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Up-regulation of receptor-ligand pairs during interaction of an MHC-presented epitope on dendritic cells (DCs) with cognate TCR may amplify, sustain, and drive diversity in the ensuing T cell immune response. Members of the TNF ligand superfamily and the TNFR superfamily contribute to this costimulatory molecule signaling. In this study, we used replication deficient adenoviruses to introduce a model tumor-associated Ag (the E7 oncoprotein of human papillomavirus 16) and the T cell costimulatory molecule 4-1BBL into murine DCs, and monitored the ability of these recombinant DCs to elicit E7-directed T cell responses following immunization. Spleenocytes from mice immunized with DCs expressing E7 alone elicited E7-directed effector and memory CTL responses. Coexpression of 4-1BBL in these E7-expressing DCs increased effector and memory CTL responses when they were used for immunization. 4-1BBL expression up-regulated CD80 and CD86 second signaling molecules in DCs. We also report an additive effect of 4-1BBL and receptor activator of NF-κB receptor activator of NF-κB ligand coexpression in E7-transduced DC immunogens on E7-directed effector and memory CTL responses and on MHC class II and CD80/86 expression in DCs. Additionally, expression of 4-1BBL in E7-transduced DCs reduced nonspecific T cell activation characteristic of adenovirus vector-associated immunization. The results have generic implications for improved or tumor Ag-expressing DC vaccines by incorporation of exogenous 4-1BBL. There are also specific implications for an improved DC-based vaccine for human papillomavirus 16-associated cervical carcinoma. The Journal of Immunology, 2003, 170: 2912–2922.

Dendritic cells (DCs) have assumed the central role as the key initiators of adaptive immunity, and play a pivotal role in eliciting antitumor immunity (1). Their potent efficacy in inducing and directing immune responses in vivo is due to their efficiency in capturing and processing Ags, and migrating to the T cell areas where they present Ags to T cells via the TCR-MHC epitope synapse. Induction of CD8 T CTL is an essential component of the immune response for the control of tumors. Ex vivo transduction of DCs to express tumor-associated Ags (TAAs) before (re)infusion into the patient is an attractive approach to DC-based immunotherapy (2), though difficulties in introduction of TAA-expressing foreign genetic material into DCs using traditional methodologies, e.g., lipofection and electroporation, have limited the widespread adoption of this approach (3).

Gene transfer with adenovirus vectors is a powerful way to induce cellular immune responses to a transgene product (4), and this approach has been used to derive vaccines for infectious diseases and cancers, with some success in animal models (5). Their efficacy is due (at least in part) to the ability of adenovirus to infect DCs, and to high expression of the transgene product. Thus, a vaccine strategy which involves immunization for cellular immune response induction with DCs transduced ex vivo with transgene-expressing adenovirus vectors is a logical inference, and has met with some success (4).

DC signaling through the TCR for T cell activation requires “second signal” ligation of CD28 on T cells with CD80/86 (B7-1/2) on DCs. Additionally, a number of further receptor-ligand pairs are up-regulated on T cells and DCs, respectively, and their coligation may amplify, sustain, and drive diversity in the ensuing immune response. Members of the TNF ligand (L) superfamily which include 4-1BBL (CD137L), FasL, CD27, CD30, CD154 (CD40L), receptor activator of NF-κB (RANK) ligand (RANKL), lymphotoxin, TRAIL, and members of the TNFR superfamily including 4-1BB (CD137), RANK, and CD40, contribute to this additional signaling (6).

Data from many sources indicate that immunological outcome depends on the milieu in which a T cell encounters its Ag (self or foreign), and that altered expression of costimulatory molecules on DCs (and exposure to immunoregulatory cytokines) may determine for example, whether DCs transmit an activating or a tolerizing signal to T cells, and the magnitude of the signal transmitted (reviewed in Ref. 7).

4-1BB is an inducible molecule expressed predominantly on activated CD4 and CD8 T cells (8, 9). Its ligand (4-1BBL) is expressed on activated APCs including DCs and macrophages (9, 10). DC-T cell interaction facilitates signaling through 4-1BB/4-1BBL ligation which costimulates CD4 and CD8 cells via activation of NF-κB, c-Jun, and p38 downstream pathways (10, 11).

*Department of Molecular Biotechnology, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; †Sir Albert Sakzewski Virus Research Center, Royal Children’s Hospital, and Clinical Medical Virology Center, University of Queensland, Brisbane, Australia.

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2 Address correspondence and reprint requests to Dr. Robert Tindle, Sir Albert Sakzewski Virus Research Center, Royal Children’s Hospital, Herston Road, Herston, QLD 4021 Australia. E-mail address: r.tindle@mailbox.uq.edu.au

3 Abbreviations used in this paper: DC, dendritic cell; TAA, tumor-associated Ag; RANK, receptor activator of NF-κB; L, ligand; HPV, human papillomavirus; eGFP, enhanced green fluorescent protein; MOI, multiplicity of infection; Ad, adenovirus; IRES, internal ribosomal entry site.

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4-1BB ligation enhances T cell expansion, augments T cell effector function (12), and prevents activation-induced apoptosis of CD8 T cells (13). These findings indicate a critical role for signaling through the 4-1BB pathway in the expansion and functional maturation of CTLs (12, 14).

Temperal studies of CD4 and CD8 survival, expansion, and development of effector function suggests that 4-1BB ligation exerts its effect subsequent to initial T cell activation by the conventional CD28 pathway. Indeed, 4-1BB stimulates T cell responses independently of signals through the CD28 molecule (15) because both CD28+/− and CD28−/− CD4 and CD8 T cells respond to 4-1BB-mediated stimulation.

In the present study, we investigated whether immunization with DCs expressing a model TAA delivered by an adenoviral vector would elicit TAA-directed effector and memory CTL responses in mice, and whether these responses would be enhanced by coexpression with the DCs of exogenous 4-1BBL.

We demonstrate that coexpression of 4-1BBL in DCs transduced with adenovirus-encoding E7 increased effector and memory E7-directed CTL responses when these DCs were used for immunization. We show that 4-1BBL up-regulated the expression of CD80 and CD86 second signal molecules on DCs. 4-1BBL acted additively with RANK/RANKL to enhance T cell responses in mice immunized with E7-transduced DCs which coexpressed these costimulatory molecules. Additionally expression of 4-1BBL in E7-transduced DCs reduced nonspecific T cell activation characteristic of immunization with adenovirally modified DCs.

These results have generic implications for the derivation of improved foreign Ag- or TAA-expressing DC vaccines by incorporation in the DCs of exogenous 4-1BBL. There are also specific implications for an improved DC-based vaccine for HPV16-associated cervical carcinoma (17).

Materials and Methods

Cell lines

A549 cells were a gift from T. Adrian (MHH, Hannover, Germany) and were grown in high-glucose DMEM (Life Technologies, Grand Island, NY) with 10% FCS, 293LP cells, an adenovirus-transformed human embryonic kidney cell line which provides phenotypic complementation of the E1 genes, were purchased from Microbix Biosystems (Ontario, Canada). The E7-expressing EL4.E7 cell line was derived from EL4 cells (a H-2b thymoma) transfected to stably express the full-length HPV16 E7 oncoprotein, we used the E7 oncoprotein of human papillomavirus (HPV) type 16 (16).

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loosely adherent cells (DCs) were washed and used for flow cytometry analysis or immunization.

Flow cytometry analysis

DCs were incubated with the primary Abs for 30 min on ice. A second incubation with Cy5-coupled streptavidin (Dianova) was performed when necessary. The following Abs were used (all obtained from BD Pharmingen): hamster IgG monoclonal anti-mouse CD11c (clone HL3), rat IgG monoclonal anti-mouse I-A<sup>i</sup>/I-E<sup>e</sup> MHC II (clone 2G9), hamster IgG monoclonal anti-mouse CD80 (clone 16-10A1), rat IgG monoclonal anti-mouse CD86 (clone GL1), and rat IgG monoclonal anti-mouse 4-1BB/CD137 (clone 1H12). Cells were fixed with 4% paraformaldehyde. Samples were analyzed on a FACSVerse (BD Biosciences, Mountain View, CA) with CellQuest 3.3 software.

Immunization

Mice (four per group) were immunized either with 5 × 10<sup>5</sup> DCs or peptide s.c. at the base of the tail. For peptide immunization, the mice received 30 μg of peptide, 10 μg of Quil A adjuvant, and 2.5 μg of tetanus toxoid (CSL, Melbourne, Australia) as a source of Th epitope(s). Ten days later, splenocytes were harvested and either used in ELISPOT assays or depleted of APCs as described (28). APC-depleted splenocytes (1 × 10<sup>6</sup>/well) were restimulated in vitro with irradiated E7-expressing EL4.E7 cells (1 × 10<sup>5</sup> well) for 6 days, and then used in CTL or ELISPOT assay.

CTL assays

51Cr-release CTL assays were conducted as previously described (19).

IFN-γ ELISPOT

Cells secreting IFN-γ were detected using ELISPOT assay (29). In brief, serially diluted ex vivo or restimulated splenocytes (2 × 10<sup>4</sup> to 5 × 10<sup>5</sup> cells/well) were incubated overnight with or without cognate peptide (1 μg/ml), in 96-well nitrocellulose plates (Multiscreen HA; Millipore, Bedford, MA) coated with capture anti-mouse IFN-γ mAb R4-6A2 (BD Pharmingen)/well in medium supplemented with human recombinant IL-2 (50 U/ml; Sigma-Aldrich). Controls included spleen cells from irrelevantly immunized mice. After washing, plates were incubated with biotinylated anti-mouse IFN-γ mAb XM12.1 (BD Pharmingen)/well for 3 h at room temperature, washed and incubated with avidin alkaline phosphatase conjugate (BD Pharmingen). Spots were detected by adding an alkaline phosphatase substrate (Sigma-Aldrich). The number of spots corresponding to IFN-γ-secreting cells was determined by using automatic AID-ELISPOT-Reader (Autoimmun-Diagnostika). The E7/D<sub>β</sub>-specific T cell response, monitored as IFN-γ-secreting T cells per 10<sup>6</sup> splenocytes, was determined as ((number of spots with peptide minus number of spots without peptide)/(number of cells per well)) × 10<sup>6</sup>.

Results

Recombinant adenoviruses

Three recombinant adenoviruses with mono- or bicistronic expression cassettes were constructed containing either a gene encoding a mutated E7 protein of HPV16 (AdE7mut), green fluorescent protein (AdGFP), or 4-1BBL (Ad4-1BBL) driven from a CMV promoter (Fig. 1A). Each bicistronic recombinant adenovirus also contained an eGFP gene driven from an IRES.

Expression of E7 was confirmed in Ad-E7mut-infected A549 cells by Western blotting (Fig. 1B) and by immunohistochemical staining with E7-specific mAbs (Fig. 1C). Expression of 4-1BBL was confirmed by immunofluorescence in A549 cells infected with Ad4-1BBL (Fig. 1D). eGFP fluorescence of either infected or uninfected A549 was used as a convenient marker for recombinant gene expression (Fig. 1D).

In this study, we used the recombinant adenoviruses to infect murine bone marrow-derived DCs. eGFP fluorescence of DCs infected with AdE7mut or Ad4-1BBL was used as a marker for recombinant gene expression. 4-1BBL expression was confirmed directly by positive immunofluorescence with anti-4-1BBL Ab (Fig. 1E).

Immunization with DCs expressing AdE7 elicits E7-directed CTL

We wished to establish whether mice immunized with autologous bone marrow-derived DCs infected with rAdE7 would develop E7-directed CTL responses. C57BL/6 mice were injected once s.c. with DCs which had been infected in vitro with rAdE7 at MOIs of 50, 125, or 250. Control mice were infected with DCs infected with AdGFP (MOI 300) or uninfected DCs. As a positive control, mice were also immunized with E7/D<sub>β</sub> peptide using conditions previously established in our laboratory to elicit an E7 CTL response (19). Ten days later, splenocytes were harvested and IFN-γ-secreting T cells were quantified by ELISPOT, either immediately (ex vivo) or after 6 days of restimulation with endogenously E7-expressing EL4 cells (EL4.E7). Mice immunized with AdE7-infected DCs showed increasing numbers of both ex vivo effector splenocytes (Fig. 2A) and restimulated memory splenocytes (Fig. 2B) secreting IFN-γ in response to E7/D<sub>β</sub> peptide with increasing MOI used for infection.

Restimulated splenocytes were also examined for CTL activity against syngeneic target cells presenting the E7/D<sub>β</sub> CTL epitope. Splenocytes from mice immunized with DCs infected with AdE7 specifically killed both target cells pulsed with E7/D<sub>β</sub> peptide (Fig. 2C) or EL4.E7 target cells which endogenously process the E7/D<sub>β</sub> epitope (Fig. 2D). The amount of killing increased with increasing MOI (Fig. 2, C and D).

These results indicate that immunization of mice with DCs infected with AdE7 elicited E7-directed effector and memory CTL responses in a dose-dependent fashion in which a MOI of 250 was required to achieve a maximal response equivalent to that elicited by peptide immunization.

Immunization with DCs coexpressing 4-1BBL enhances E7-directed effector and memory responses

We examined the effect of 4-1BBL on the E7-directed CTL response induced by immunization with bone marrow-derived DCs infected with AdE7. Groups of mice were immunized with DCs infected with AdE7 at MOIs of 50, 125, or 250 which either had or had not been coinfected with Ad4-1BBL at MOI 200. Control mice were immunized with DCs infected with Ad4-1BBL alone. IFN-γ ELISPOT assays were conducted on ex vivo splenocytes 10 days after immunization (effector response) and after 6 days of further restimulation in vitro with EL4.E7 cells (memory response). As expected from the previous experiments (Fig. 2), in mice receiving DCs expressing E7 alone, the proportion of ex vivo and restimulated splenocytes secreting IFN-γ in response to E7/D<sub>β</sub> peptide tended to increase the higher the MOI of Ad7 used to infect the DC immunogen, reaching maximal levels at MOI 250 (Fig. 3, A and B). In mice receiving DCs coexpressing E7 and 4-1BBL, the proportion of splenocytes secreting IFN-γ in response to E7/D<sub>β</sub> peptide was significantly higher in comparison to mice receiving DCs expressing E7 alone, but only where suboptimal MOIs for eliciting a maximal E7-directed response (MOIs 50, 125) had been used. This observation held true for both effector (ex vivo) and memory (restimulated) responses (Fig. 3, A and B). The proportion of splenocytes secreting IFN-γ was not increased in mice immunized with DCs coexpressing 4-1BBL and E7 compared with those immunized with DCs expressing E7 alone, where the MOI of AdE7 was 250 (i.e., the MOI capable of eliciting a maximal E7 response) (Fig. 3, A and B).

Similarly, an increase in DC immunogen efficacy related to 4-1BBL coexpression was observed when memory CTL responses against E7/D<sub>β</sub>-pulsed target cells (Fig. 3C) or EL4.E7 target cells constitutively processing E7/D<sub>β</sub> (not shown) were measured in 51Cr-release cytotoxicity assays. Thus, coexpression of 4-1BBL...
enhanced the E7-directed response but only when MOIs of AdE7, which elicited submaximal killing, were used to infect the DC immunogens. The observed effect of 4-1BBL coexpression was not due to the increased amount of adenovirus used for DC infection (i.e., AdE7 plus Ad4-1BBL) because coinfection of DCs with AdE7 at MOI 125 and “irrelevant” AdeGFP at MOI 600 did not
significantly increase the E7-specific immune response following DC immunization, when compared with infection of DCs with AdE7 alone at MOI 125 (Fig. 3).

Together these data indicate that immunization with DCs coexpressing 4-1BBL enhances E7-directed effector and memory responses when compared with immunization with DCs expressing E7 alone.

**Transduction of DCs with Ad4-1BBL up-regulates the proportion of DCs expressing high levels of CD80 and CD86**

Enhanced stimulation of T cells by costimulatory molecules on mature DCs likely occurs by more than one pathway. Thus, CD80 (B7.1) and CD86 (B7.2) on mature DCs ligate to CD28 on T cells and this interaction is crucial for initial T cell expansion. In contrast, ligation of 4-1BBL on mature DCs with 4-1BB constitutively expressed on T cells affects T cell numbers much later in the response and is essential for the survival and/or responsiveness of the memory CD8 T cell pool (30). Though distinct, these pathways are not mutually independent (30). Therefore, we inquired whether provision of 4-1BBL specifically up-regulates the expression of “second signal” molecules CD80 and CD86 on DCs.

**Coexpression of 4-1BBL with RANK and RANKL in DCs acts additively to enhance E7-directed effector and memory CTL responses**

We have shown that provision of 4-1BBL with E7 in DCs will not enhance E7-directed responses, when the MOI of infecting AdE7 is high (MOI 250) (Fig. 3). We infer that a high MOI equates with a nonlimiting amount of E7 Ag available for immune response induction. Our observation is in keeping with other work showing that 4-1BBL costimulation plays a more important role in CD8+ T cell activation in conditions of limiting Ag (14). We have recently found that immunization of mice with DCs coinfected with AdE7 and recombinant adeno- viruses expressing RANK and RANKL together (RANK/RANKL) improved on E7-directed CTL responses elicited by immunization with DCs, but only when MOIs for AdE7 were low (MOIs 50, 125) (RANK is a costimulatory molecule expressed on mature DCs with high homology to CD40. Signaling through its receptor, RANKL, on T cells enhances survival of DCs and stimulates T cell growth and differentiation (6)). At a high MOI of AdE7, (MOI 250) coexpression of RANK/RANKL in the DC immunogen barely improved upon the responses elicited by DCs transduced with AdE7 alone (C. Wiethe, K. Dittmar, T. Doan, W. Lindenmaier, and R. Tindle, manuscript in preparation). In the present study, we wished to inquire whether coprovision of 4-1BBL and RANK/RANKL to DCs infected with AdE7 at a high MOI would augment E7-directed CTL responses compared to DCs infected with AdE7 at high MOI alone. Groups of mice were immunized with DCs infected with recombinant adenoviruses, with uninfected DCs, or with E7/D0 peptide as indicated. Splenocyte effector cells were reacted with E7/D0 peptide-pulsed target cells (C), or with endogenously E7-expressing EL4.E7 target cells (D) at the E:T ratios indicated. (Data are pooled from three independent experiments, except ELISPOT MOI 50 data which is from one experiment)

**FIGURE 2.** DCs expressing E7 induce effector and memory T cell responses. A and B, IFN-γ-secreting splenocytes from mice (four per group) immunized with bone marrow-derived DCs infected with recombinant adeno- viruses, with uninfected DCs, or with E7/D0 peptide as indicated, were measured by ELISPOT assay either ex vivo (A), or after restimulation for 6 days in vitro with EL4.E7 cells (B). ELIS- POTs were developed after a 20-h incubation in vitro with E7/D0 peptide. C and D. 51Cr-release CTL assay of restimulated splenocytes from mice immunized with DCs infected with re- combinant adeno- viruses, with uninfected DCs, or with E7/D0 peptide as indicated. Splenocyte effector cells were reacted with E7/D0 peptide-pulsed target cells (C), or with endogenously E7-expressing EL4.E7 target cells (D) at the E:T ratios indicated. (Data are pooled from three independent experiments, except ELISPOT MOI 50 data which is from one experiment)
presenting EL4.E7 cells. As previously observed, mice immunized with DCs infected with AdE7/H11001 Ad4-1BBL, or with AdE7/H11001 AdRANKL showed little or no augmentation of ex vivo or

restimulated splenocyte ELISPOT responses compared with mice immunized with DCs infected with AdE7 alone, when a high MOI (250) of AdE7 was used (Fig. 5, A and B). However, mice immunized with

FIGURE 3. Coexpression of 4-1BBL in E7-expressing DCs enhances T cell responses. A and B, IFN-γ-secreting splenocytes from mice (four per group) immunized with bone marrow-derived DCs cotransduced with AdE7 at various MOIs as indicated and with 4-1BBL, or with AdE7 alone were measured by ELISPOT assay either ex vivo (A; *, p < 0.05; **, p < 0.001), or after specific restimulation with E7/D9 peptide (B; *, p < 0.002; **, p < 0.004). ELISPOTs were developed after a 20-h incubation in vitro with E7/D9 peptide. C, 51Cr-release CTL assay of restimulated splenocytes from mice immunized with DCs infected with AdE7 at various MOIs as indicated and Ad4-1BBL, or with AdE7 alone. Effector cells were reacted with E7/D9-pulsed EL4.A2 target cells at the E:T ratios indicated (*, p < 0.05; **, p < 0.002; ***, p < 0.02). Pooled data from three independent experiments are shown; significant differences were determined with the paired Student’s t test. Data are pooled from three independent experiments.

FIGURE 4. Expression of 4-1BBL in DCs enhances CD80 and CD86 expression. FACS analysis of bone marrow-derived DCs (pooled from two mice) infected on day 4 with Ad4-1BBL (MOI 200), with AdGfp (MOI 300) or uninfected, and cultured for 2 more days before double-staining with Abs to CD11c and MHC class II, or CD80 or CD86 as indicated. No staining was recorded using isotype control primary Abs anti-B220 (rat IgG2a) for CD86 and MHC II, and anti-CD3 (hamster IgG2) for CD80 (data not shown).
DCs infected with AdE7 + Ad4-1BBL + AdRANKL showed significantly enhanced ELISPOT responses both ex vivo (Fig. 5A) and following restimulation (Fig. 5B). Similarly, restimulated splenocytes from mice immunized with DCs infected with AdE7 + Ad4-1BBL + AdRANKL + AdRANKL showed significantly enhanced killing of E7/Dβ-pulsed target cells in a 31Cr-release assay (Fig. 5C).

In control groups of mice immunized with DCs infected with AdeGFP (MOI 600) (Fig. 5) or with AdE7 (MOI 125) + AdeGFP (MOI 600) (not shown), no ELISPOT or 31Cr-release data significantly above the background of splenocytes from mice immunized with DCs expressing 4-1BBL/RANK/RANKL, but no E7, were recorded. This indicates that increasing total Ad vector MOIs for DCs per se did not overtly contribute to the above findings.

These results indicate that 4-1BBL and RANK/RANKL coexpression in DCs transduced with optimal amounts of AdE7 for immune response induction acts additively to further enhance the response.

Coexpression of RANK/RANKL with 4-1BBL up-regulates the proportion of DCs expressing high levels of MHC class II, CD80 and CD86.

A possibility is that the additive effect on the specific immune response reported above was due to enhanced Ag presentation and/or immunostimulatory capability by up-regulation of MHC and second signal molecules on DC immunogens expressing both 4-1BBL and RANK/RANKL. Therefore, we asked whether coexpression of 4-1BBL and RANK/RANKL in DCs would up-regulate MHC class II, CD80, and CD86 when compared with expression of either 4-1BBL or RANK/RANKL alone (Fig. 6). DCs infected with Ad4-1BBL + AdRANK + AdRANKL showed a higher proportion of MHC class II-expressing cells (69.9% MHC class II° CD11c° cells) than DC infected with AdRANK + AdRANKL (49.7% MHC class II° CD11c° cells) or with Ad4-1BBL alone (42.7% MHC class II° CD11c° cells). Similarly, DCs infected with Ad4-1BBL + AdRANK + AdRANKL showed a higher proportion of CD80-expressing cells (70.4% CD80° CD11c° cells) and CD86 (69.4% CD86° CD11c° cells) than DCs infected with AdRANK + AdRANKL (56.3% CD80° CD11c° cells, 51.8% CD86° CD11c° cells) or with Ad4-1BBL alone (42.2% CD80° CD11c° cells, 46.5% CD86° CD11c° cells). These data indicate that coexpression of RANK/RANKL with 4-1BBL up-regulates the proportion of DCs expressing high levels of MHC class II, CD80, and CD86.

Coprovision of 4-1BBL reduces nonspecific T cell activation

Throughout our experiments, we recorded elevated background levels of ex vivo IFN-γ-secreting splenocytes following immunization with DCs infected with AdE7, AdeGFP, AdRANK, or AdRANKL, but not with Ad4-1BBL, compared with immunization with uninfected DCs or with free peptide. These observations are exemplified by the data in Fig. 7A which shows ELISPOT wells containing ex vivo splenocytes from mice immunized with DCs infected with AdE7 at various MOIs either with or without coinfection with Ad4-1BBL. Where 4-1BBL is provided, there is a markedly lower proportion of IFN-γ-secreting splenocytes in the absence of E7/Dβ peptide, compared with mice which have received DCs infected with AdE7, but not Ad4-1BBL (Fig. 7A). This was particularly true of splenocytes from mice immunized with DCs infected at the two higher MOIs (125, 250) of AdE7. Similarly, ex vivo splenocytes from mice immunized with DCs infected...
with AdE7 + AdRANK + AdRANKL + Ad4-1BBL (Fig. 7C) show a lower proportion of IFN-γ-secreting cells in the absence of E7/Dβ peptide, than splenocytes from mice immunized with DCs infected with AdE7 + AdRANK + AdRANKL alone (Fig. 7B).

These data indicate that coprovision of 4-1BBL reduces non-specific T cell activation in mice immunized with DCs infected with adenovirus vector, as measured by the proportion of splenocytes secreting IFN-γ.

**FIGURE 6.** Coexpression of 4-1BBL and RANK/RANKL in DCs acts synergistically to up-regulate MHC class II, CD80, and CD86 expression. FACS analysis of DCs infected with Ad4-1BBL, with AdRANK/RANKL, or with Ad4-1BBL + AdRANK/RANKL, and double-stained with Abs to CD11c and MHC class II, or CD80 or CD86 as indicated. No staining was recorded using isotype control primary Abs anti-B220 (rat IgG2a) for CD86 and MHC II, and anti-CD3 (hamster IgG2) for CD80 (data not shown).

**FIGURE 7.** Coexpression of 4-1BBL reduces non-specific T cell activation. Individual ELISPOT wells containing ex vivo splenocytes from mice immunized with DCs infected with (A) AdE7 at various MOIs as indicated with (+) or without (−) coinfection with Ad4-1BBL. B, AdE7 (MOI 250) + AdRANK/RANKL; C, AdE7 + AdRANK/RANKL + Ad4-1BBL, reacted in an IFN-γ ELISPOT assay with or without E7/Dβ peptide. Controls (D) show splenocytes from mice immunized with uninfected DCs, with E7/Dβ peptide or with DCs infected with Ad4-1BBL alone or with Ads RANK/RANKL + Ad4-1BBL. MOIs of 200 were used for Ad4-1BBL, AdRANK, and RANKL.
Discussion

DCs are the predominant APC of the immune system. The role of “mature” DCs is particularly important in the activation of T cell-mediated immune responses against tumors (1). At least in some cases Ag presentation by DCs is the limiting factor in antitumor immunity (31). Vaccines comprising DCs pulsed with TAA-derived peptides, recombinant tumor proteins, virus-like particles containing TAA, and apoptotic tumor cells have proved effective in generating tumor protection in animal models (3, 32–34), and in generation of tumor Ag-specific T cell responses in vitro in humans (35–36). DC immunogens genetically modified to express TAA can improve on the pulsing approach by providing more prolonged Ag stimulation (37, 38). Thus, a goal has been to optimize transduction and expression of TAA within DCs and to maximize tumor-directed T cell responses when the genetically modified DCs are used for immunization.

The costimulatory molecule 4-1BB is induced on naive T cells following TCR-MHC/peptide and CD80/86/CD28 ligation following interaction of cognate T cells with DCs. 4-1BB/4-1BBL signaling is one of several receptor-ligand pathways which come into play when an Ag-presenting DC encounters a cognate T cell. Others include CD40 and RANK on DCs which ligate to CD40L and RANKL on T cells, respectively. The result is bidirectional signaling which enhances the maturation of DCs and the activation of T cells. Signaling through 4-1BB by 4-1BBL enhances T cell expansion, and augments T cell effector function, (including cytokine release, CD4-mediated “help”, and CTL activity (11). 4-1BB signaling has an additional immune enhancing effect by prevention of activation-induced apoptosis of CD8 T cells by enhancing the expression of the survival gene bcl-xL (13). 4-1BB signaling overcomes activation-induced nonresponsiveness (39) in CD8 T cells in vitro (40), can break immunological ignorance (promoting regression of poorly immunogenic tumors in vivo) (41), can convert an otherwise tolerogenic peptide vaccine into a formulation capable of CTL priming (42), and can enhance cross presentation (43).

Recently, immature DCs have been reported to constitutively express 4-1BB (44), raising the possibility of DC-DC reciprocal and/or autocrine stimulation via the 4-1BB/4-1BBL pathway. Thus, 4-1BBL may be involved in DC activation in a T cell-independent, as well as a T cell-dependent, fashion (44).

In the present study, we investigated whether provision of exogenous 4-1BBL in DC vaccines transduced with an adenviral vector encoding a TAA would enhance TAA-directed effector and memory CTL responses. To do this we generated adenovirus vectors expressing E7 or 4-1BBL (Fig. 1A) and confirmed expression of these transgenes in infected A549 tissue culture cells (Fig. 1, B–D). We also confirmed expression of 4-1BBL in infected DCs used for immunization (Fig. 1E) We were unable to detect E7 expression in rAdE7-infected DCs even though the DC recipients subsequently mounted E7-specific T cell responses. Lack of detection of E7 in DCs presumably relates to the short half-life of E7 in cells (45), and the increased instability of the mutated Cys-x-x-Cys E7 variant we used compared with wild-type E7 (22). Furthermore, E7 is likely to be more rapidly processed in DCs than in A549 cells.

The magnitude of TAA-directed effector and memory responses in splenocytes from mice immunized with DCs transduced with AdE7 alone increased with the MOI of AdE7 used for DC infection until the responses equaled or exceeded those of splenocytes from mice immunized with E7 peptide (Fig. 2). This may suggest that the amount of E7 available for presentation to T cells was limiting in mice immunized with DCs transduced with AdE7 at lower MOIs, leading to suboptimal responses. Immunization of mice with DCs cotransduced with a suboptimal amount of AdE7 and with Ad4-1BBL, augmented the number of IFN-γ-secreting T cells both ex vivo and after in vitro restimulation, compared with mice immunized with DCs transduced with AdE7 alone (Fig. 3, A and B). Similarly, restimulated splenocytes from mice immunized with DCs cotransduced with a suboptimal amount of AdE7 and with Ad4-1BBL showed enhanced specific cytotoxicity of E7-target cells in a 51Cr-release assay (Fig. 3C). In contrast, mice immunized with DCs cotransduced with an optimal amount of AdE7 and with 4-1BBL did not exhibit augmented numbers of IFN-γ splenocytes, either ex vivo or after restimulation, or augmented specific killing of E7-target cells compared with mice immunized with DCs transduced with an optimal MOI of AdE7 alone. These results are compatible with the notion that coprovision of exogenous 4-1BBL to TAA-expressing DCs enhances suboptimal, but not optimal, TAA direct effector and memory T cell responses evoked by recombinant DC immunization. These findings are congruent with those of other workers indicating that 4-1BB costimulation plays a more important role in T cell activation where Ag is limiting (12, 14). The mechanism presumably relates to the strength of signaling through the TCR (46). Thus, where lower amounts of Ag are presented, the optimal number of TCRs required for T cell activation may not be available, and consequently costimulatory pathways such as 4-1BBL/4-1BBL (and CD80/86/CD28) may be required for optimal T cell responses (46).

We observed an ∼4- to 10-fold increase in the proportion of IFN-γ-secreting cells following restimulation of splenocytes from both mice immunized with DCs infected with AdE7 alone, or with DCs coinfected with AdE7 and Ad4-1BBL (Fig. 3, A and B). Although the numbers following restimulation depend on several interrelated factors (i.e., number of memory cells in the ex vivo splenocyte population, their avidity for cognate peptide-MHC, and their capacity to proliferate in response to activation), the observation that the outcome (i.e., a 4- to 10-fold increase) was the same with or without the presence of added 4-1BBL in the initial DC immunogen suggests that the effect of 4-1BBL is on the generation of primary effector and memory cells rather than the capacity of memory cells to be restimulated.

We found that expression of 4-1BBL in DCs increased the numbers of DCs expressing “professional” APC molecules CD80 and CD86 (Fig. 4). These second signal molecules are ligands for CD28 expressed on T cells. It is reported that 4-1BB signaling works synergistically with CD28-CD80/86 signaling to drive activation of T cells stimulated by TCR-peptide/MHC ligation (41, 47). Up-regulation of CD80/86 by 4-1BBL expression in DCs provides a possible mechanism for this synergy.

We have demonstrated that immunization with DCs cotransduced with RANK/RANKL and E7 gave enhanced effector and memory E7-directed CTL responses compared with immunization with DCs transduced with E7 alone (C. Wiethe, K. Dittmar, T. Doan, W. Lindenmaier, and R. Tindle, manuscript in preparation). This is in keeping with reports that RANK/RANKL ligation enhances survival of DCs, induces expression of cytokines, and T cell growth and differentiation factors (6), and has a direct effect on T cells by stimulating c-Jun N-terminal kinase activity (6). In the present studies, we asked whether 4-1BBL and RANK/RANKL expressed in E7-transduced DCs would enhance E7-directed T cell responses in DC-immunized mice. Under conditions of high amounts of E7 (AdE7 MOI 250) at which neither 4-1BBL alone nor AdRANK/AdRANKL alone augmented E7-directed CTL responses, cotransduction of both sets of costimulatory molecules (i.e., 4-1BBL and RANK/RANKL) into DCs used for immunization resulted in up-regulation of E7-specific effector and memory IFN-γ-secreting cells, and the memory killing response (Fig. 5). These data indicate...
that costimulatory molecules 4-1BBL and RANK/RANKL coexpressed with E7 in DCs act additively to enhance induction of E7-directed T cell responses. Additionally, we report up-regulation in the numbers of 4-1BBL and RANK/RANKL-transduced DCs expressing MHC class II, CD80, and CD86 compared with DCs transduced with either of these entities alone. This finding is congruent with the former DCs having enhanced class II pathway Ag presentation and CD28 pathway costimulatory capabilities. Studies elsewhere indicate that a single Ag responsive (CD4+) T cell may express multiple costimulatory molecules simultaneously (11), and that knockout mice lacking any one costimulatory molecule display down-regulated immune responses (14, 48, 49). This suggest that different costimulatory molecules do not have redundant functions, but rather the norm may be for them to act additively or synergistically during particular immune responses in vivo, and this is congruent with our findings.

We were concerned that that enhanced T cell responses may be caused by transduction of DCs per se and/or its downstream sequelae. For this reason we included control groups of mice which received DCs transduced with “irrelevant” gene (eGFP), or with costimulatory molecules without E7, or with E7 without costimulatory molecules. We were at pains to ensure that the conditions of transduction were “normalized” so that total MOs with adenovirus vector per DC were similar for these negative control groups and for groups receiving DCs expressing both E7 and costimulatory molecule(s). These control groups obviate the possibility that the enhanced T cell responses we observe resulted from transduction per se.

Immunization with Ad-transduced DCs produced a high background of nonspecific IFN-γ-secreting splenocytes compared with immunization with uninfected DCs or peptide (Fig. 7). However, we consistently recorded that immunization with DCs coinfected with Ad4-1BBL substantially reduced this nonspecific T cell activation (Fig. 7). Nonspecific immune activation likely relates to the fact that adenovirus itself induces an inflammatory response (50), and slightly enhances MHC class I, CD80, and CD86 expression (Fig. 4). Ag presentation and the T cell stimulatory capacity of transduced DCs (51, 52). Although the mechanism(s) of reduction of nonspecific T cell activation by 4-1BBL is not clear, the more highly focused T cell response which occurs when our DC immunogens expressed 4-1BBL is likely to be beneficial in terms of specific vaccine efficacy. In the absence of “danger signals” in a noninflammatory tumor environment, DCs fail to mature (41) and may transmit a tolerizing rather than activating signal to T cells (53). An advantage of delivering TAA to DCs via adenovirus vectors is that adenovirus infection predisposes DCs to mature and therefore to activate rather than tolerate TAA-directed T cells. Further, incorporation of 4-1BBL in the DC immunogen enhances this activating response, while holding bystander activation in check.

Strategies that use DCs to deliver TAAs have shown considerable promise in inducing TAA-directed CTL responses. Clearly, our findings are pertinent to more effective and focused vaccines using the DC approach. Specifically, our findings have implications for an E7-based therapeutic vaccine for cervical cancer. We have experiments planned to test whether immunization with DCs expressing E7 and costimulatory molecules will prevent the development of carcinomas in mice expressing an E7 transgene in squamous epithelium (54), which constitute a model for human cervical cancer (55). The generation of HPV-specific CTLs in cervical cancer patients, even after E7 immunization with whole E7 protein, E7 peptides and vaccinia-E7 recombinants has been problematic, although immunization with E7-pulsed or E7-transfected DCs elicited E7-specific CD4+ and CD8+ T cells (35). Clinical trials are underway using adoptive transfer of HPV tumor lysate-pulsed DCs (56). Our results suggest that incorporation of 4-1BBL, with or without other costimulatory molecules, e.g., RANK/RANKL, along with DC-vectored E7 may improve on these approaches (57).

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References
