pooled splenocytes of six mice immunized with HBe-Fc-DCs were also cocultured in triplicate with the HBe/cAg-pulsed DCs at a ratio of 1000:1 (T:DC) in the presence of an anti-CD4 or anti-CD8 Ab (30 \( \mu \text{l/well; PharMingen} \) for 2 days (B), or with DCs pulsed with recombinant HBsAg proteins (200 \( \mu \text{g/ml} \)) for 2 days (C). Cytokine levels in the culture media were determined. Data represent the mean ± SE of two independent assays of one representative experiment of three using five to six mice per group. D. T cell responses induced by DCs cocultured with supernatants of transduced DCs. Nontransduced mouse DCs were cultured in the culture medium of HBe-Fc-tranduced DCs (HBe-Fc-TDC) for 4 days. The cultured DCs (HBe-Fc-TDC-supernatant-DCs), and transduced DCs (HBe-Fc-TDC) (\( 5 \times 10^{5} \) per mouse) were then administered IP into mice. Four to 6 wk later, CD4^{+} T cells isolated from the mouse splenocytes were cocultured with HBe/cAg-pulsed DCs. On day 2, the levels of IFN-\( \gamma \) in media were measured.
FIGURE 4. In vivo induction of CTL responses. Pooled splenocytes taken from six to eight mice per group 8 mo after immunization were restimulated in vitro with a low concentration synthetic peptide HBcAg13-27 for 4 days. The restimulated splenocytes (E) were cocultured for 3 h with the 51Cr-labeled target cells, EL-4 or p815 (T), which were pulsed with the HBcAg13-27. Percentages of target cell killing by the splenocytes from different immunized mice are shown (A), $p < 0.05$, HBe-Fc compared with HBeAg, HBcAg, or Fc. Percentages of cell killing by the splenocytes from the mice immunized with HBe-Fc-DCs in the presence of the anti-CD4 or -CD8 Ab (30 μl/well; PharMingen) or culture medium control are also shown (B). Data represent the mean ± SE of triplicate samples from one representative experiment of three using six to eight mice per group.
reduce strong CTL responses, a chromium release cytotoxic assay specific T cells in the mice had differentiated past the Th0 stage. mice were detected (data not shown), suggesting that the HBe/cAg-DCs for 4 days and then administered into mice. Four to 6 wk later, DCs were cultured in the culture medium of HBe-Fc-tranduced transduced DCs could be uptaken by other DCs. Nontransduced transduced with HBe-Fc can more efficiently activate Th cells, HBe-Fc-DCs immunization. These results indicate that the DCs shown that the cocultured, nontransduced DCs induced CD4+ T cell responses, but were much less potent than HBe-Fc-transduced DCs (Fig. 3D). The superior ability of transduced DCs is likely due to the enhanced Th and the direct MHC-I presentation of internalized HBe-Fc by DCs (10–16).

To determine whether HBe-Fc-DC immunization can induce Ab responses, we measured anti-HBe/cAg Ab titers in the pooled sera of mice immunized with different vectors. As shown in Fig. 5, anti-HBe/cAg Abs were detected in the sera of mice as early as 2 wk after HBe-Fc-DC immunization and persisted for at least 6 months, indicating that a long-lasting Ab response was induced by the HBe-Fc-DC immunization. The specificity of the Ab responses was demonstrated by the lack of Ab against HBsAg in the immunized mice (data not shown). The IgG subtypes in the immunized mice were of the IgG1 and IgG 2a classes (data not shown), in agreement with the dual Th1 and Th2 helper cytokine profiles (Fig. 3A). By contrast, significantly lower Ab titers were detected in mice immunized with HBeAg- or HBcAg-DCs (Fig. 5). Taken together, we conclude that HBe-Fc is significantly superior to the native HBeAg and HBcAg in inducing CD4+ helper and CD8+ cytotoxic T cell, as well as B cell, responses.

HBsAg-pulsed DCs were unable to stimulate the CD4+ T cells of HBe-Fc-DC-immunized mice in the described assay (Fig. 3C), demonstrating the specificity of CD4+ T cell responses induced by HBe-Fc-DCs immunization. These results indicate that the DCs transduced with HBe-Fc can more efficiently activate Th cells, especially Th1 (36, 37) than can the native Ags.

We also assessed whether the fusion proteins secreted from transduced DCs could be uptaken by other DCs. Nontransduced DCs were cultured in the culture medium of HBe-Fc-tranduced DCs for 4 days and then administered into mice. Four to 6 wk later, CD4+ responses of the immunized mice were monitored. It was shown that the cocultured, nontransduced DCs induced CD4+ T cell responses, but were much less potent than HBe-Fc-transduced DCs (Fig. 3D). The superior ability of transduced DCs is likely due to the continuous Ag production and presentation by DCs in an autocrine and paracrine manner. Interestingly, IL-4 and IL-5 were also detected in the cocultures with the T cells from the mice with HBe-Fc-DCs, although their levels were significantly lower than IL-2 and IFN-γ. Because IL-2 and IFN-γ are mainly produced by Th1 cells, and IL-4 and IL-5 by Th2 (36, 37), the results suggest that HBe-Fc-DCs can induce both Th1 and Th2 responses. Although a Th0 response could also produce the dual Th1 and Th2 cytokine profile, primed CD4+ T cell responses in the immunized mice were detected (data not shown), suggesting that the HBe/cAg-specific T cells in the mice had differentiated past the Th0 stage.

To determine whether immunization with HBe-Fc-DCs can induce strong CTL responses, a chromium release cytotoxic assay was performed. Splenocytes from different immunized mice were restimulated in vitro for 4–6 days in medium containing synthetic peptide HBcAg13-27 and then cocultivated with radiolabeled peptide (HBcAg13-27)-pulsated target cells EL-4 (H-2b) and p815 (H-2b) at varied E:T ratios to measure target cell lysis (17). As shown in Fig. 4A, splenocytes from mice immunized with HBe-Fc-DCs demonstrated significantly higher target cell killing than those from mice immunized with HBeAg or HBcAg. The specificity of the killing was demonstrated by the inability of the splenocytes to kill HBcAg-pulsed p815 target cells with an H-2b background and unpulsed EL-4 cells (not shown) and the inhibition of the killing by the anti-CD8, but not anti-CD4 Ab (Fig. 4B). Furthermore, HBsAg was also used to restimulate splenocytes from HBe-Fc-DC-immunized mice, and no significant killing to HBcAg-pulsed target cells was observed by the HBsAg-restimulated splenocytes (data not shown). The superior CTL responses induced by HBe-Fc-DCs may be due to the enhanced Th and the direct MHC-I presentation of internalized HBe-Fc by DCs (10–16).

Protective immunity induced by fusion construct immunization
As there is no HBV animal model readily available to investigate the ability of HBe-Fc to confer protection against HBV infection, we have developed a tumor challenge model that allows us to investigate the ability of the anti-HBV immune responses induced by HBe-Fc to control HBV Ag-bearing cells in vivo. Because both humoral and cellular immunity are typically involved in tumor rejection (1, 38), an immunization strategy that can elicit immune responses against a tumor-borne Ag would in effect establish a protective in vivo immune response. We used a common tumor cell line (EL-4) that grows rapidly in syngeneic mice (23, 39) as the target cell line for transfection and challenge experiments. EL4 clones transfected with the HBcAg expression vector were generated and shown to express HBcAg by PCR and immunoprecipitation assays (data not shown).

The tumor growth potential of EL4-HBcAg cells was assessed by intradermal implantation of 3 × 106 cells into syngeneic C57BL/6 mice. EL4-HBcAg cells showed aggressive tumor growth similar to WT EL-4 cells, producing visible tumors in mice only 3 days after inoculation and resulting in mouse death usually within 3 wk after inoculation. To test the ability of fusion construct immunization to protect these mice from EL4-HBcAg tumor growth, we immunized mice i.v. twice (3-day interval) with 1 × 105 DCs transduced with HBe-Fc, HBeAg, HBcAg, or Fc, before challenging them with the EL4-HBcAg cells (3 × 105). As shown in Fig. 6, A and B, tumor growth was inhibited to a much greater
extent in mice immunized with HBe-Fc-DCs, although immunization with the HBeAg-DCs or HBcAg-DCs also did confer some degree of protection. The mice immunized with HBe-Fc-DCs also survived longer (Fig. 6C). In the HBe-Fc-immunized group, 33.3% mice (8 of 24 total immunized mice) were tumor free and completely protected. Although 66.7% mice still got tumors, they survived longer in comparison with mice immunized with other constructs. All mice (100%) that were immunized with HBeAg-DCs (24 mice), HBcAg-DCs (22 mice), Fc-DCs (22 mice), or PBS (20 mice) got tumors and were dead within 1 month after the tumor challenge. The potency of the antitumor activity shown by these constructs correlated with their abilities to induce immune responses (Figs. 3 and 4). The antitumor activity induced by the HBe-Fc-DCs was specific because HBe-Fc-DC-immunized mice challenged with WT EL4 cells also developed lethal tumors and died within 3 wk (data not shown). The ability of HBe-Fc-DCs to partially inhibit the growth of established EL4-HBcAg tumors in mice was also observed (data not shown), despite that this aggressive tumor model may not allow sufficient response time of the immune system to efficiently control the rapid, lethal tumor growth.

**Discussion**

Inadequate Ag presentation by APCs contributes to the failure of the human immune system to mount effective immune responses against chronic infection and tumors (1–4, 38, 40). Accumulating evidence indicates that a vaccine or immunotherapy, which can induce combined CD4+ and CD8+ T cell and B cell immune responses, is likely the most effective one to prevent or control chronic infections such as HIV-1, hepatitis virus infection, or *Mycobacterium tuberculosis*, and tumors (15, 34, 35, 38, 40–42). The results of this study demonstrate that this receptor-mediated Ag presentation strategy, which uses a unifying mechanism to efficiently present Ags to both MHC-I and -II, potently activates Ag-specific Th cells, CTLs, and B cells. Thus, the receptor-mediated Ag presentation strategy with the ability to induce all arms of the adaptive immunity may have broad applications for the treatment and prevention of cancer, infection, and even autoimmune diseases (43).

Many existing vaccines, except live, attenuated vaccines, lack an efficient Ag presentation mechanism to induce potent CD8+ CTL and CD4+ Th responses (44–52). DCs are the most potent
APCs for initiating primary and secondary immune responses (1). Thus, for vaccines or immunotherapies to be effective, Ags must be acquired and displayed by DCs. Many investigators have tried to use the potential efficacy of DCs to develop effective immunotherapies and vaccines. For example, several groups have demonstrated that DCs pulsed with peptides from tumor-associated Ags (TAA) can induce Ag-specific immune responses in vivo in murine tumor models (1, 2). However, the efficacy of peptide-pulsed DCs would be limited in vivo, because peptides pulsed onto DCs stay bound to the MHC molecules only transiently. Moreover, the use of peptide-pulsed DCs is dependent on the knowledge of the HLA haplotypes of the patients and the restriction element of the peptide epitopes for any particular Ag. Subsequently, many investigators have transduced Ag genes into DCs, which allow the constitutive expression of the Ag proteins leading to prolonged Ag presentation of multiple or unidentified epitopes in the context of MHC (44, 45). Because the Ag-presenting pathway to MHC-I is distinctively different from that to MHC-II, it is difficult for an Ag to be presented to both MHC-I and MHC-II by DCs. For example, an intracellular Ag expressed by transduced DCs can be efficiently processed and presented to MHC-I, but not to MHC-II. A secretory protein expressed from transduced DCs cannot be efficiently presented to MHC-I. Thus, developing a strategy for DCs to present an Ag to both MHC-I and -II may lead to more effective immunotherapies and vaccines, because Th cells play a central role for the activation of CTLs, B cells, NK cells, and macrophages (1–4, 49–52).

There have been attempts to facilitate MHC-II-restricted Ag presentation, for example, using a sequence derived from the lysosomal transmembrane proteins to target Ags to the endosomal pathway (53). However, our strategy, which efficiently activates not only Th cells, but also cytotoxic T cells and B cells, has unique and superior features. First, by using the receptor-mediated endocytosis pathway, fusion proteins can be efficiently captured, processed, and presented to MHC-II by DCs in both autocrine and paracrine modes to vigorously induce Th cells. Interestingly, dual Th1 and Th2 responses, which have been shown to collaborate in directing an effective antitumor response (49–52), can be induced by this strategy. Moreover, high levels of cytokines produced by primed Th cells can be directly responsible for the control of viral infection and tumor growth (49–52, 54). Second, this strategy can efficiently induce CTLs, because FcγR-mediated internalization can directly present internalized Ags to MHC-II (cross-primming) as well as activate DCs (5–16). Third, fusion constructs can elicit strong Ag responses because of the efficient protein secretion from transduced cells and enhanced Th (55, 56). Fourth, this strategy should be superior to transient peptide-pulse DC strategies because transduced DCs can continuously produce, as well as process, Ags. Finally, this strategy is versatile because of its adaptability for use with any Ag or many cell-binding domains and for incorporation into the design of almost any vaccine and immunotherapy. Indeed, this strategy has been shown to significantly enhance the potency of DNA vaccines to induce immune responses (Z. You, X. F. Huang, J. Hester, and S.-Y. Chen, unpublished data). Thus, the receptor-mediated Ag presentation strategy may provide a generic and powerful means for the development of effective immunotherapies, and therapeutic and preventive vaccines.

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References


