Adenovirus Vector-mediated Overexpression of a Truncated Form of the p65 Nuclear Factor κB cDNA in Dendritic Cells Enhances Their Function Resulting in Immune-mediated Suppression of Preexisting Murine Tumors

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ABSTRACT

Purpose: The purpose of this study was to evaluate the effect of the Rel homology domain (RHD) of the transcription factor, nuclear factor κB (NFκB), on proinflammatory gene expression in bone marrow-derived dendritic cells (BMDCs).

Experimental Design: We used an adenovirus vector encoding only the RHD of the NFκB (p65 family member) cDNA (AdRHD) to transduce murine BMDCs ex vivo. Endpoints measured included BMDC expression of activation markers, cytokine secretion, peptide antigen presentation, and the ability of these transduced cells to induce antitumor immunity in vivo.

Results: AdRHD-transduced BMDCs secreted higher levels of the cytokines interleukin (IL) 1β, IL-6, and IL-12 (p40) compared with sham-transduced BMDCs or those transduced with an empty vector. AdRHD induced heightened surface expression of the activation markers CD40, B7.1, B7.2, and MHC class II on BMDCs, and these cells were able to present a peptide antigen to a T-lymphocyte hybridoma more efficiently than controls in vitro. Growth of syngeneic, established tumors (CT26 and B16.F10) was inhibited, and survival was prolonged in the mice that received intratumoral AdRHD-modified BMDCs compared with controls. Splenocytes from CT26 tumor-bearing animals that received intratumoral AdRHD-modified BMDCs were able to lyse CT26 target cells more efficiently than controls. Similar experiments using host mice harboring targeted mutations in CD4 and CD8, as well as BMDCs from mice lacking MHC class I, MHC class II, or IL-12 revealed that this tumor immunity was dependent on the presence of CD4+ and CD8+ cells in the tumor-bearing host, as well as MHC class I, MHC class II, and IL-12 expression by the administered BMDCs. Furthermore, induction of IL-12 (p40) expression by AdRHD was completely abrogated in BMDCs lacking the c-Rel NFκB family member.

Conclusions: We made the following conclusions: (a) gene transfer-mediated overexpression of the RHD of NFκB activates BMDCs; (b) AdRHD-modified BMDCs induce antitumor immunity when administered intratumorally, an effect mediated by both the CD4+ T cell/MHC class II and the CD8+ T cell/MHC class I pathways, as well as IL-12; and (c) IL-12 (p40) up-regulation by the RHD transgene in BMDCs is dependent on the presence of the c-Rel NFκB family member.

INTRODUCTION

DCs3 are APCs that initiate and modulate host immune responses. Immature DCs, located in peripheral nonlymphoid tissues, capture and process antigens, and migrate to lymphoid tissues where they play a vital role in activating T lymphocytes (1–4). After antigen capture, DCs mature, a phenotype manifested by their capacity to efficiently prime T cells in association with the release by the DCs of proinflammatory cytokines, such as IL-1, IL-6, IL-12, and tumor necrosis factor α, as well as increased expression of costimulatory molecules, including CD40, CD80, CD86, major histocompatibility complex class II, major histocompatibility complex class I, and intercellular adhesion molecule-1 (1–4).

The NFκB/Rel proteins are a family of ubiquitous eukaryotic transcription factors found in the cytoplasm of most cell types in an inactive form bound by the IκB family of proteins (5–7). On triggering by a variety of activation signals such as infection, inflammation, necrosis, and apoptosis, the Rel protein is released from IκB, migrates to the nucleus, and binds to specific DNA binding sites (κB) that function as promoters for many inducible genes, such as acute phase reactants, cytokines,
growth factors, and leukocyte adhesion molecules (5-7). The activation of the NFκB/Rel proteins is an important process in the maturation of DCs (8-15). The five subunits that comprise the mammalian Rel family include p65 (RelA), RelB, c-Rel, p50 (NFκB1), and p52 (NFκB2), all of which exist as homo- or heterodimers (5-7, 16, 17). All of the Rel proteins contain a highly conserved, 300 amino acid, NH2-terminal region called the RHD required for dimer formation, nuclear localization, DNA binding, and kB binding (5-7, 16, 17). In contrast, only the RelA, RelB, and c-Rel proteins contain the TD thought to be responsible for the induction of DNA transcription (5-7, 16, 17).

Although the TD of NFκB proteins is generally thought to be responsible for the activation of transcription, some studies have shown that the p50 homodimer is transcriptionally active, suggesting that the RHD, in and of itself, may possess transcriptional regulatory capacity (18, 19). On this basis, along with the knowledge that mature DCs have the capacity to induce an immune response in vivo and that mature DCs are typically characterized by an up-regulation of the NFκB proteins (8-15), we hypothesized that: (a) ex vivo modification of the DCs to overexpress just the RHD coding sequences of the NFκB/p65 cDNA using an Ad vector will induce DC maturation/activation; and (b) these RHD-activated DCs will enhance the efficacy of DC tumor vaccines in vivo as manifested by the ability to induce a tumor-specific immune response when administered intratumorally. To evaluate this concept, BMDCs were modified by Ad-mediated transfer of the truncated human p65 NFκB cDNA, containing only the RHD (AdRHD; Ref. 20), and were administered to preexisting murine tumors in vivo. The data demonstrate that AdRHD-modification of murine DCs: (a) induces DCs to produce high levels of proinflammatory cytokines and up-regulates DC surface costimulatory markers; (b) enhances the capacity of DCs to present model peptide antigen to an antigen-specific T-cell line; (c) elicits a marked tumor-specific CTL response when administered into preexisting murine tumors, resulting in inhibition of tumor growth and prolonged animal survival; and (d) the antitumor effect of AdRHD-modified DCs is dependent on their production of IL-12, which, in turn, is dependent on the presence of the c-Rel subunit of the NFκB family of proteins.

MATERIALS AND METHODS

Mice. Six to 8-week-old male wild-type BALB/c (H-2d), wild-type C57BL/6 (H-2b), wild-type B6129P2/J (H-2b), CD4+ T-cell deficient (C57BL/6-CD4tm1Mas), CD8+ T-cell deficient (C57BL/6-CD8tm1Mas), MHC class I-deficient (C57BL/6-B2mtm1Mas), MHC class II-deficient (C57BL/6-Abbtm1Mas), and IL-12-deficient (C57BL/6-Il12tm1Mas) mice were obtained from the Jackson (Bar Harbor, ME) or Taconic (Germantown, NY) laboratories. The respective reagents of the CD4 -/-, CD8 -/-, MHC I -/-, and MHC II -/- mice were confirmed by subjecting splenocytes to flow cytometric analysis (data not shown). Mice harboring targeted mutations in the NFκB p50 (B6129P-Nkbptm1Boj) and RelB [C57BL/6-Tg(H2KkGH1)106Bri Relbtm1Boj] genes were obtained from The Jackson Laboratory, whereas mice lacking the c-Rel subunit (c-Rel -/-) of NFκB were provided as a generous gift from Hsiou-Chi Liou (Weill Medical College of Cornell University, New York, NY). All of the animals were housed under specific pathogen-free conditions.

Cell Culture. CT26 is a colon adenocarcinoma (H-2d, syngeneic to BALB/c mice) originally derived by intrarectal injections of N-nitro-N-methyurethane in a female BALB/c mouse (provided by Nicholas P. Restifo, National Cancer Institute, Bethesda, MD). The SVBalb fibroblast cell line is also syngeneic to BALB/c (H-2d, provided by Linda Gooding, Emory University, Atlanta, GA). The B16 clone F10 melanoma (H-2b, syngeneic to C57BL/6), was obtained from ATCC (Manassas, VA). The OVA-specific T-lymphocyte hybridoma, D011.10, was a generous gift from Marcel van den Brink (Sloan-Kettering Institute, New York, NY). The cell lines were cultured in complete (10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin) RPMI 1640 (CT26, D011.10) or DMEM (SVBalb) unless otherwise specified. The B16,F10 line was cultured and maintained as instructed by the ATCC.

DCs were generated from bone marrow precursors of syngeneic mice as described previously (21, 22). Briefly, the bone marrow derived cells were cultured for 6 days in complete RPMI 1640 (10% FBS) supplemented with 100 units/ml recombinant GM-CSF (R&D Systems, Minneapolis, MN) and 2 ng/ml recombinant mouse IL-4 (R&D systems). As demonstrated by flow cytometric analysis (Table 1), 54–87% of the resulting nonadherent cell population displayed the characteristic murine DC phenotype (CD11c+, CD3-), which remained consistent throughout all strains of mice used in this study, with <7% contaminating T cells.

Ad Vectors and Transduction of DCs. All of the Ad vectors used in this study are replication-deficient, E1-, E3-, serotype 5 vectors produced in human embryonic kidney (293) cells (ATCC, Bethesda, MD) and purified through two cesium chloride gradient ultracentrifugations as described previously (23, 24). Viral particle concentration was determined by UV absorbance at 260 nm (25). Each Ad vector contains an expression cassette with the cytomegalovirus early/immediate gene promoter/enhancer driving the cDNA. The AdRHD vector was a generous gift of Josef Anrather (Weill Medical College of Cornell University, New York, NY; Ref. 20).

<table>
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<th>Table 1</th>
<th>Comparison of percentage of DC (CD11c+, CD3-) in bone marrow preparations after 6 days in culture for knockout mice and respective parental control strains</th>
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<tr>
<td>Knockout</td>
<td>% DC in bone marrow preparation from knockout</td>
</tr>
<tr>
<td>IL-12 -/-</td>
<td>60% C57BL/6</td>
</tr>
<tr>
<td>CD4 -/-</td>
<td>63% C57BL/6</td>
</tr>
<tr>
<td>CD8 -/-</td>
<td>63% C57BL/6</td>
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<tr>
<td>p50 -/-</td>
<td>87% B6129P2J</td>
</tr>
<tr>
<td>RelB -/-</td>
<td>64% C57BL/6</td>
</tr>
<tr>
<td>c-Rel -/-</td>
<td>76% C57BL/6</td>
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encodes for aa 2–320 of the human p65/RelA preceded by a 13 aa sequence containing 10 aa from the human c-myc gene used as a recognition sequence by the antihuman c-myc mAb (20, 26). AdNull is a similar vector but contains no transgene in the expression cassette (27).

Unless otherwise stated, DCs were transduced with Ad vectors after 6 days of \textit{ex vivo} culture at a moi of 100 for 24 h in complete RPMI 1640 (without GM-CSF or IL-4). Before \textit{in vivo} administration, the transduced DCs were washed using PBS (pH 7.4) to remove any remaining, viable vector.

\textbf{Cytokine Production by DCs.} To evaluate cytokine production, DCs were transduced with Ad vectors as described above. After transduction, cells were plated in 96-well plates (5 \times 10^5 cells, 200 \mu l complete medium without GM-CSF or IL-4). After 72 h of incubation (37\circ C), the supernatants (150 \mu l/well) were harvested and centrifuged to remove debris. The levels of cytokines (p40 IL-12, IL-6, MIP-2, MIP-1\alpha, RANTES, and IL-1\beta) released into the culture medium were quantified by a commercially available ELISA kit (R\&D Systems).

\textbf{DC Surface Marker Expression.} To evaluate expression of DC activation surface markers, DCs from BALB/c mice were transduced with Ad vectors as described above, and incubated (48 h, 37\circ C) in complete RPMI 1640 (without GM-CSF or IL-4). The DCs were then stained with FITC- and phycoerythrin-conjugated mAbs against the activation markers CD40, and I-A\beta (MHC class II), CD86 (B7.2), and CD80 (B7.1), or appropriate isotype-controlled matched control Abs (PharMingen, San Diego, CA) as described previously (28). Cells were then subjected to flow cytometric analysis (FACScalibur; Becton Dickinson, San Jose, CA). For coexpression analysis, the labeled DCs were analyzed by a density plot of 530 nm for FITC and 585 nm for phycoerythrin; 1\% false positive events was accepted for isotype control Ab staining.

\textbf{Presentation of Peptide Ag by DC to a T-Cell Hybridoma in Vitro.} To assess Ag presentation by DCs modified by AdRHD, the OVA-specific T-cell hybridoma D011.10 was used. This cell line specifically recognizes the OVA\textsubscript{323–339} peptide and secretes IL-2 when the peptide is presented by APCs in an MHC-restricted fashion (29). This peptide requires no internal processing by the APC, and, thus, the D011.10 cells secrete IL-2 even when the APCs have been placed in fixative agents before encountering the peptide (29).

After transduction with either AdRHD or AdNull for 24 h, DCs were fixed in 2\% paraformaldehyde for 1 min to eliminate any stimulatory effect of the D011.10 cells on the BMDC in coculture. Sham-transduced DCs served as an additional control. DCs were then washed in PBS and resuspended in complete RPMI 1640 that contained 5 \mu g/ml OVA\textsubscript{323–339} peptide. After a 2-h incubation at 37\circ C, excess peptide was removed, and the DCs were washed in complete RPMI 1640. Varying numbers of DCs were transduced with Ad vectors after 6 days of \textit{in vitro} incubation at 37\circ C, excess peptide was removed, and the DCs were washed in complete RPMI 1640. Varying numbers of DCs were then cocultured with 10^5 D011.10 cells/well in complete RPMI 1640 in 96-well plates. After 24 h of incubation at 37\circ C, the supernatants were harvested, and IL-2 was quantified using a commercially available ELISA kit (R\&D Systems). Experiments were performed in triplicate and averaged.

\textbf{In Vivo Tumor Model.} Tumor cells (2 \times 10^5 CT26 or 3 \times 10^5 B16.F10 in 100 \mu l PBS) were injected s.c. into the flanks of syngeneic mice. When tumor growth reached \~30 mm\textsuperscript{2} (day 5–8), AdRHD-modified DC (DC-AdRHD) or AdNull-modified DC (DC-AdNull) were administered intratumorally in 100 \mu l PBS (5 \times 10^5 DC for CT26 tumors or 10^5 DC for B16.F10 tumors). Untreated tumors served as additional controls. The size of the flank tumor was assessed every 2–3 days, and recorded as average tumor area (mm\textsuperscript{2}) \pm SE by measuring the largest perpendicular diameters using micrometers. When the animals appeared moribund or the tumor growth reached 15 mm in diameter, the mice were sacrificed; this time point was defined as death for survival analysis.

\textbf{Cytotoxic T Lymphocytes.} To demonstrate the induction of tumor-specific CTL response by administration of AdRHD-modified DCs to the tumors, 14 days after intratumoral administration of AdRHD, DC-AdNull or DC-sham, splenocytes (8 \times 10^6) of 3 mice from each group were harvested, pooled, and restimulated with mitomycin C (100 \mu g/ml for 90 min; Sigma, St. Louis, MO) treated CT26 cells (2 \times 10^5) for 5 days in 4 ml of complete RPMI 1640. After restimulation, viable effector cells at various effector to target ratios (6:1, 1:1, and 1:6) were analyzed for CTL activity by evaluation of their ability to lyse 	extsuperscript{51}Cr-labeled CT26 target cells (2 \times 10^5/200 \mu l/well) during a 4-h incubation at 37\circ C as described previously (22, 28). The percentage of specific 	extsuperscript{51}Cr release was calculated as [(experimental release – spontaneous release)/(maximal release – spontaneous release)] %.

\textbf{Statistical Analysis.} All of the data are reported as mean \pm SE. Statistical comparison between means was performed using the unpaired two-tailed Student’s \textit{t} test. Survival evaluation was performed using the Kaplan-Meier analysis with statistical significance determined using the generalized Wilcoxon test.

\section*{RESULTS}

\textbf{Expression of Activation Cytokines and Surface Activation Markers by DCs Modified by AdRHD.} Evaluation of the secreted cytokine profile by DCs revealed that AdRHD-mediated delivery of the truncated p65 CDNA to DCs markedly enhanced the production of IL-12 (p40 subunit), IL-6, and IL-1\beta compared with controls (AdRHD versus AdNull; Fig. 1A, \textit{P} < 0.005; Fig. 1B, \textit{P} < 0.001; Fig. 1C, \textit{P} < 0.01). In contrast, DC secretion of MIP-2, MIP-1\alpha, and RANTES did not increase after modification with AdRHD when compared with either AdNull or sham-transduced DCs (data not shown).

In a similar fashion, DCs modified with AdRHD expressed higher levels of surface activation markers compared with those transduced with PBS (sham transduction) or AdNull (Fig. 2). The DCs activated by AdRHD exhibited surface levels of CD40, MHC class II, B7.1, and B7.2 that approached twice that seen with that in the PBS group (DC-sham). Transduction of DCs with AdNull slightly increased activation marker expression, but not to the degree seen with AdRHD.

\textbf{Ability of DCs Modified with AdRHD to Present a Model Peptide Antigen.} To assess the antigen-presenting capacity of DCs and to determine whether AdRHD modification affects this function, the ability of DCs to present the OVA\textsubscript{323–339} peptide to the OVA-specific D011.10 T-lymphocyte line was evaluated. At low DC:T cell ratios (1:100), AdRHD modification of the DCs clearly increased IL-2 production by
The Survival in Mice with Established Tumors. Reduced with AdRHD to Inhibit Tumor Growth and Prolong
DCs Is Dependent on the CD4 and CD8 T-Cell Subtypes in the Mechanism of the Antitumor Immunity Induced by AdRHD-modified DCs. To determine whether systemic CTLs were induced after intratumoral administration of DCs modified by AdRHD, splenocytes from treated BALB/c mice were evaluated in a standard 51Cr release assay using CT26 tumor cells as targets. Splenocytes from DC-AdRHD-treated mice were able to lyse significantly more CT26 targets compared with splenocytes from the controls, even at low E:T ratios (Fig. 5A; P < 0.0001; 6:1 ratio; P < 0.01; 1:1 ratio). The CTL response was dose-dependent as demonstrated by the increasing specific 51Cr release with increasing E:T ratios. Minimal CTL activity was demonstrated for the control SVBalb cell line for all of the groups (Fig. 5B; P > 0.05).

The Antitumor Effect Induced by AdRHD-modified DCs Is Dependent on the CD4+ Cell/MHC Class II and CD8+/MHC Class I Pathways. To evaluate the role of T-cell subtypes in the mechanism of the antitumor immunity induced by AdRHD-modified DCs, the efficacy of this treatment was evaluated in B16.F10 tumor-bearing C57BL/6 wild-type, CD4−/−, and CD8−/− mice. DCs for these experiments were obtained from bone marrow from CD4−/− and CD8−/− mice to rule out any potential role of contaminating T cells in the DC preparation. Tumor growth was not inhibited in either the CD4−/− and CD8−/− mice that received DC-AdRHD, suggesting that both T-cell subtypes are required for antitumor immunity (Fig. 6A; CD4−/− versus CD8−/− wild-type/untreated).

Because CD4+ cells recognize Ag in the context of MHC class II and CD8+ cells in the context of class I, the role of these two DC surface receptors in the antitumor immunity induced by DC-AdRHD was evaluated. DCs obtained from MHC class I−/− or class II−/− donor mice were transduced with AdRHD and administered to C57BL/6 wild-type mice bearing B16.F10 tumors. Tumor growth was only partially inhibited when MHC class I or II−/− DCs transduced by AdRHD were administered intratumorally (Fig. 6B, P < 0.01, MHC class I or II−/− versus untreated group). There was no difference in tumor growth between the MHC class I−/− and II−/− DC-AdRHD groups (P > 0.1).
Dependency of the Antitumor Effect of AdRHD-modified DCs on IL-12. In light of the marked production of IL-12 by DCs modified with AdRHD, the role of IL-12 secreted by the injected DCs in the antitumor effect of DC-AdRHD was assessed. BMDCs were harvested from IL-12−/− mice, transduced with AdRHD, and provided as therapy to B16.10 tumor-bearing C57BL/6 wild-type mice. Inhibition of tumor growth was significantly attenuated in the animals that received DC-AdRHD from IL-12−/− mice compared with DCs from wild-type mice (Fig. 6C; P < 0.005). However, tumors still grew more rapidly in the untreated control group compared with those treated with DC-AdRHD obtained from IL-12−/− mice (Fig. 6C; P < 0.01).

**DISCUSSION**

This study is based on the hypothesis that Ad-mediated gene transfer of only the RHD region of NFκB (lacking the TD) to BMDCs may induce DC activation and enhance specific DC immune functions, thereby augmenting the antitumor efficacy of DC therapies in vivo. The data show that AdRHD-modification of DCs induced the secretion of high levels of proinflammatory cytokines (p40 IL-12, IL-6, and IL-1), as well as up-regulation of surface activation and costimulatory markers [CD40, MHC class II, CD86 (B7.2), and CD80 (B7.1)]. In addition, in vitro peptide Ag presentation by AdRHD-modified DCs to an Ag-
specific T-lymphocyte hybridoma was enhanced at low DC:T cell ratios. As a result of AdRHD modification and subsequent in vivo intratumoral delivery, DCs induced a tumor-specific CTL response, inhibited the growth of preexisting tumors in two different mouse strains, and enhanced animal survival. The presence of the endogenous c-Rel, but not the p50 or RelB subunits of NFκB, was necessary for AdRHD-mediated up-regulation of IL-12 (p40) secretion by DCs, which in turn was needed for in vivo inhibition of tumor growth.

**DC Activation by AdRHD.** DCs are professional APCs that play a critical role in the activation of the immune response to antigen (1–4). Production of cytokines by DCs, particularly IL-12, has been shown to correlate with the maturation status of DCs (1–4). In the present study, modifying DCs to produce a truncated form of the p65 NFκB protein (RHD) induced DC maturation, as demonstrated by the enhanced production of the proinflammatory cytokines IL-12, IL-6, and IL-1β. IL-12 is thought to be an important polarizing cytokine for Th1 cells (30, 31), a critical mediator in the cellular immune responses to tumors (30, 31). When secreted by mature DCs, the presence of IL-12 induces the differentiation of Th0 cells into Th1 cells, which produce IFN-γ (30, 31).

In addition to cytokine secretion, mature DCs express high levels of costimulatory molecules, necessary components of T-cell activation by APCs, which must occur in conjunction with MHC-restricted presentation of the antigenic peptide to the T-cell receptor.

The costimulatory molecules identified on DCs with their respective T-cell receptors are CD54 (ICAM-1) and CD11a/18 (LFA-1), B7.1/B7.2, CD28, CD40, and CD154 (CD40 ligand; Refs. 1–4). In the present study, the costimulatory molecules B7.1, B7.2, and CD40 were clearly up-regulated after AdRHD modification of DCs compared with controls. Although other markers of DC maturation exist, such as CD83, we chose to monitor CD86, CD80, and CD40 because these are known to directly be involved in the antigen presentation process.

In addition to the elaboration of cytokines, which may act locally or distantly, the primary purpose of DCs is to initiate the immune response to foreign antigen by presenting these antigen to quiescent T lymphocytes (1–4). The efficiency at which this function is performed correlates with the maturation status of the DCs, with immature DCs being poor antigen presenters.

To evaluate the ability of DCs to present peptide antigen to T lymphocytes, the T-cell hybridoma, D011.10, was chosen. This hybridoma exclusively recognizes the OVA323–339 peptide but does not require the peptide to be ingested and processed by DCs. The DCs were fixed in 2% paraformaldehyde before exposure to the peptide and T cells to eliminate any stimulatory effect of the T cells on the DCs (through such interactions as CD40L-CD40; Ref. 32). The data clearly show that at low DC:T-cell ratios (1:100), AdRHD significantly enhanced Ag presentation by DC over AdNull or sham-transduction. However, at high DC:T-cell ratios (1:1), AdRHD had an inhibitory effect on T-cell stimulation, an effect which may be because of interaction between activated DCs and themselves, because activated DCs tend to clump in culture. This finding is similar to a study by Jonuleit et al. (33) reporting that DCs transduced with Ad vector encoding a marker gene inhibited T-cell proliferation at high DC:T-cell ratios. The in vivo ratio of DC to T lymphocytes more closely approaches 1:100, because murine lymph nodes typically contain <1% DC.

**Role of the RHD Transgene.** Initial studies evaluating AdRHD examined its effect on cultured endothelial cells (20, 26). These reports demonstrated that the RHD transgene func-
spleocytes were harvested, restimulated with mitomycin C-treated CT26 cells for 5 days in vitro, and evaluated for cytotoxicity against parent CT26 cells or the syngeneic control cell line, SVBalb, using a standard 51Cr release assay. Untreated tumor-bearing mice as well as intratumoral administration of sham-transduced DCs served as additional controls. Each point represents the mean; bars, ±SE. A, CT26 cells; B, SVBalb cells.

Fig. 5 Assessment of CTL response in tumor-bearing mice induced by intratumoral injection of AdRHD-modified DCs. Fourteen days after injection of DCs transduced with either AdRHD (n = 3) or AdNull (n = 3; 5 × 10^6 cells) into established CT26 tumors (BALB/c mice), total splenocytes were harvested, restimulated with mitomycin C-treated CT26 cells for 5 days in vitro, and evaluated for cytotoxicity against parent CT26 cells or the syngeneic control cell line, SVBalb, using a standard 51Cr release assay. Untreated tumor-bearing mice as well as intratumoral administration of sham-transduced DCs served as additional controls (n = 3). Data points represent the mean for each group; bars, ±SE. A, CT26 cells; B, SVBalb cells.

The discrepancy in the pro versus inhibitory function of AdRHD between these reports and the present study is unclear, and is likely explained by the cell type and situation-specific manner in which the NFκB homodimers are known to act (5–7, 16, 17). Given that the RHD transgene product does not possess the TD of NFκB, two possibilities exist as potential mechanisms for the effect of AdRHD on DCs. First, the RHD segment may have some nuclear activating capacity for certain genes in DCs independent of the TD. Along these lines, although the p50 homodimer and the p52 homodimer (both lacking the TD) were initially thought to be transcriptionally repressive (5–7, 16, 17), subsequent investigations have suggested that the RHD segment may combine with one or more of the TD-containing NFκB family members (p65, RelB, and c-Rel) forming transcriptionally active dimers. The data from the present study support this hypothesis, because AdRHD had no effect on IL-12 (p40) secretion in c-Rel−/− mice, implying that a c-Rel/RHD dimer is responsible for IL-12 up-regulation.

This finding is consistent with a previous study demonstrating that a c-Rel/RHD dimer is responsible for IL-12 up-regulation.
that c-Rel is necessary for IL-12 (p40) gene induction in macrophages (34) but conflicts with another report implicating c-Rel as a regulator in the induction of transcription of the p35, but not p40, subunit of IL-12 in CD8+ DCs (35). These potentially conflicting findings suggest that the various NFκB homo- and heterodimers probably function in a cell type and situation-specific manner.

An additional, interesting finding in the present study is the presence of CD11c+, CD3− cells in the bone marrow of RelB−/− mice. Previously published data have shown that mice lacking RelB possess a severe functional defect in splenic APCs, suggesting lack of development of mature DCs (36). However, the present study demonstrates not only the presence of CD11c+, CD3− cells in the bone marrow of these mice, but also that these cells secrete high levels of IL-12 (p40) in response to AdRHD transduction, suggesting that these DCs may have matured, at least to some degree. Whether or not these BMDCs possess antigen presenting ability was not evaluated in this study.

Unfortunately, the effect of AdRHD on DCs could not be studied in mice lacking p65 given the lethality of the homozygous form of this mutation (37).

"Preactivating" DCs with AdRHD Elicits Tumor-specific Immunity. The present study demonstrates that DCs, activated *ex vivo* with AdRHD, induce tumor-specific immunity resulting in tumor growth inhibition and prolonged animal survival when administered intratumorally. The antitumor immune response generated by AdRHD-modified DCs was dependent on CD4+ as well as CD8+ cells. In addition, DC expression of both MHC class I and II was necessary, at least in part, for this response. These data suggest that DCs, which are activated by AdRHD transduction, act not only by priming CD4+ "helper" cells, which in turn may activate CTL, but also by directly priming CTLs themselves, a class I-dependent pathway.

Although recent literature has suggested that culturing DCs in FBS may result in the presentation and recognition of miscellaneous xenogeneic antigens, which may result in tumor cell lysis (if the target tumor cells are cultured in FBS as well; Ref. 38), this phenomenon is probably not playing a role in the present study because DC culture conditions remained constant throughout the experimental and control groups. In addition, BALB/c BMDCs are difficult to culture in the absence of serum (39, 40). However, in the clinical setting, culture of DCs in either serum-free medium or with autologous serum is potentially the safer technique.

Activating DCs before their encounter with antigen, described in the present study, at first seems to represent a counterintuitive strategy, because DCs *in situ* become activated only after antigen contact. However, our data and that of others, clearly show that antitumor immunity is achieved using therapies consisting of preactivated DCs (41–43). These activated DCs are able to present a model peptide antigen more efficiently to T cells at low DC:T-cell ratios, such as those that are probably seen in the regional lymph nodes where antigen presentation occurs. In addition, AdRHD-transduced DCs elaborate significant amounts of IL-12, a cytokine that appears to be at least partially responsible for the antitumor property of the RHD genetically modified DCs. IL-12 is an important cytokine involved in the development of antitumor immunity by a variety of mechanisms including the activation and generation of CTLs and promotion of a Th1-T-cell response (30, 31). Given the significant *in vivo* antitumor qualities of preactivated DCs, it seems likely that the beneficial effects of increased antigen presentation as well as cytokine release far outweigh any potentially negative effects on antigen uptake capacity. As a result, DC preactivation strategies warrant additional investigation when designing DC-based antineoplastic vaccines. Future investigation will need to focus on the optimal preactivation strategy and involve experiments comparing AdRHD to other DC activators, including, but not limited to, tumor necrosis factor α, lipopolysaccharide, and CD40 ligation. In this regard, optimal activation may not necessarily mean producing the most extremely activated DC, but instead producing a “balanced” DC that is efficient at both antigen uptake and antigen presentation.

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Adenovirus Vector-mediated Overexpression of a Truncated Form of the p65 Nuclear Factor κB cDNA in Dendritic Cells Enhances Their Function Resulting in Immune-mediated Suppression of Preexisting Murine Tumors


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