The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2000

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J Immunol (2000) 165 (8): 4581–4591.
https://doi.org/10.4049/jimmunol.165.8.4581

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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[1]

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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 an Ag for presentation 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.**

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The adaptive immunity is maintained by cellular and humoral immune responses that are initiated and modulated by professional APCs such as dendritic cells (DCs)[3] (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/HBcAg 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′-TTAAGCTTATGCAACTTTTTCACCTCT GCCTAATC-3′, corresponding to the nucleotide sequence 1904–2020 of the HBV genome with an additional *Hin*dIII restriction site, and 3′-primer (P-B): 5′-TTTCTAGAATCGATTAACATTGAGATTCCCGAGA-3′, corresponding to the nucleotide sequence 2437–2457 of the HBV genome with additional *Xba*I and C1a I sites). The truncated HBeAg gene with the

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Received for publication April 4, 2000. Accepted for publication July 28, 2000.

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[1] This work was supported by National Institutes of Health Grants AI41959 and RR13272 and a North Carolina Baptist Hospital Technology Developmental Award.

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[3] Abbreviations used in this paper: DC, dendritic cell; FcγRs, IgG Fc receptors; MHC-I, MHC class I; MHC-II, MHC class II; HBV, hepatitis B virus; BM, bone marrow; mSCF, murine stem cell factor; KO, knockout; NGFR, nerve growth factor receptor; WT, wild type.

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0022-1767/00/$02.00

deletion of the arginine-rich, C′-terminal sequence of HBeAg (aa 150–185) that is cleaved during viral infection was generated by PCR amplification with a pair of primers (5′-primer: P-A; 3′-primer: 5′-GTGCGGCCG CTCTAACAACAGTAGTTTCCGGAAGTGT-3′, corresponding to the nucleotide sequence 2324–2350 of the HBV genome with an additional *Not*I restriction site). The full-length HBcAg gene was generated by PCR amplification with a pair of primers (5′-primer: 5′-TTAAGCTTATGGA CATTGACCCTTATAAAGAATTTGGAGC-3′, corresponding to the nucleotide sequence 1901–1932 of the HBV genome with an additional *Hin*dIII restriction site and the primer P-B). The human IgG cDNA Fc fragment was generated by PCR amplification with the plasmid pEE6/CLL-1 containing human IgG heavy chain cDNA (18) as a template. The pair of primers for the PCR are 5′-primer: 5′-ATAAGCGGCCGCTA AAACTCACACATGCCCA-3′, corresponding to the nucleotide sequence 785–802 of the heavy chain with an additional *Not*I site and 3′-primer: 5′-TATTCTAGATCGATCACTCATTTACCCGGAGACAGG-3′ (P-C), corresponding to the nucleotide sequence 1447–1468 of the heavy chain with a *Cla*I 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 HBe-Fc, which expresses the secretory HBe-Fc fusion protein consisting of the truncated HBeAg fused in frame to the IgG Fc, was constructed by a three-piece ligation of the truncated HBe fragment, IgG Fc, and *Hin*dIII/*Cla*I-cut pLNCX-ΔNGFR (19). The retroviral vector HBeAg, which expresses a secretory HBeAg protein, was constructed by inserting the *HBeAg* gene into the *Hin*dIII/*Cla*I cut pLNCX-ΔNGFR. The retroviral vector HBcAg, which expresses a cytosolic HBcAg protein, was constructed by inserting the *HBcAg* gene into the *Hin*dIII/*Cla*I cut pLNCX-ΔNGFR. To construct the IgG Fc expression vector, the human IgG Fc cDNA fragment was linked with a mouse V_H signal leader sequence by two PCRs. In the first PCR, the IgG Fc cDNA was used as a template for the amplification with a pair of primers (5′ primer, 5′GCAGCTCCCAGATG GGTCCTGTCCAAAACTCACACATGCCCACCGTGCCCAGCAC-3′, corresponding to the nucleotide sequence 785–815 of the heavy chain and a partial V_H 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′-TTAAGCTTCATATGGGAA CATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGATGGGTC CTGTCC-3′, corresponding to the N-terminal nucleotide sequence of the V_H leader sequence with additional *Hin*dIII and *Nde*I sites, and the 3′ primer P-C). The Fc cDNA with a leader sequence was cloned into the *Hin*dIII/*Cla*I 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 (Life Technologies, Grand Island, NY) and transfected with 10–15 μg of retroviral vector plasmids (prepared by endotoxin-free kits; Qiagen, Chatsworth, CA) by lipofectin (Life Technologies). After overnight incubation, the medium was replaced with DMEM containing 5% FBS. Forty-eight hours later, the culture medium containing recombinant retroviruses was harvested and filtered (0.45 μm), as described previously (20). To generate DCs, bone marrow (BM) cells were flushed from the bones of mouse limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI 1640, cells were incubated with rabbit complements (Calbiochem, La Jolla, CA), and a mixture of mAbs consisting of anti-CD4, anti-CD8, anti-CD45R/B220, and anti-MHC-II (PharMingen, San Diego, CA and BioSource International, Camarillo, CA) in RPMI 1640 at 37°C for 40–60 min. After extensive washing with RPMI 1640, cells (5×10^5 cells/ml) in RPMI 1640 supplemented with 6% FBS, 80 ng murine stem cell factor (mSCF)/ml (R&D Systems, Minneapolis, MN), and 20 U mIL-6/ml (BioSource International) were plated in 12-well culture plates (2.5 ml/well), incubated at 37°C, 5% CO_2 overnight, and then refed with fresh medium. After a 48-h incubation, the cells were spun down, resuspended in 1.5 ml of the retrovirus supernatants, and placed onto 24-well culture plates coated with Retronectin (Panvera, Madison, WI) at a concentration of 10–20 ng/ml. The cells were incubated at 37°C, 5% CO_2 for 3–4 h. The supernatants were then replaced with 1.5 ml of RPMI 1640 supplemented with 5% FBS, 10 ng mSCF/ml, 60 ng mGM-CSF/ml (BioSource International), and 100 U mIL-4/ml (R&D Systems) overnight. The transduction procedure was repeated 2–3 times. After the final transduction, the cells were washed and cultured in Opti-MEM (Life Technologies) containing mGM-CSF and mIL-4 for several days to allow further DC differentiation. DCs were further enriched by using a 50% FCS-RPMI 1640 sedimentation procedure as described previously (21).

Mice and in vitro T cell analyses

C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) or Taconic Farms (Germantown, NY). MHC-II knockout (KO) and IgG Fc receptors (FcγRs) KO C57BL/6 mice were obtained from Taconic Farms. Mice were housed in animal facilities of the institution, and all animal experiments were performed according to an animal protocol approved by Institutional Animal Care and Use Committee. $CD4^+$ or $CD8^+$ T cells were isolated from pooled mouse spleen suspension by using a $CD4^+$ or $CD8^+$ T cell enrichment column (R&D Systems). Isolated $CD4^+$ or $CD8^+$ T cells were cultured for 24–48 h in RPMI 1640 supplemented with 10% FBS and plated in duplicates (2–5 $\times 10^5$ cells/well) in 96-well plates. The transduced BM-derived DCs were added to the wells of T cells at different ratios. After several days of coculture, the cell proliferation was observed, and culture medium was collected for determination of various cytokine concentrations by ELISA (R&D Systems).

Analysis of primed T cells

C57BL/6 mice were injected i.p. with 5×10^5 of the transduced DCs in 0.2 ml PBS containing 50,000 U IL-2 (Chiron Therapeutics, Emeryville, CA) per mouse. At different times after immunization, mice were sacrificed and peripheral blood, spleens, and other organs were collected. $CD4^+$ or $CD8^+$ T cells were isolated from spleen suspensions using $CD4^+$ or $CD8^+$ T cell-enriched columns (R&D Systems) and were cultured in RPMI 1640 supplemented with 10% FBS for 24–48 h before coculture with Ag-pulsed DCs. 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 HBeAg (100 μg/ml) and HBcAg (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-2^b) and p815 (H-2^d) target cells were incubated with a synthetic peptide, HBcAg13–27 (Chiron), at a concentration of 10 μg/ml overnight and labeled with 150 μCi of sodium ^{51}Cr chromate solution (Amersham International, Arlington Heights, IL) for 90 min. Different numbers of effector cells were incubated with a constant number of target cells (1×10^4/well) in 96-well V-bottom plates (200 μl/well) for 3 h at 37°C. The supernatants (100 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 defined as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum release was determined by cell lysis by 1% Triton X-100. Spontaneous release was always $<$5% of the maximum release in the assays.

ELISA

Anti-HBc/eAg Abs in the sera of immunized mice were determined by ELISA. Briefly, microtiter plates (Dynatech Laboratories, Chantilly, VA) coated with a mixture of recombinant HBeAg 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/eAg Ab (Dako, Carpinteria, CA) was used as a positive control, and nonimmunized mouse sera as a negative control. The Ab titer was defined as the highest dilution with an OD_{A450} or OD_{A405} of $>$0.2. The background OD_{A450} or OD_{A405} of 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% CO_2 in DMEM containing 10% heat-inactivated horse serum and 1 mg/ml G418. In the tumor protection experiments, C57BL/6 mice were immunized by i.v. injection with 1×10^5 transduced DCs on days 0 and 3, and then intradermally challenged with 3×10^6 exponentially growing EL4-HBcAg cells 1

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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 1×10^8 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 HBc/eAg (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 polyvinylidene difluoride membrane (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-HBc/eAg 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 then 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 FcγRs, which mediate a privileged Ag internalization 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. 1*A*). Although both HBcAg and HBeAg 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 HBcAg (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 retroviral vector under the CMV promoter control (HBe-Fc) (Fig. 1*A*) (19). Several control retroviral vectors expressing HBeAg (secretory), HBcAg (cytosolic), or the Fc fragment with a signal sequence (secretory) were constructed and are schematically represented in Fig. 1*A*. 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, HBcAg, 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, HBcAg, or Fc (Fig. 1*A*). 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. 1*B*). The HBe-Fc, HBeAg, HBcAg, 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, HBcAg, and HBeAg proteins were expressed in the transduced DCs, and both HBe-Fc and HBeAg were efficiently secreted (Fig. 1*C*).

Priming of naive CD4$^+$ T cells by fusion construct in vitro

First we evaluated whether this receptor-mediated Ag presentation strategy is capable of priming naive murine CD4$^+$ 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 DC activation, we examined surface markers of DCs transduced with HBe-Fc, HBcAg, or HBeAg by flow cytometric assays. As shown in Fig. 2*A*, 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 HBcAg or HBeAg. This result suggests that the secretion and subsequent interaction of the fusion protein Fc with FcγR activate DCs.

The transduced DCs were then cocultured with naive CD4$^+$ T cells to monitor T cell proliferation and cytokine production. When CD4$^+$ T cells were cocultured with the DCs transduced with either HBcAg, 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. 2*B*). In contrast, after CD4$^+$ 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. 2*B*). The primed CD4$^+$ 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 CD4$^+$ T cells than HBeAg and HBcAg.

Role of MHC-II and FcγRs for CD4$^+$ 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 Fcγ RI, II, and III genes, or wild-type (WT) C57BL/6 mice were transduced with the retroviral vector HBe-Fc. The derived DCs were cocultured with naive CD4$^+$ 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. 2*C*). 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. 2*D*). Collectively, these results suggest that the priming of CD4$^+$ Th cells by HBe-Fc DCs is MHC-II dependent and greatly assisted by FcγRs. No apparent CD8$^+$ T cell activation was detected in the in vitro coculture with DCs transduced with all these constructs, although CD8$^+$ T cells were efficiently induced in the mice immunized with the DCs transduced with HBe-Fc (see below). The failure to prime naive CD8$^+$ T cells in this culture condition may

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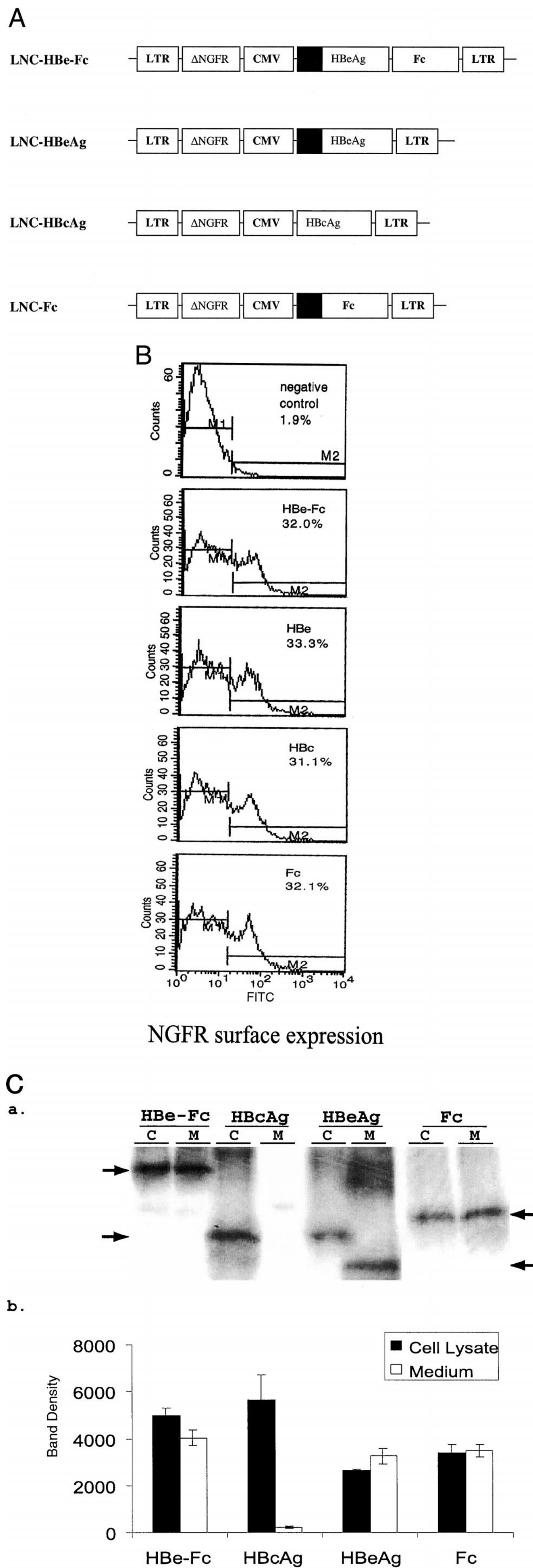

FIGURE 1. Construction and expression of HBe-Fc fusion proteins. *A*, Schematic representation of recombinant retroviral vectors. The HBe-Fc fusion gene was cloned into a retroviral vector with a truncated NGFR marker (LNC-ΔNGFR) (19). The control HBcAg (cytosolic) gene, HBeAg (secretory) gene, or Fc 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, HBcAg, or Fc, and differentiated into DCs in vitro. After 4 days of culture with mGM-CSF and mIL-4, a total of 1×10^8 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.

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-, HBcAg- or Fc-DC did not actively proliferate, and only low levels of IL-2, GM-CSF, and IFN-γ were detected in the coculture media (Fig. 3*A*). 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-γ, 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 HBcAg-DCs (Fig. 3*A*). Anti-CD4, but not anti-CD8 Abs, dramatically blocked the production of these cytokines by the cocultured cells (Fig. 3*B*). 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

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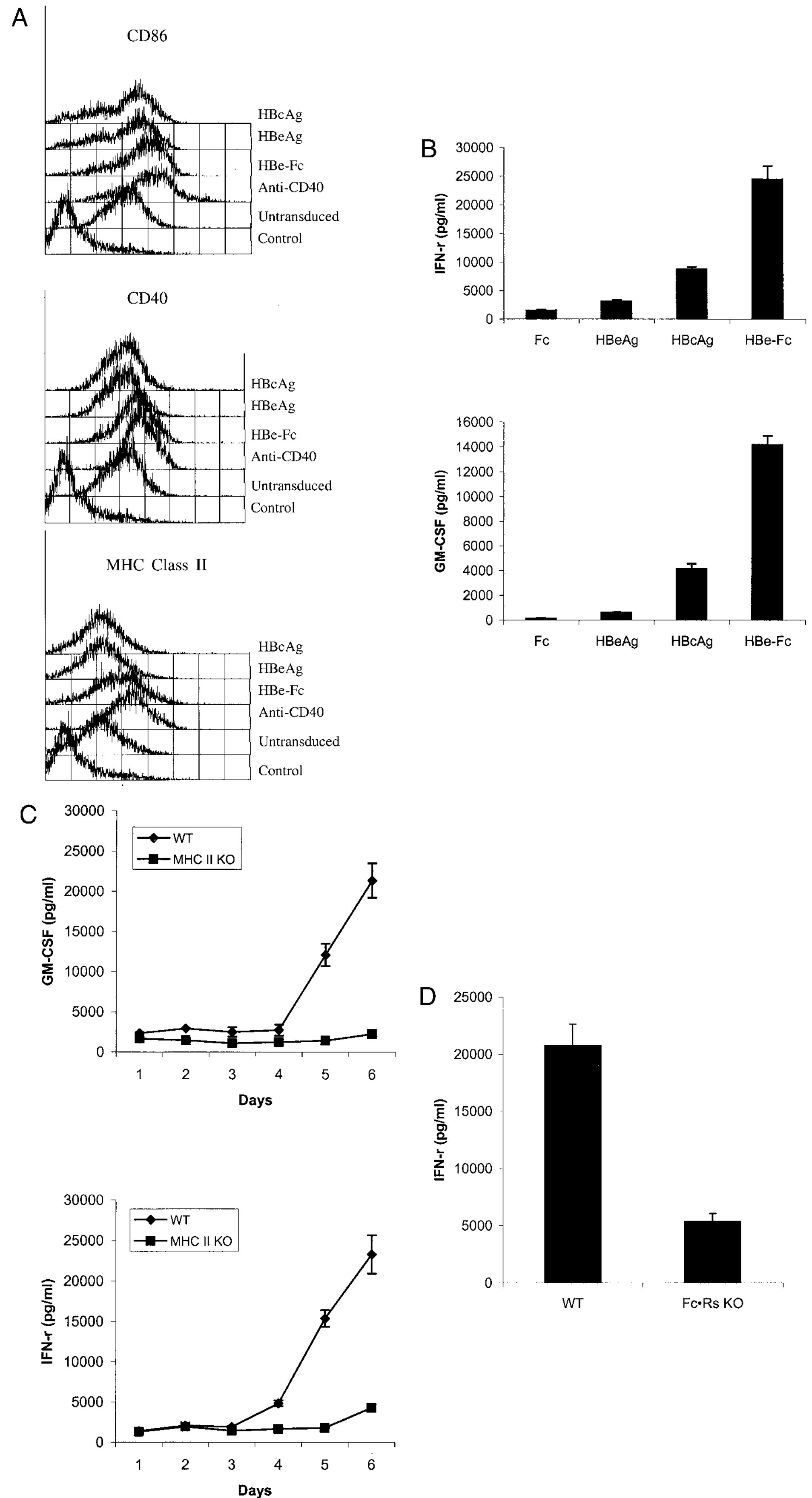

FIGURE 2. In vitro priming of naive T cells by HBe-Fc-transduced DCs. *A*, Surface markers of transduced DCs. BM-derived DCs transduced with each construct were stained for MHC-II (M5/114.15.2), CD40 (HM40-3), and CD86/B7.2 (GL1) (PharMingen) on day 6 of DC culture, and analyzed by FACScan. Nontransduced 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 mouse spleens 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 from WT mice (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 from WT mice (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, $p < 0.05$.

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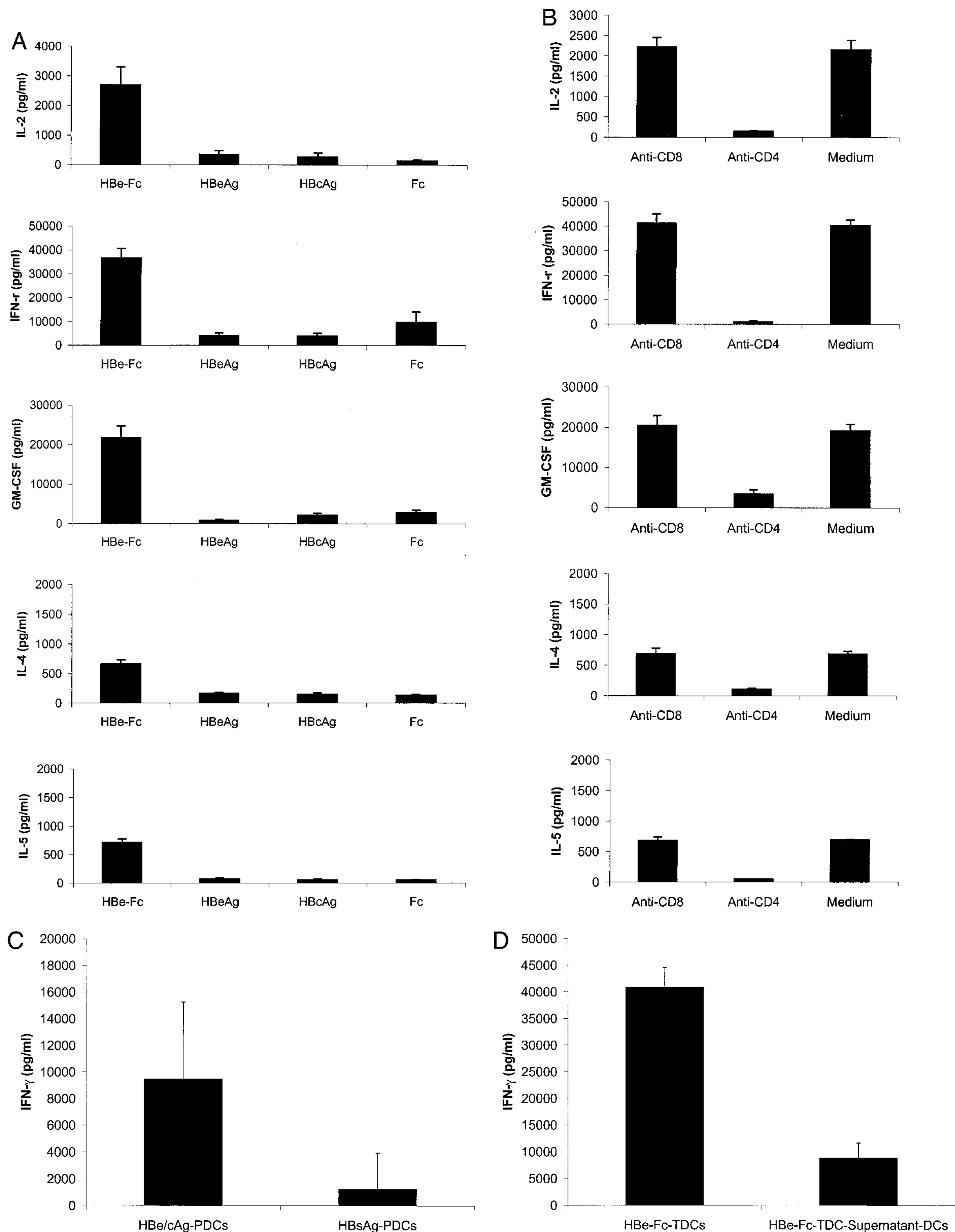

FIGURE 3. *A–C*, In vivo induction of $CD4^+$ T cell responses. $CD4^+$ T cells were isolated from pooled splenocytes of five to six mice (each group) 4 mo after immunization by using a column (R&D Systems). The cells were then cocultured in duplicate with HBe/cAg-pulsed DCs at a ratio of 1000:1 (T:DC), and the concentrations of IL-2, IFN-γ, 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 μl/well; PharMingen) for 2 days (*B*), or with DCs pulsed with recombinant HBsAg proteins (200 μg/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) (5×10^5 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-γ in media were measured.

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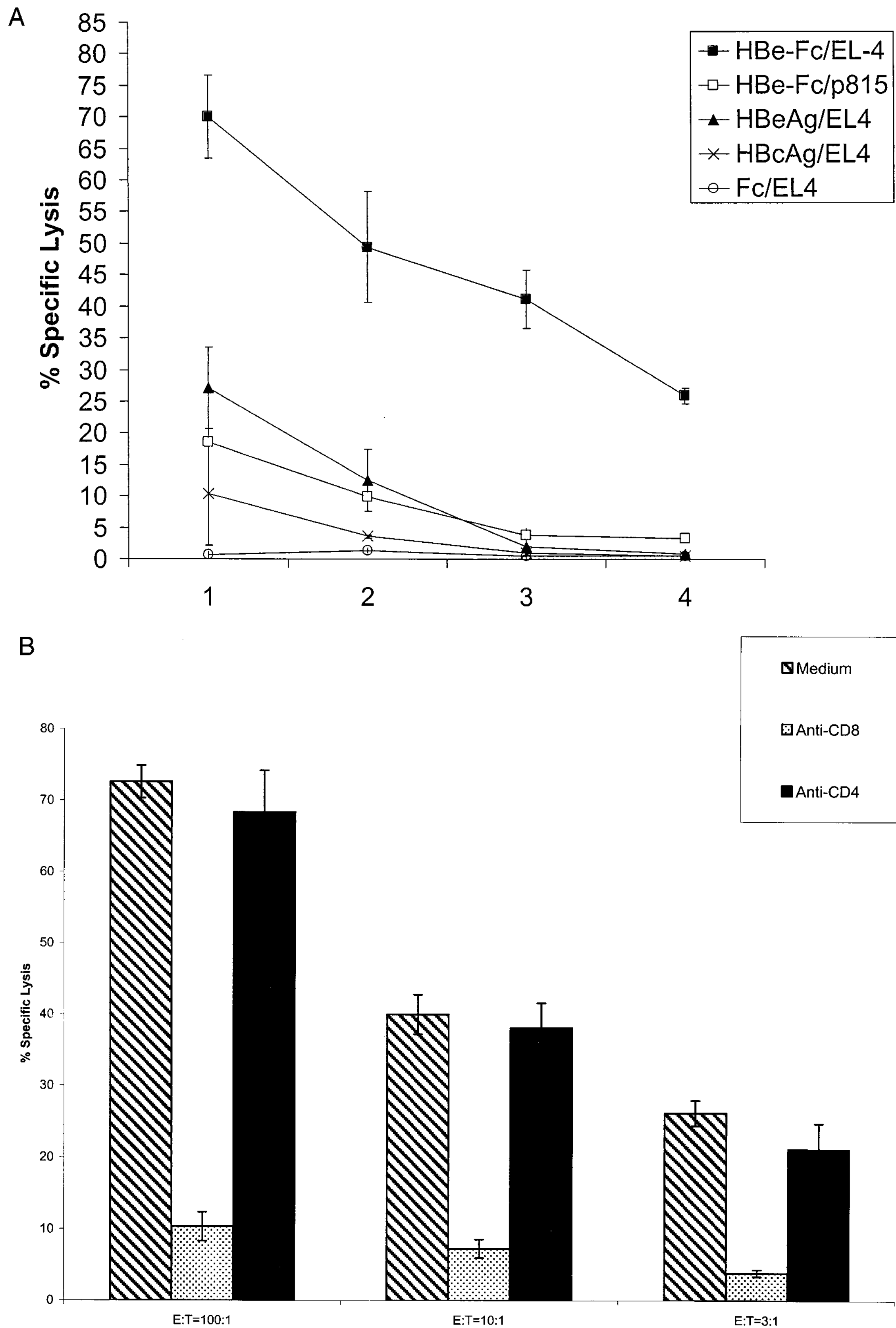

FIGURE 4. In vivo induction of CTL responses. Pooled splenocytes taken from six to eight mice per group 8 mo after immunization were restimulated in vitro with a low concentration synthetic peptide HBcAg13-27 for 4 days. The restimulated splenocytes (E) were cocultured for 3 h with the ^{51}Cr-labeled target cells, EL-4 or p815 (T), which were pulsed with the peptide $HBcAg_{13-27}$. 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.

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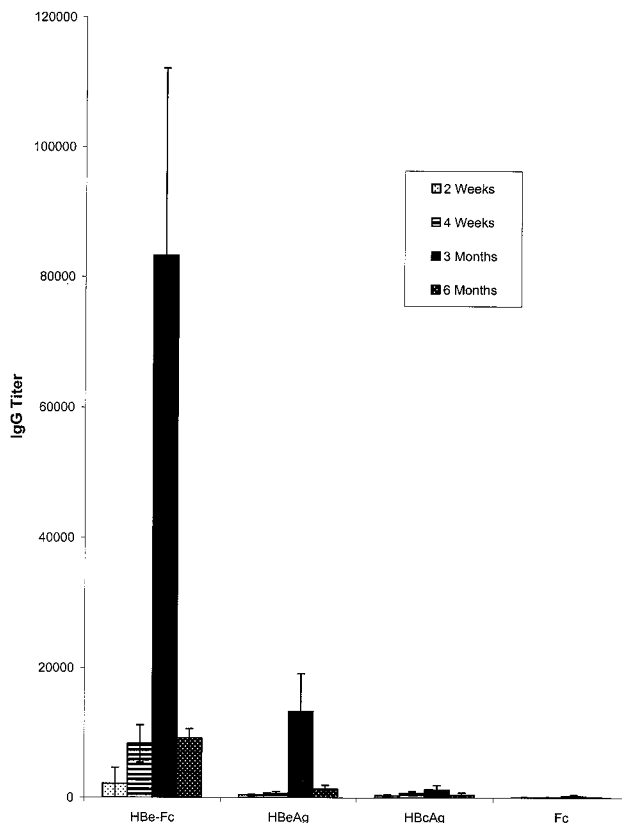

FIGURE 5. Induction of high-titer, long-lasting Ab responses. The titers of HBe/cAg-specific IgG Abs from the pooled sera of six to eight immunized mice per group at different times after DC immunization were determined by ELISA. Data represent the mean ± SE of three independent experiments using five to six mice per group, $p < 0.05$, HBe-Fc compared with HBeAg, HBcAg, or Fc.

HBsAg-pulsed DCs were unable to stimulate the $CD4^+$ T cells of HBe-Fc-DC-immunized mice in the described assay (Fig. 3*C*), 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. 3*D*). 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)-pulsed target cells EL-4 (H-2^b) and p815 (H-2^d) at varied E:T ratios to measure target cell lysis (17). As shown in Fig. 4*A*, 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-2^d background and unpulsed EL-4 cells (not shown) and the inhibition of the killing by the anti-CD8, but not anti-CD4 Ab (Fig. 4*B*). 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).

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 IgG_1 and IgG_{2a} classes (data not shown), in agreement with the dual Th1 and Th2 helper cytokine profiles (Fig. 3*A*). 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.

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 3×10^6 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 1×10^5 DCs transduced with HBe-Fc, HBeAg, HBcAg, or Fc, before challenging them with the EL4-HBcAg cells (3×10^6). As shown in Fig. 6, *A* and *B*, tumor growth was inhibited to a much greater

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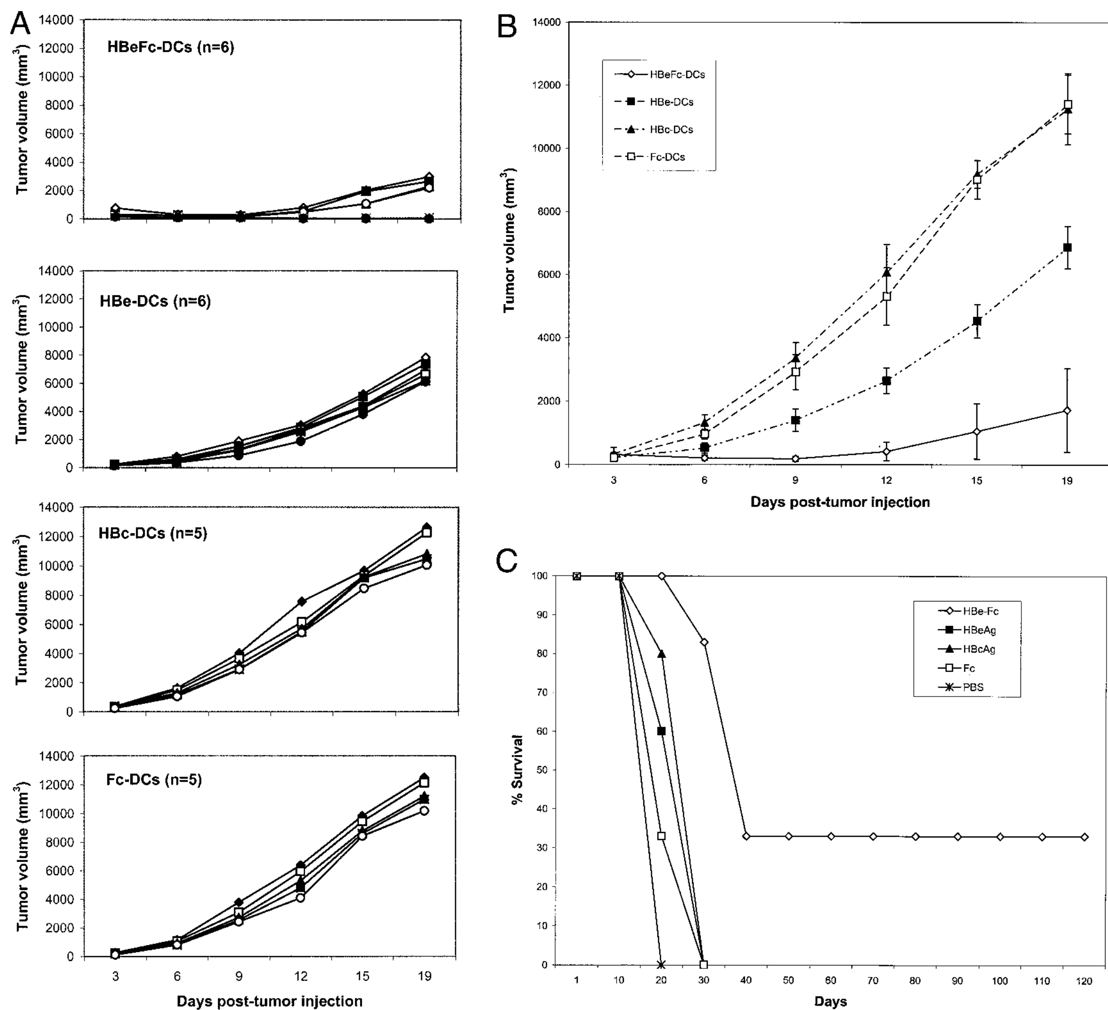

FIGURE 6. Antitumor immunity. C57BL/6 mice (five to six mice per group) were immunized by i.v. injection with 1×10^5 DCs transduced with different constructs on days 0 and 3. On day 7 after the first immunization, the mice were intradermally inoculated with 3×10^6 exponentially growing EL4-HBcAg tumor cells expressing HBcAg. Tumor sizes were measured every 2–3 days. Tumor volumes in individual mice (*A*) and mean tumor volumes (*B*) of each group ± SE are presented from one of four independent experiments using five to six mice per group, $p < 0.05$, HBe-Fc compared with HBeAg, HBcAg, or Fc. The survival rates were calculated from the survival data of a total of four independent experiments (HBe-Fc group: 24 mice; HBeAg: 24 mice; HBcAg: 22 mice; Fc: 22 mice; and PBS: 20 mice) (*C*), $p < 0.05$, HBe-Fc compared with HBeAg, HBcAg, or Fc.

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. 6*C*). 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

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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-I (cross-priming) as well as activate DCs (5–16). Third, fusion constructs can elicit strong Ab 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.

Acknowledgments

We thank Xia Zhang and Angang Yang for excellent technical assistance, Anna Frolov for her assistance in statistical analyses, and Dr. M. Brenner for his suggestions. We also thank R. Cook, J. Rodgers, and M. Barry for their comments.

References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
2. Bona, C. A., S. Casares, and T. D. Brumeanu. 1998. Towards development of T-cell vaccines. *Immunol. Today 19:126.*
3. Mellman, I., S. J. Turley, and R. M. Steinman. 1998. Antigen processing for amateurs and professionals. *Trends Cell Biol. 8:231.*
4. Zajac, A. J., K. Murali-Krishna, J. N. Blattman, and R. Ahmed. 1998. Therapeutic vaccination against chronic viral infection: the importance of cooperation between $CD4^+$ and $CD8^+$ T cells. *Curr. Opin. Immunol. 10:444.*
5. Daeron, M. 1997. Fc receptor biology. *Annu. Rev. Immunol. 15:203.*
6. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol. 55:186.*
7. Steinman, R. M., and J. Swanson. 1995. The endocytic activity of dendritic cells. *J. Exp. Med. 182:283.*
8. Watts, C. 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol. 15:821.*
9. Serre, K., P. Machy, J. C. Grivel, G. Jolly, N. Brun, J. Barbet, and L. Leserman. 1998. Efficient presentation of multivalent antigens targeted to various cell surface molecules of dendritic cells and surface Ig of antigen-specific B cells. *J. Immunol. 161:6059.*
10. Albert, M. L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature 392:86.*
11. Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science 264:961.*
12. Kovacsovics-Bankowski, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science 267:243.*
13. Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark, and C. V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature 361:359.*
14. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Fcγ receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med. 189:371.*
15. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480.*
16. Sigal, L. J., S. Crotty, R. Andino, and K. L. Rock. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature 398:77.*
17. Townsend, K., M. Sallberg, J. O'Dea, T. Banks, D. Driver, S. Sauter, S. M. Chang, D. J. Jolly, S. J. Mento, D. R. Milich, and W. T. Lee. 1997. Characterization of $CD8^+$ cytotoxic T-lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. *J. Virol. 71:3365.*
18. Hu, P., M. S. Glasky, A. Yun, M. M. Alauddin, J. L. Hornick, L. A. Khawli, and A. L. Epstein. 1995. A human-mouse chimeric Lym-1 monoclonal antibody with specificity for human lymphomas expressed in a baculovirus system. *Hum. Antibodies Hybridomas 6:57.*
19. Chen, J. D., X. Bai, A. G. Yang, Y. Cong, and S. Y. Chen. 1997. Inactivation of HIV-1 chemokine co-receptor CXCR-4 by a novel intrakine strategy. *Nat. Med. 3:1110.*
20. Chen, S. Y., A. G. Yang, J. D. Chen, T. Kute, C. R. King, J. Collier, Y. Cong, C. Yao, and X. F. Huang. 1997. Potent antitumour activity of a new class of tumour-specific killer cells. *Nature 385:78.*
21. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 176:1693.*
22. Brunda, M. J., L. Luistro, R. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med. 178:1223.*
23. De Veerman, M., C. Heirman, S. Van Meirvenne, S. Devos, J. Corthals, M. Moser, and K. Thielemans. 1999. Retrovirally transduced bone marrow-derived dendritic cells require $CD4^+$ T cell help to elicit protective and therapeutic antitumor immunity. *J. Immunol. 162:144.*
24. Gallagher, S., S. E. Winston, S. A. Fuller, and J. G. R. Hurrell. 1998. Immunoblotting and immunodetection. In *Current Protocols in Immunology* (Supplement 26), Vol. 2. R. Coico, ed. Wiley, New York, pp. 8.100.101–108.110.121.
25. Ferrari, C., A. Penna, A. Bertoletti, A. Valli, A. D. Antoni, T. Giuberti, A. Cavalli, M. A. Petit, and F. Fiaccadori. 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J. Immunol. 145:3442.*
26. Ou, J. H., O. Laub, and W. J. Rutter. 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. *Proc. Natl. Acad. Sci. USA 83:1578.*
27. Tiollais, P., C. Pourcel, and A. Dejean. 1985. The hepatitis B virus. *Nature 317:489.*
28. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods 223:77.*

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29. Fields, R. C., J. J. Osterholzer, J. A. Fuller, E. K. Thomas, P. J. Geraghty, and J. J. Mule. 1998. Comparative analysis of murine dendritic cells derived from spleen and bone marrow. *J. Immunother. 21:323.*
30. Ribas, A., L. H. Butterfield, W. H. McBride, S. M. Jilani, L. A. Bui, C. M. Vollmer, R. Lau, V. B. Dissette, B. Hu, A. Y. Chen, et al. 1997. Genetic immunization for the melanoma antigen MART-1/Melan-A using recombinant adenovirus-transduced murine dendritic cells. *Cancer Res. 57:2865.*
31. Pierre, P., S. J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R. M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature 388:787.*
32. Haeffner-Cavaillon, N., M. Klein, and K. J. Dorrington. 1979. Studies on the Fc γ receptor of the murine macrophage-like cell line P388D1. I. The binding of homologous and heterologous immunoglobulin G_1. *J. Immunol. 123:1905.*
33. Haeffner-Cavaillon, N., K. J. Dorrington, and M. Klein. 1979. Studies on the Fc γ receptor of the murine macrophage-like cell line P388D1. II. Binding of human IgG subclass proteins and their proteolytic fragments. *J. Immunol. 123:1914.*
34. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature 393:474.*
35. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*
36. Romagnani, S. 1996. Th1 and Th2 in human diseases. *Clin. Immunol. Immunopathol. 80:225.*
37. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
38. Pardoll, D. M., and S. L. Topalian. 1998. The role of $CD4^+$ T cell responses in antitumor immunity. *Curr. Opin. Immunol. 10:588.*
39. Stremmel, C., E. A. Greenfield, E. Howard, G. J. Freeman, and V. K. Kuchroo. 1999. B7-2 expressed on EL4 lymphoma suppresses antitumor immunity by an interleukin 4-dependent mechanism. *J. Exp. Med. 189:919.*
40. Bennett, S. R., F. R. Carbone, F. Karamalis, J. F. Miller, and W. R. Heath. 1997. Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. *J. Exp. Med. 186:65.*
41. Dittmer, U., D. Brooks, and K. Hasenkrug. 1999. Requirement for multiple lymphocyte subsets in protection by a live attenuated vaccine against retroviral infection. *Nat. Med. 5:189.*
42. Hasenkrug, K. J., D. M. Brooks, and U. Dittmer. 1998. Critical role for $CD4^+$ T cells in controlling retrovirus replication and spread in persistently infected mice. *J. Virol. 72:6559.*
43. Matsue, H., K. Matsue, M. Walters, K. Okumura, H. Yagita, and A. Takashima. 1999. Induction of antigen-specific immunosuppression by CD95L cDNA-transfected 'killer' dendritic cells. *Nat. Med. 5:930.*
44. Specht, J. M., G. Wang, M. T. Do, J. S. Lam, R. E. Royal, M. E. Reeves, S. A. Rosenberg, and P. Hwu. 1997. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J. Exp. Med. 186:1213.*
45. Song, W., H. L. Kong, H. Carpenter, H. Torii, R. Granstein, S. Rafii, M. A. Moore, and R. G. Crystal. 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med. 186:1247.*
46. Tang, D. C., M. DeVit, and S. A. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature 356:152.*
47. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, and A. Friedman. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science 259:1745.*
48. Wang, R., D. L. Doolan, T. P. Le, R. C. Hedstrom, K. M. Coonan, Y. Charoenvit, T. R. Jones, P. Hobart, M. Margalith, J. Ng, et al. 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science 282:476.*
49. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of $CD4^+$ T cells in the antitumor immune response. *J. Exp. Med. 188:2357.*
50. Kalams, S. A., and B. D. Walker. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med. 188:2199.*
51. Levitsky, H. I., J. Montgomery, M. Ahmadzadeh, K. Staveley-O'Carroll, F. Guarnieri, D. L. Longo, and L. W. Kwak. 1996. Immunization with granulocyte-macrophage colony-stimulating factor-transduced, but not B7-1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. *J. Immunol. 156:3858.*
52. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. *Science 278:1447.*
53. Wu, T. C., F. G. Guarnieri, K. F. Staveley-O'Carroll, R. P. Viscidi, H. I. Levitsky, L. Hedrick, K. R. Cho, J. T. August, and D. M. Pardoll. 1995. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. *Proc. Natl. Acad. Sci. USA 92:11671.*
54. Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari. 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science 284:825.*
55. Parker, D. C. 1993. T cell-dependent B cell activation. *Annu. Rev. Immunol. 11:331.*
56. Cyster, J. G., S. B. Hartley, and C. C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature 371:389.*

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