

## Ligand-Independent Toll-like Receptor Signals Generated by Ectopic Overexpression of MyD88 Generate Local and Systemic Antitumor Immunity

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### Abstract

Although critical for initiating and regulating immune responses, the therapeutic use of individual cytokines as anticancer immunotherapeutic agents has achieved only modest clinical success. Consequently, many current strategies have focused on the use of specific immunotherapeutic agonists that engage individual receptors of innate immune networks, such as the Toll-like receptor (TLR) system, each resulting in specific patterns of gene expression, cytokine production, and inflammatory outcome. However, these immunotherapeutics are constrained by variable cellular TLR expression and responsiveness to particular TLR agonists, as well as the specific cellular context of different tumors. We hypothesized that overexpression of MyD88, a pivotal regulator of multiple TLR signaling pathways, could circumvent these constraints and mimic coordinated TLR signaling across all cell types in a ligand-independent fashion. To explore this hypothesis, we generated an adenoviral vector expressing MyD88 and show that Ad-MyD88 infection elicits extensive Th1-specific transcriptional and secreted cytokine signatures in all murine and human cell types tested *in vitro* and *in vivo*. Importantly, *in vivo* intratumoral injection of Ad-MyD88 into established tumor masses enhanced adaptive immune responses and inhibited local tumor immunosuppression, resulting in significantly inhibited local and systemic growth of multiple tumor types. Finally, Ad-MyD88 infection of primary human dendritic cells, tumor-associated fibroblasts, and colorectal carcinoma cells elicited significant Th1-type cytokine responses, resulting in enhanced tumor cell lysis and expansion of human tumor antigen-specific T cells. Thus, Ad-MyD88 initiated robust anti-tumor activity in established murine tumor microenvironments and in human contexts, suggesting its potential effectiveness as a clinical immunotherapeutic strategy. *Cancer Res*; 70(18); 7209–20. ©2010 AACR.

### Introduction

Immunotherapy strategies either frequently target single tumor antigens or, alternatively, nonspecifically induce anti-tumor immunity through nonspecific approaches, such as the systemic delivery of purified cytokines (1, 2). The development of gene transfer techniques has expanded focus on individual effector cytokine, chemokine, or receptor genes delivered to specific tissues, such as granulocyte macrophage

colony-stimulating factor (GM-CSF) gene delivery (3). These advances focused attention on the need for therapeutic synergy obtained from coordinated sets of cytokines (4), typically induced after stimulation of different pattern recognition receptors (PRR).

One of the best-characterized PRR families is the Toll-like receptor (TLR) family, which plays an instrumental role in the generation of inflammatory and adaptive responses to a wide range of pathogen- or danger-associated molecular patterns (PAMP and DAMP; ref. 5). TLRs are activated by ligand-mediated dimerization of different TLR family members, which in turn recruit adaptor proteins to initiate a unique collection of signaling pathways. Thus, TLR signaling elicits a pattern of cytokines and chemokines that result in a characteristic immune response, not often achieved by single cytokine delivery (6). Significantly, as different TLR agonists are combined and multiple TLR receptors are engaged, cellular immune responses often become more synergistically complex and, in some cases, polarize cells toward a Th1 phenotype (7).

Although many different TLR-specific agonists are currently being tested (8), most are being used in isolation, thus negating the synergistic advantages afforded by the stimulation of multiple pathways. Furthermore, the effectiveness of single

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Microarray data were submitted to the National Center for Biotechnology Information's Gene Expression Omnibus Web site (<http://www.ncbi.nlm.nih.gov/geo/>), deposited as accession numbers GSE18957 and GSE19006 using platform GP6524.

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TLR agonists is highly dependent on the responsiveness of tumor cells to TLR agonists (9, 10), which is dependent on cellular TLR expression that varies by tissue and can be repressed in certain tumor microenvironments (11). We hypothesized that an alternative strategy to overcome these obstacles could be to stimulate coordinated TLR signaling in multiple cell types by overexpressing adaptor proteins, which serve as pivotal signaling scaffolds for all TLRs.

TLR signaling interfaces with diverse sources of input, enabling responses to a large diversity of PAMPs, and generates broad responses through a conserved core consisting of a limited number of adaptor genes (12). Chief among these is MyD88, which is critical for signaling in all TLRs (with the exception of TLR3). On recruitment, MyD88 homodimerizes and initiates a broad cascade of coordinated immune responses from a wide range of TLR combinations (13, 14). Overexpression of MyD88 has been shown to mimic these responses, as MyD88 accumulates and homodimerizes in various cellular compartments to elicit cellular innate immune responses (15, 16). Multiple studies have confirmed that MyD88 expression is critical in initiating cellular immune responses to a wide range of pathogens in a variety of different cell types (6). Although MyD88-mediated inflammation has been shown to be protumorigenic in certain models of tumorigenesis, it has also been proven critical for initiating multiple types of antitumor immunity (17, 18).

Given the importance of MyD88 in mediating immune responses, we hypothesized that specific overexpression of MyD88 by adenoviral (Ad) vectors could initiate broad innate cellular and subsequently adaptive immune responses in a variety of cell types found in the tumor microenvironment. This strategy would overcome lack of TLR expression or responsiveness, initiate extensive TLR signaling, and be independent of TLR ligands. We further hypothesized that delivery of this gene *in vivo* would stimulate proinflammatory responses and alter the immunosuppressive tumor microenvironment to produce therapeutic antitumor immunity *in vivo*.

## Materials and Methods

### Vector preparation

Mouse and human MyD88 [plasmids 7502654 and 10700610; American Type Culture Collection (ATCC)] were cloned into E1 and E3 shuttle plasmids and used to generate [E1-E3-] Ad vectors using pAdEasy (19). Dual-expressing Ad vector was generated by inserting cytomegalovirus-driven LacZ into the E3 region of pAd-MyD88 constructs. Ad vector stocks were evaluated for replication-competent adenovirus via real-time PCR (RT-PCR) and titered using the AdEasy Titer kit (Stratagene).

### *In vitro* tumor cell lines and procedures

CT26.CL25 (LacZ-expressing mouse colon carcinoma), CT26.WT (mouse colon carcinoma), B16-F10 (mouse melanoma), and 4T1 (mouse breast carcinoma) cells were obtained from ATCC and cultured accordingly. The CpG ODN 1826 (5'-TCCATGACGTTCCCTGACGTT) was purchased from

Coley Pharmaceutical Group and used *in vitro* at a concentration of 5  $\mu$ g/mL. Primary colon cancer cells and tumor-associated fibroblasts from surgically resected tumors, C57/BL6 wild-type mouse embryonic fibroblasts (MEF), and HLA-A\*0201 peripheral blood mononuclear cells (PBMC) were kind gifts from Drs. David Hsu, Joseph Nevins, and H. Kim Lyerly, respectively (Duke University, Durham, NC). Mouse dendritic cells (DC) were prepared by culturing bone marrow progenitors in GM-CSF (10 ng/mL) and interleukin (IL)-4 (10 ng/mL) for 5 days, whereas human DCs (hDC) were prepared from PBMCs cultured in GM-CSF (10 ng/mL) and IL-4 (10 ng/mL) for 5 to 7 days (20). Mouse DCs and hDCs were stained using CD80, CD86, CD83, CD40, HLA-DR, and CD11c antibodies (BD Biosciences). Tetramer staining was performed using labeled CD3, CD4, CD8, MART1, and pp65 tetramer mixes (BD Biosciences). Chromium release assays were performed by culturing activated T cells (10-day culture in 600 units/mL IL-2) with targets and incubating with chromium-labeled target cells for 5 hours. Western blots were performed using MyD88 and  $\beta$ -actin antibodies from Abcam. Assessment of cell viability and proliferation was performed using a MTT assay.

### Animal procedures

C57BL/6J, BALB/c, SCID-B6.129S7-Rag1(tm1Mom), and NOD CB17-Prkdc SCID/J mice were purchased from The Jackson Laboratory. All tumors were injected at the indicated doses by s.c. administration in PBS. Tumor size was measured at the indicated time points by caliper measurement, and tumor volume was calculated as (small diameter)<sup>2</sup>  $\times$  (large diameter/2). Footpad viral injections were performed by anesthetizing animals with a ketamine/xylazine/atropine mix and injecting via the footpad. The CpG ODN 1826 (5'-TCCATGACGTTCCCTGACGTT-3') was injected intraperitoneally as a single dose of 20  $\mu$ g in PBS. Intratumoral viral injections were performed by direct injection of virus into the tumor in PBS. Animal studies were approved by the Duke University Institutional Animal Care and Use Committee.

### Microarray methods

RNA was extracted using TRI-Reagent (Molecular Reagents Center), purified using an RNeasy kit (Qiagen), and assessed for quality using both UV spectroscopy and an Agilent Lab-on-a-Chip 2100 Bioanalyzer (Agilent Technologies). RNA was directly labeled and hybridized using the MO36k 70-mer probe sets from Operon (Mouse Genome Set version 4.0) by the Duke University microarray core. Analysis was performed using GeneSpring v7.2 and DAVID v6.0 (21) with data sets deposited at National Center for Biotechnology Information's Gene Expression Omnibus in a MIAME-compliant form (along with complete details of all procedures and analyses) as accession numbers GSE18957 and GSE19006 using the platform GP6524.

### Quantitative RT-PCR and cytokine/chemokine assays

RNA was reverse transcribed using iScript (Bio-Rad), and RT-PCR was performed using FastStart Universal SYBR Green (Roche) with intron-spanning primers, normalizing

to the geometric mean of housekeeping genes (*β-actin*, *HMBS*, and *GAPDH*). We calculated relative expression differences with the comparative  $C_T$  method (22). Serum and supernatants were assayed using Bio-Rad and Bio-Plex 23-Plex mouse and 28-Plex human cytokine kits, according to the manufacturer's recommendations.

### Statistics

Statistics for ELISA, quantitative RT-PCR (qRT-PCR), enzyme-linked immunospot (ELISPOT), and MTT assays were performed using two-tailed homoscedastic Student's *t* test with Bonferroni multiple testing correction for Bio-Plex ELISA samples. For animal studies, statistical differences were calculated with a mixed-effects regression model using autoregressive covariance.

### ELISA and ELISPOT procedures

Anti-adenovirus and anti-LacZ antibodies were quantified using ELISA. Plates were coated with  $1 \times 10^9$  viral particles (vp) or 2  $\mu$ g LacZ (Sigma) per well in a bicarbonate solution [200 mmol/L NaHCO<sub>3</sub>, 81 mmol/L Na<sub>2</sub>CO<sub>3</sub> (pH 9.5)] overnight at 4°C and washed five times before secondary sheep anti-mouse IgG H+L antibody (Jackson ImmunoResearch Laboratories) and *O*-phenylenediamine dihydrochloride substrate (Sigma) application.

Alloantigen-primed IFN- $\gamma$ -producing T cells were quantified using an ELISPOT assay. Capture and detection anti-IFN- $\gamma$  monoclonal antibodies were purchased from MABtech. Stimulatory LacZ peptide (TPHPARIGL, 1  $\mu$ g/mL) and green fluorescent protein (GFP) peptide (HYSTQSAL, 1  $\mu$ g/mL) were purchased from GenScript, whereas LacZ protein (50  $\mu$ g/mL) and amyloglucosidase protein controls (50  $\mu$ g/mL) were purchased from Sigma. HIV irrelevant overlapping peptide mixes were purchased from BD Biosciences. Plates were developed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich), and the number of spots per well was determined using a KS ELISPOT Automated Reader System with KS ELISPOT 4.2 Software (Carl Zeiss, Inc.).

### Results

Although contemporary immunotherapeutic strategies use single defined molecular interventions (23, 24), we sought to deliver the TLR adaptor protein MyD88 as a means to initiate broad innate and adaptive antitumor immune responses within the tumor microenvironment.

#### Ad-MyD88 infection elicits unique patterns of immune-stimulatory gene expression in bmDCs and MEFs and enhances adaptive immune responses *in vivo*

We first investigated the transcriptional effect of Ad-mediated mouse MyD88 overexpression on mouse bone marrow-derived DCs (bmDC). Ad-MyD88 infection resulted in significant MyD88 protein expression as determined by Western blot (Supplementary Fig. S1), and microarray assessment revealed that ~3.1% of gene transcripts were significantly dysregulated by Ad-MyD88 compared with Ad-GFP (25, 26) or mock-infected counterparts (Fig. 1A), mainly in

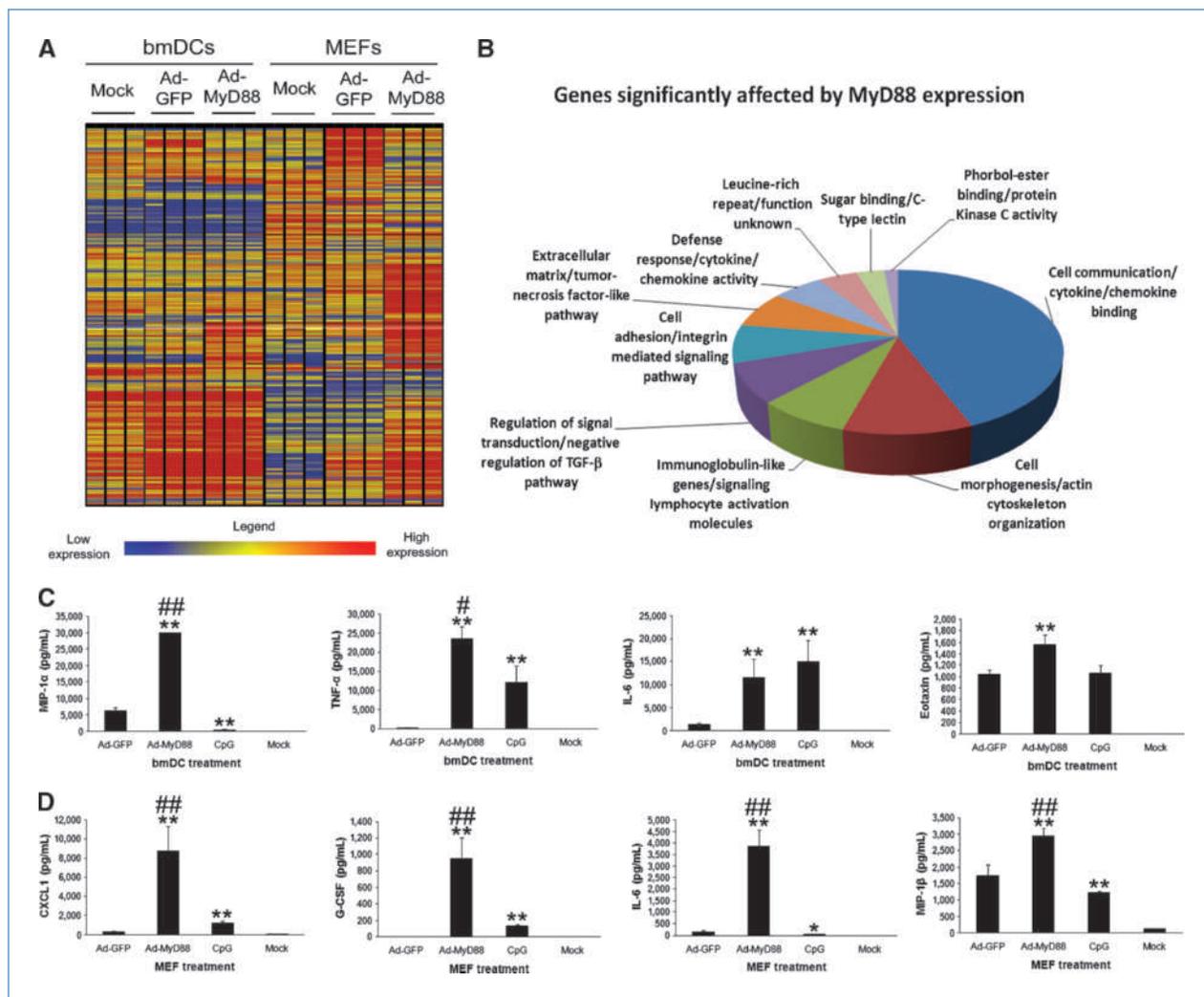
gene groups involving inflammation and myeloid development (using DAVID analysis).

To determine if nonimmune cells infected with Ad-MyD88 would show similar responses, primary MEFs were infected with Ad-MyD88 and transcriptional assessment revealed that an even larger fraction of genes (~4.3%) were significantly dysregulated compared with controls (Fig. 1A and B; Supplementary Fig. S1), mainly in gene clusters involved in inflammation (using DAVID analysis). While revealing differences in specific gene signatures between cell types, these results showed a conserved MyD88 inflammatory response in two primary cell types relevant to the tumor microenvironment.

When all differences between control- and Ad-MyD88-infected MEFs and bmDCs were assessed together by one-way ANOVA ( $P = 0.05$  with Benjamini and Hochberg multiple testing correction), it was revealed that ~8.0% of the total transcriptome was dysregulated by MyD88 overexpression. Functional analysis of these dysregulated genes revealed significant induction of the expression of cytokine/chemokine, lymphocyte activation, actin cytoskeleton, negative regulation of transforming growth factor- $\beta$  (TGF- $\beta$ ), as well as leucine-rich repeat gene clusters (Fig. 1C; Supplementary Table S2). A qRT-PCR analysis of multiple genes in MEFs and bmDCs (Supplementary Table S1) confirmed these differences, and comparison with a MyD88-dependent CpG TLR9 agonist (ODN 1826) determined that Ad-MyD88 largely elicited greater transcriptional responses compared with CpG stimulation (Supplementary Table S1). These transcriptional patterns indicated that MyD88 overexpression elicited a unique and broad Th1-biased immune-stimulatory response in different cell types.

In addition to mRNA differences, alterations in protein expression were determined by Bio-Plex ELISA analysis of supernatants from Ad-MyD88-infected and CpG-treated cells. These assays showed significantly higher secretion of proinflammatory and Th1-related cytokines and chemokines in Ad-MyD88-infected bmDCs (Fig. 1C; Supplementary Fig. S2) and Ad-MyD88-infected MEFs (Fig. 1D; Supplementary Fig. S2). Compared with CpG, we found that Ad-MyD88-infected cells secreted significantly different levels of multiple cytokines and chemokines dependent on cell type (Fig. 1C and D; Supplementary Fig. S2), suggesting differences in the mechanism of MyD88-dependent activation between these treatments. Collectively, these results show that Ad-MyD88 infection induces a selective Th1 inflammatory profile in different primary murine cell types *in vitro* in comparison with control Ad- or CpG-stimulated counterparts.

To determine if Ad-MyD88 could enhance adaptive immune responses, as has previously been reported for a MyD88 plasmid-based strategy (27), we injected mice with a comixture of Ad-MyD88 and Ad-LacZ or with a dual LacZ- and MyD88-expressing Ad versus control Ad vectors. Expression of MyD88, either as an Ad comixture or in the dual-expressing Ad, elicited significantly higher numbers of LacZ-specific IFN- $\gamma$ -secreting functional T cells by ELISPOT (Fig. 2A), indicating an Ad-MyD88 Th1 adaptive immune phenotype. In contrast, MyD88 overexpression did not significantly enhance IgG responses to LacZ or Ad antigens (Fig. 2B and C)



**Figure 1.** Ad-MyD88 infection elicits Th1 inflammatory profiles in primary mouse DCs and fibroblasts. A, microarrays performed for bmDCs and MEFs ( $n = 5$ ). Hierarchical clustered heat map of significant genes. B, gene groups affected by Ad-MyD88 overexpression from both MEFs and bmDCs. Assessment of significantly different cytokines and chemokines at 16 h after infection in bmDCs ( $n = 4$ , multiplicity of infection (MOI) = 2,000; C) and MEFs ( $n = 4$ , MOI = 150; D). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Ad-MyD88- and CpG-treated cells versus Ad-GFP-infected controls; #,  $P < 0.05$ ; ##,  $P < 0.01$ , Ad-MyD88-treated versus CpG-treated cells. Bars, SD.

### Ad-mediated MyD88 overexpression alters gene expression in tumor cells and suppresses growth of established tumors

In contrast to the characteristic patterns of gene expression we observed in various untransformed cell types, we hypothesized that the genetic instability and potential loss of TLR signaling mediators in different tumor types could significantly hinder immune MyD88 pathway activation (28). Therefore, we compared Ad-mediated MyD88 overexpression with CpG (ODN 1826) treatment in murine tumor cell lines CT26.CL25, 4T1, and B16-F10. As before, Western blots revealed that MyD88 was strongly overexpressed in Ad-MyD88-infected tumor cells (Supplementary Fig. S3), and different types of tumor cells were highly responsive to MyD88 overexpression, with multiple inflammatory genes be-

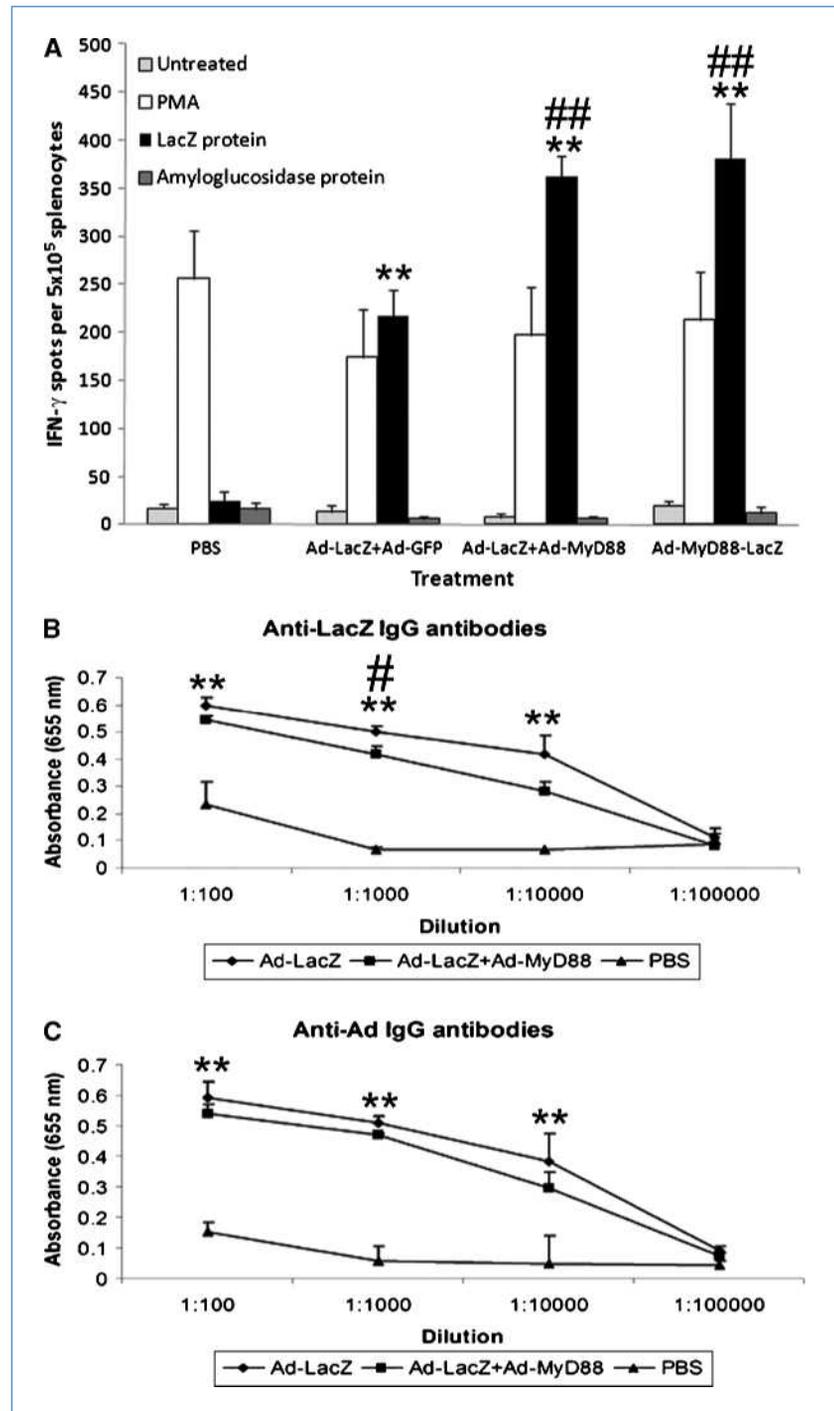
ing strongly activated (Fig. 3A). In contrast, these tumor lines were not highly responsive to CpG stimulation (Fig. 3A). Significantly, Ad-MyD88 infection elicited enhanced expression of multiple cytokines and chemokines in supernatants (ELISA; Fig. 3B; Supplementary Fig. S4), thus indicating largely intact functional signaling pathways downstream of MyD88 in different tumor types.

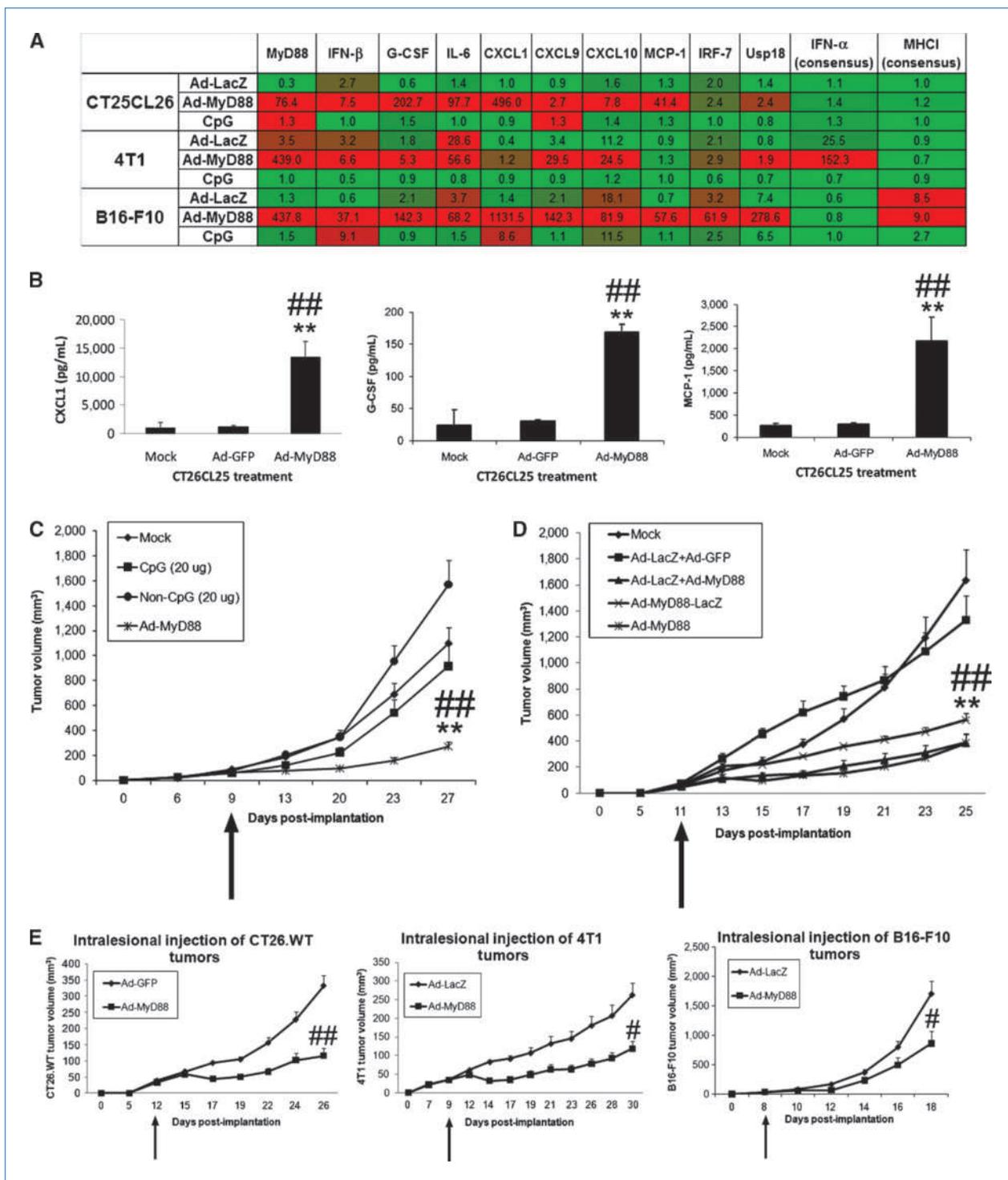
We next compared the therapeutic efficacy of Ad-MyD88 with a TLR9 agonist (CpG 1826; ref. 29) that could activate innate responses in primary cells but not in tumor cells. As established tumors are therapeutically resistant (30), 11-day-old (mean tumor volume,  $\sim 50 \text{ mm}^3$ ) CT26.CL25 LacZ<sup>+</sup> tumors were injected intrasiesionally with Ad-MyD88, CpG, or vehicle or non-CpG controls (Fig. 3C). Tumor masses injected with Ad-MyD88 had significantly retarded growth in contrast to

the minor effects of CpG. To determine if these responses could be enhanced by targeting the tumor-specific antigen LacZ, we performed intrasplenic injections of Ad-MyD88, Ad-LacZ, Ad-MyD88-LacZ, Ad-GFP, comixtures of these vectors, or vehicle buffer alone. Notably, we found that Ad-LacZ injection did not significantly affect tumor growth (Fig. 3D),

although the tumors continued to express LacZ (data not shown). In contrast, all tumors injected with a MyD88-expressing vector had significantly reduced growth, independent of Ad-LacZ administration (Fig. 3D). To eliminate the immunologic effects of LacZ expression, we tested a non-LacZ-expressing model. As before, a single intrasplenic

**Figure 2.** Ad-MyD88 infection elicits enhanced activation of adaptive immune responses *in vivo*. A, C57/BL6 mice were vaccinated ( $2.6 \times 10^{10}$  vp) via footpad and adaptive immune responses were assessed at 2 wk after infection by ELISPOT [using control phorbol 12-myristate 13-acetate (PMA), LacZ (50  $\mu$ g/mL), control amyloglucosidase protein (50  $\mu$ g/mL), or mock stimulation ( $n = 5$ )]. B and C, ELISA was performed on vaccinated mice (at 14 d after injection) to determine LacZ-specific (B) and Ad-specific (C) IgG antibodies in mice treated as above ( $n = 5$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus mock-infected controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus Ad control-infected counterparts. Bars, SD.





**Figure 3.** Ad-MyD88 activates innate immune responses in tumor cells and strongly suppresses tumor growth *in vivo*. A, CT26.CL25, B16-F10, and 4T1 cells were infected (MOI = 200) with Ad-MyD88, Ad-GFP, CpG (5  $\mu$ g/mL), or mock and transcription was assessed at 48 h after infection by qRT-PCR. The average fold change is displayed by color-coded gradient (red for higher expression and green for lower expression;  $n = 3$ ). B, using conditions as in A, supernatants were harvested from CT26.CL25-infected cells and cytokines/chemokines were assessed at 24 h after infection ( $n = 3$ ). Several representative cytokines/chemokines are displayed. C and D, BALB/c mice with CT26.CL25 tumors were intrasionaly treated as shown at 9 to 11 d after infection (indicated by arrow) and tumor growth was measured over time ( $n = 5$ ). Bars, SE. E, BALB/c bearing CT26.WT (no LacZ), 4T1, or B16-F10 tumors were vaccinated at 8 to 12 d after infection (indicated by arrow) as in C ( $n = 5$ ). Bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus mock controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus Ad control- or CpG-treated counterparts.

injection of Ad-MyD88 into established (day 12,  $\sim 50 \text{ mm}^3$ ) non-LacZ-expressing CT26.WT colon carcinomas resulted in significant growth suppression (Fig. 3E).

We extended these studies to different tumor types, testing the relatively nonimmunogenic B16-F10 melanoma (31) and 4T1 breast carcinoma lines (32, 33). Established 4T1 and B16-F10 tumors (days 8 and 9,  $\sim 50 \text{ mm}^3$ ) were injected intralesionally with Ad-MyD88 or Ad-GFP. As in the CT26 model, a single injection of Ad-MyD88 significantly suppressed the growth of both 4T1 and B16-F10 tumors (Fig. 3E). *In vitro* infection of these tumors revealed that Ad-MyD88 did not affect tumor growth (Supplementary Fig. S5), thus showing that antigrowth effects were not mediated by alteration of cellular growth properties. Thus, Ad-MyD88 elicits immune responses in tumors of distinct histologies that translate into repressed growth *in vivo*.

#### **Ad-MyD88 injection elicits systemic and local immunity, which suppresses tumor growth through T cells and natural killer cells**

To determine if Ad-MyD88 had a systemic effect on tumor growth, intralesional injection of Ad-MyD88 was tested in a bilateral CT26.CL25 tumor model. In addition to repression of growth in the Ad-MyD88-injected tumor, we observed growth repression in the contralateral uninjected CT26.CL25 tumor (Fig. 4A), suggesting a systemic response against tumor antigens (34, 35). This was confirmed by ELISPOT analysis of splenocytes, which showed enhanced T-cell responses against LacZ in mice with Ad-MyD88-injected tumors (Fig. 4B). However, there was no enhancement of LacZ-specific IgG antibodies in Ad-MyD88-injected mice (Supplementary Fig. S6).

Although we showed that Ad-MyD88 elicited genes associated with Th1-type inflammation and TGF- $\beta$  repression *in vitro* (Fig. 1A and B), it was unclear if these responses could alter local tumor immunity *in vivo*. Because larger established tumors are immunosuppressive (36), we investigated if Ad-MyD88 could alter this phenotype by assessing transcription levels of several known immunosuppressive genes after Ad-MyD88 infection of CT26.CL25 cells *in vitro* or intralesional injection *in vivo*. Surprisingly, we found that intralesional administration of Ad-MyD88 was able to repress the expression of four immunosuppressive genes (*CSF-1*, *IL-10*, *PGE2*, and *TGF- $\beta$* ) tested in our panel (Fig. 4C; refs. 23, 37). This transcriptional suppression of immunosuppressive genes was not seen after tumor cell infection *in vitro*, suggesting that the *in vivo* effect is indirectly mediated through stromal cell infection, resulting in an altered inflammatory environment that promotes antitumor immunity (30, 38, 39).

As Ad-MyD88 could significantly enhance systemic T-cell responses *in vivo*, we hypothesized that Ad-MyD88-mediated tumor growth repression was largely T-cell mediated. To test this hypothesis, established CT26.CL25 tumors were intralesionally injected with Ad-MyD88 or Ad-GFP in mice deficient for T cells. In comparison with immunocompetent mice, the degree of Ad-MyD88 tumor repression was highly diminished ( $P = 0.04$ ) but still significant compared with Ad-GFP-injected T-cell-deficient control mice (Fig. 4D). These results

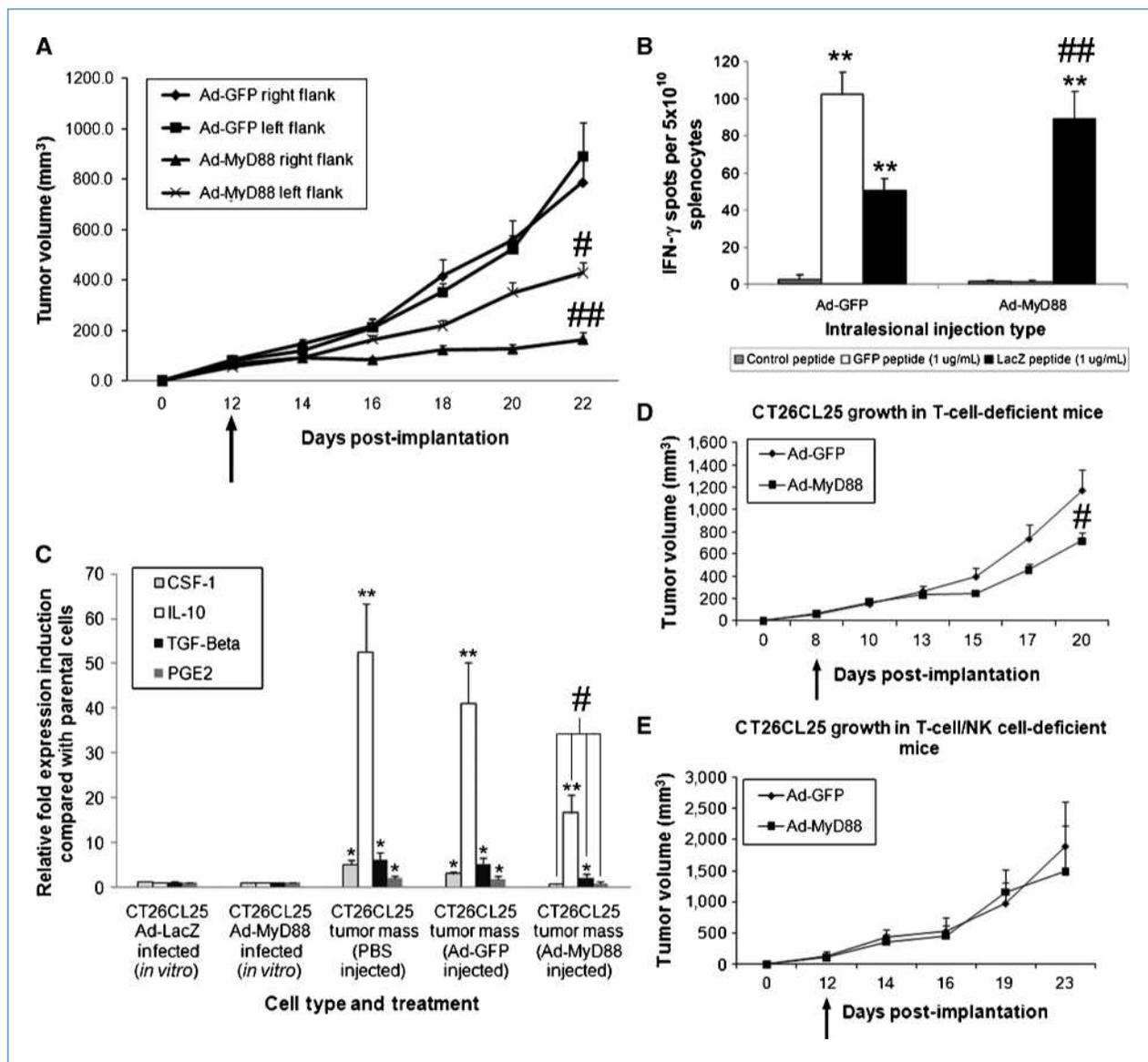
suggest that T cells play a critical role in Ad-MyD88-mediated immunity but are not the sole effectors. The broad cytokine and chemokine repertoire elicited by MyD88 overexpression also suggested that natural killer (NK) cells could be playing a role in Ad-MyD88-mediated immunity (40, 41), and this was confirmed in mice deficient for both T and NK cells, where Ad-MyD88 had no effect on tumor growth (Fig. 4E).

#### **Systemic delivery of Ad-MyD88 elicits activation of innate immune responses *in vivo* that are temporally regulated and well tolerated**

As intratumoral viral administration can be partially systemic via vascular uptake (42), we next investigated the effect of systemic Ad-MyD88 delivery in an *in vivo* model, exploiting the ability of adenovirus to effectively transduce hepatocytes *in vivo* after i.v. delivery (26). In this model, mice were i.v. injected with a therapeutically relevant dose ( $7.5 \times 10^{10}$  vp; equivalent to  $\sim 2.1 \times 10^{14}$  vp in a 70-kg human) of Ad-MyD88, Ad-GFP control, or vehicle control with hepatocytes harvested at 6 or 24 hours after infection for transcriptional assessment by qRT-PCR. Mice tolerated these injections well, and qRT-PCR assessment revealed that the expression of multiple effector and regulatory immune genes peaked at 6 hours after infection (Fig. 5A). Assessment of cytokine and chemokines at 1, 6, and 24 hours after infection revealed that multiple cytokines/chemokines in the serum of Ad-MyD88-infected mice also peaked at 6 hours after infection (Fig. 5B and C), with only G-CSF still significantly induced compared with controls at 24 hours after infection (Fig. 5B). Comparison of genome copy number by RT-PCR at 6 and 24 hours after infection revealed no difference in the infectivity or elimination of these viruses (data not shown). These results thus show that although Ad-MyD88 can strongly induce local and systemic innate immune responses *in vivo* after i.v. delivery, systemic exposure to very high doses of Ad-MyD88 and its induced cytokines/chemokines is well tolerated and still subject to immune-regulatory control mechanisms.

#### **Expression of human MyD88 in Ad vectors elicits proinflammatory patterns of gene expression in human cells and enhances tumor lysis and the expansion of tumor antigen-specific T cells**

To determine if these results were relevant to human cancers, we tested human MyD88 overexpression in different primary and transformed human cells. Primary hDCs, tumor-associated fibroblasts cultured from primary human colon cancer metastases (CRC-TAF), multiple colon cancer cell lines, as well as two primary colorectal tumors surgically resected from patients (CRC) were infected with a human MyD88-expressing Ad-MyD88, and immune pathway activation was examined by qRT-PCR, as previously performed in mouse cells. Infection of primary immune and nonimmune human cells with Ad-MyD88 vectors elicited a characteristic proinflammatory gene expression profile (Fig. 6A), although, as before, responses did vary between cell types. Assessment of multiple cytokines and chemokines after hDC infection confirmed Ad-MyD88 induction of 12 different proinflammatory



**Figure 4.** Intralesional injection of Ad-MyD88 represses tumor growth by systemically enhancing Th1 responses and alleviating immunosuppression in injected tumors. **A**, mice bearing CT26.CL25 bilateral tumors were treated by a right flank tumor injection of the indicated Ad vectors 12 d after infection ( $n = 6$ ). Bars, SE. **B**, ELISPOT assays using LacZ, GFP, or control irrelevant peptides were performed on mice from **A** ( $n = 6$ ). Bars, SD. **C**, qRT-PCR was performed to assess the transcription of immunosuppressive genes from infected CT26.CL25 cells (MOI = 200, 48 h after infection) and in injected tumor masses as described in **A** ( $n = 5-6$ ). Bars, SD. **D** and **E**, CT26.CL25 tumor-bearing B6.129S7-Rag1(tm1Mom) mice (**D**) and NOD CB17-Prkdc SCID/J (**E**) were vaccinated with the respective Ad vectors or mock treated (at 50 mm<sup>3</sup>, indicated by arrow) and tumor growth was measured ( $n = 5$ ). Bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus mock-infected controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus Ad control-infected counterparts.

cytokines (Fig. 6B; data not shown); DC immune-stimulatory molecules CD80, CD86, and CD40; and the DC maturation marker CD83 (Supplementary Fig. S7A). Similarly, MyD88 elicited significant secretion of Th1-related cytokines, including CXCL10, in CRC-TAFs and tumor cells (Supplementary Fig. S7B-G; data not shown). Of particular significance, we found that Ad-MyD88-infected primary CRCs secreted high levels of the major Th1 cytokines tumor necrosis factor (TNF)- $\alpha$ , IFN- $\gamma$ , and CXCL10 compared with controls

(Fig. 6B). Collectively, these results reveal that overexpression of human MyD88 strongly induces a proinflammatory immune response profile in all human cell types, and thus suggest that the use of Ad-MyD88 could significantly affect immune responses in the tumor microenvironment in human patients.

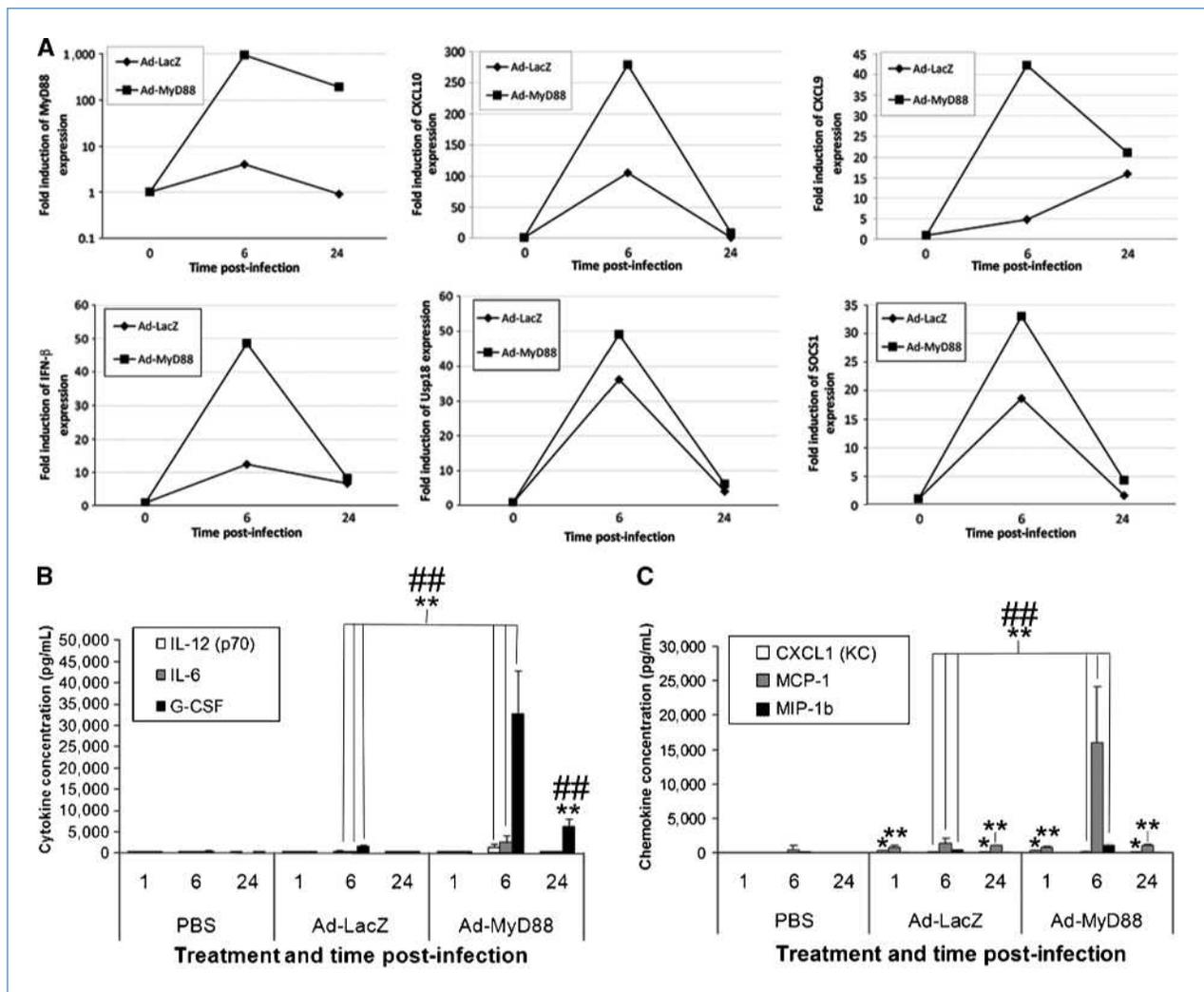
As Ad-MyD88 infection had induced the expression of multiple bioactive cytokines in different cell types, as well as the expression of costimulatory markers in DCs, we

hypothesized that these and other effector mechanisms could have an effect on tumor cell lysis and T-cell expansion. Infection of a HLA-A\*0201<sup>+</sup> colorectal cancer line (COLO205) with Ad-MyD88 followed by coincubation with IL-2-activated HLA-A\*0201<sup>+</sup> normal donor T cells revealed that Ad-MyD88-infected cells were indeed more sensitive to tumor cell lysis in comparison with Ad-LacZ-infected cells (Fig. 6C). To ascertain if Ad-MyD88 could enhance the expansion of tumor antigen-specific T cells, PBMCs were infected with Ad-MyD88 in the presence of the self-tumor antigen peptide MART1(27–35). Assessment of cytokines in culture supernatants revealed that Ad-MyD88-infected cells secreted higher levels of the Th1-like cytokines GM-CSF, IFN- $\gamma$ , and TNF- $\alpha$  (Supplementary Fig. S8). In addition, we found a 10-fold higher induction of MART1(27–35) peptide-specific T cells in these cultures using tetramer staining (Fig. 6D). Collec-

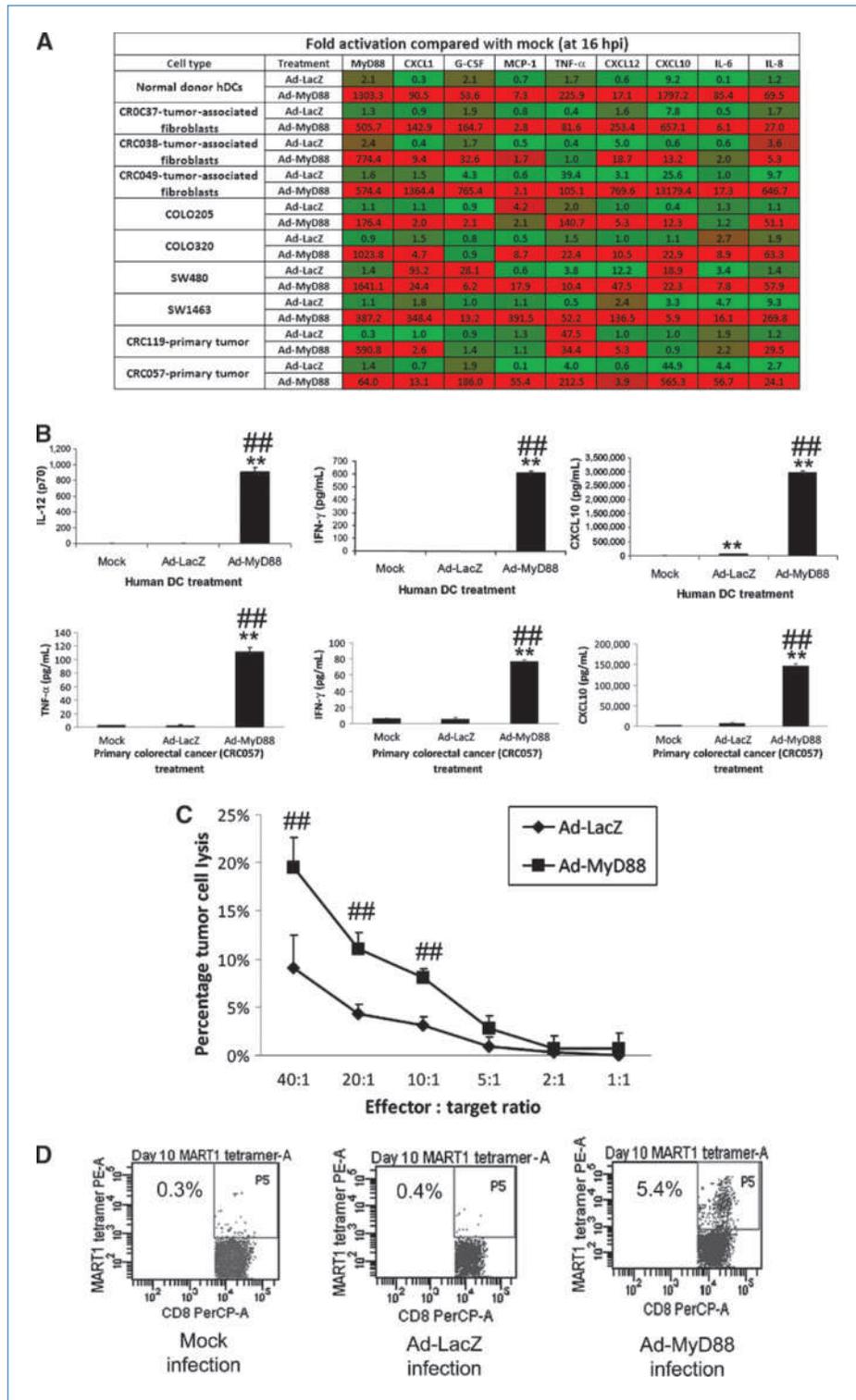
tively, these results show that Ad-MyD88 can elicit robust Th1-skewed innate immune responses in a wide range of cells present in a human colorectal tumor microenvironment, as well as functionally inducing greater tumor cell lysis and expansion of tumor antigen-specific T cells after infection of human cells *in vitro*.

## Discussion

The lack of effective immunotherapeutic strategies in patients with advanced cancer is in large part due to the inefficiency of generating immune responses coupled with the powerful immunosuppressive and immunomodulatory environment present in patients. Using Ad vectors that expressed MyD88, we sought to deliver coordinated TLR-like signals to stimulate innate host immune responses and



**Figure 5.** Systemic administration of Ad-MyD88 elicited temporally enhanced innate immune responses. A, C57/BL6 mice ( $n = 6$ ) were i.v. injected with indicated vectors ( $7.5 \times 10^{10}$  vp) and assessed for liver transcriptional dysregulation by qRT-PCR (average dysregulated in comparison with mock presented). B and C, serum at 1, 6, and 24 h after infection was assessed for inflammatory cytokines and chemokines, respectively ( $n = 6$ ). Bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus mock-infected controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus Ad control-infected counterparts.



**Figure 6.** Ad-MyD88 activates innate immune responses in multiple types of human cells and enhances human tumor cell lysis as well as the expansion of human tumor antigen-specific T cells. A, hDCs, primary CRCs, and CRC-TAFs were infected with Ad-MyD88, Ad-GFP, or mock (hDC: MOI = 2,000; CRC: MOI = 10,000; CRC-TAF: MOI = 2,000) and gene expression was assessed by qRT-PCR. The average fold change (from mock-treated cells) is displayed by color-coded gradient (red for higher expression and green for lower expression;  $n = 4$ ). hpi, hours after infection. B, representative cytokine and chemokine secretion from hDCs (top) and primary CRC057 tumor cells (bottom) at 16 h after infection treated as in A ( $n = 4$ ). C, Ad-MyD88 infection of COLO205 cells enhances tumor cell killing by activated T cells. COLO205 cells were infected with Ad-LacZ, Ad-MyD88, or mock (MOI = 10,000) and cultured with IL-2-activated T cells at indicated E:T ratios ( $n = 6$ ). D, Ad-MyD88 enhances expansion of MART1-specific CD8<sup>+</sup> cells. Human PBMCs were treated as indicated (MOI = 2500) and stimulated with MART1 peptide (100  $\mu$ g/mL). At 3 d after infection, IL-2 was added (60 units/mL) and cells were expanded for 7 additional days (10 d after infection) and assessed by tetramer staining, which was performed using labeled CD3, CD4, and CD8 antibodies and a MART1-specific tetramer. These experiments were independently performed five times in triplicate, and representative scatter plots are shown. Bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus mock-infected controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus Ad control-infected counterparts.

provoke adaptive antitumor immunity. Our results show that Ad-MyD88 infection elicits a profound induction of inflammatory genes and a unique Th1-biased gene signature in multiple primary and transformed murine and human

cell types *in vitro*. This was in contrast to the use of a specific TLR ligand, CpG, which elicited a different Th1-biased signature in primary murine cells but did not elicit robust responses in transformed murine cells. *In vivo*, we found

that a single intratumoral injection of Ad-MyD88 enhanced adaptive immune responses and suppressed local and systemic growth of multiple tumor types, in contrast to a single injection of a CpG TLR9 agonist. Finally, Ad-MyD88 infection of primary human cells *in vitro* elicited Th1-type responses and enhanced tumor cell lysis and the expansion of tumor antigen-specific T cells, suggesting that the clinical delivery of human Ad-MyD88 into a colorectal tumor microenvironment could effectively parallel the *in vivo* effectiveness observed with the CT26 mouse colorectal cancer model.

Previous approaches at cytokine therapy of cancer largely used single agents, leading to elevated systemic cytokine levels that were linked to dose-limiting toxicities (34, 43–48). The local delivery of a TLR-specific signaling adaptor protein confers two major advantages. First, delivery of the TLR adaptor to multiple cell types in the tumor microenvironment leads to a pattern of cytokine secretion and innate immune activation, regardless of TLR receptor repertoire in normal and malignant cells. Second, it activates a consistent pattern of specific cytokines to elicit robust immunity. The expression of this pattern of cytokines at coordinated levels by Ad-MyD88 could also minimize the need for high systemic levels of individual cytokines that may be associated with toxicities (34, 45–48). Thus, although cytokine-induced toxicity remains a concern in the use of Ad-MyD88 that must be addressed in animal toxicology studies, the activation of a coordinated set of cytokines in multiple cell types may indirectly counteract systemic toxicities while permitting maximal immune responses.

Finally, our study showed the effectiveness of human MyD88 expression in inducing cellular immunity in primary tumor cells and fibroblasts from metastatic colon carcinomas. Although the importance of MyD88 and TLRs in humans is largely unknown (49), with only a few studies

identifying patients with functional defects in TLR genes that translate into hindered immunity to different pathogens (50), our study found significant concordance between the responses elicited by mouse MyD88 expression in murine cells *in vitro* and *in vivo*, and human MyD88 overexpression in different types of human primary and transformed cells. These data suggest a strong functional role for MyD88 in human immunity and could further suggest the utility of MyD88 overexpression-based approaches in therapies requiring immune modulation, including infectious disease and autoimmunity.

In summary, this investigation reveals that MyD88 overexpression activates TLR pathways in established models, leading to the effective generation of innate and adaptive responses that alter immunity to allow for effective tumor immunotherapy. The capacity of human Ad-MyD88 to elicit similar immune response profiles and effective immune stimulation *in vitro* in human cells thus indicates that Ad-MyD88 warrants further development and clinical translation as a novel means of enhancing antitumor immunotherapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Ligand-Independent Toll-like Receptor Signals Generated by Ectopic Overexpression of MyD88 Generate Local and Systemic Antitumor Immunity

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