*Sensitive compounds as ACI-RCC cell inhibitors*

| Category | Compound | % DMSO |
|---|---|---|
| Antitumor (estrogen receptor) | Tamoxifen, citrate | 5.2 |
| Antitumor (DNA) | Daunorubicin, HCl | 7.4 |
| Antitumor (DNA) | Doxorubicin, HCl | 9.2 |
| Heat shock protein 90 | Radicicol | 3.5 |
| Heat shock protein 90 | 17-Allylamino-17-demethoxygeldanamycin (tanespimycin) | 14.6 |
| Protein kinase C | Bisindolymaleimide I, HCl | 9.7 |
| Protein kinase C | Go7874 | 0.5 |
| Protein kinase C, A, G, myosin light chain kinase | Staurosporine | 2.1 |
| V-adenosine triphosphatase | Bafilomycin A1 | 8.2 |
| Cathepsin G | Z-gly-leu-phe-chloromethyl ketone | 5.2 |
| Na ionophore | Monensin | 7.8 |
| K ionophore | Nigericin | 3.4 |
| Guanylate cyclase | LY 83583 | 0.8 |
| Hypoxia-inducible factor | Chetomin | 1.4 |
| Spermidine/spermine N1-acethyltransferase activator | N1, N12-doethylspermine (BESpm) | 0.5 |
| Akt | Akt inhibitor IV | 0.4 |
| Bruton's tyrosine kinase | Terric acid | 9.7 |
| Hepatocyte growth factor receptor/Met | SU11274 | 8.7 |
| Platelet-derived growth factor | SU11652 | 0.2 |

Luc can catabolize a substrate D-luciferin, which is relatively stable in vivo. Using the newly established bioluminescent rat RCC cell line we developed a novel system to test in vitro and in vivo tumor growth.

We first examined the in vitro characteristics of 2 Luc labeled ACI-RCC cell lines. ACI-RCC-cbLuc cells were superior to ACI-RCC-ffLuc cells for in vitro measurement in terms of luminescence intensity with a much lower detection limit. As reported in previous studies of other tumor models, the number of tumor cells in vitro correlated with the bioluminescence signal, indicating the quantitative value of this system for in vitro measurement (fig. 2). This appeared reproducible in solid tumors but not in an IL-3 dependent murine leukemia cell line, in which signal intensity was affected by IL-3 concentration and proliferation stage.[12–14]

We then examined in vivo the growth of these luciferase labeled ACI-RCC cell lines. ACI-RCC-cbLuc cells were rejected completely by syngeneic ACI rats while ACI-RCC-ffLuc cells were consistently taken to establish tumors. This difference may be due to the higher antigenicity of Luc protein derived from Phototinus plagiophthalamus, which predisposes ACI-RCC-cbLuc cells to increased susceptibility to attack by the host cellular immune defense system. This susceptibility may be explained by the fact that ACI-RCC-cbLuc cells grew in athymic mice. Even with syngeneic transplantation transplanted cells expressing a marker protein such as green fluorescent protein and β-galactosidase (LacZ) occasionally disappear due to immunogenicity.[15,16] Even ffLuc, which is considered less immunogenic than green fluorescent protein,[17] has been reported to induce a T-helper type 1 immune response after intradermal immunization in immune competent Balb/c mice.[18]

These factors may subsequently raise the question of the difference in antigenicity between ACI-RCC-ffLuc and ACI-RCC-cbLuc cells. The quantity or quality of Luc protein expressed in each cell line must be considered, although it is hard to accurately compare the differences. We assume that the former is less likely to be the reason for the different fates in vivo of the 2 cell lines. This assumption is supported by the finding that enhanced ffLuc expression in mouse T cells permitted sensitive tracking of adoptively transferred T cells infiltrating tumor sites without increasing immunogenicity in immunocompetent murine models.[19] Thus, immune responses involving the different Luc proteins may have to be considered cautiously. Thereafter we focused on ACI-RCC-ffLuc cells to investigate in vivo tumor growth using an in vivo imaging system.

A significant correlation between luminescence intensity and subcutaneous tumor volume verified the quantifiable nature of our Luc labeled tumor model system (fig. 5, *B*). Because of the impossibility of measuring tumor size using calipers in orthotopic RCC models, therapeutic experiments are usually assessed by animal symptoms and end point variables, such as survival time, kidney weight and histological analysis of explanted kidneys. However, our system enabled noninvasive real-time extracorporeal monitoring of orthotopic tumors beneath the renal capsule. Notably subsequent metastasis of the orthotopic tumor to the lung could also be monitored (fig. 6, *B*). This metastatic pattern, similar to the RENCA model, mimics that in human RCC, which is clinically meaningful for studying RCC progression.

Our use of rats rather than mice was also advantageous for BLI. Easy intravenous delivery of the substrate D-luciferin enabled rapid, stable measure-

ment of bioluminescence intensity. Photon counts measured by IVIS peaks 1 minute after intravenous injection of D-luciferin had fewer individual differences in our models (data not shown). The window of reliable maximum photon emission in murine models requiring intraperitoneal injection of Luc varies among studies and is reportedly much longer, usually 15 minutes or more.[12,20,21]

Finally, we examined the use of our Luc labeled rat RCC cell line ACI-RCC-cbLuc as an in vitro screening tool for anticancer agents. The results of drug sensitivity using the SCADS inhibitor kit combined with Luc labeled cells provided a useful screening tool with rapid high throughput. Screened compounds with an inhibitory effect on ACI-RCC cells included molecules related to RCC progression, ie heat shock protein 90, AKT, Met and platelet-derived growth factor, which are molecular targets of recent RCC therapies (see table).[3,22,23] These results imply the practical accuracy and feasibility of our systems for in vitro RCC investigation. The exact efficacy and toxicity of such screened compounds in vivo as well as appropriate combinations are under investigation at our laboratory.

## CONCLUSIONS

We engineered Luc expressing cancer cells from a newly established rat RCC cell line arising from immunocompetent rats, and noted easy quantitative detection of orthotopic tumor growth and metastatic spread in real time using the Luc based bioluminescent systems. We also introduced a feasible method of in vitro screening for anticancer compounds using Luc labeled rat RCC cells. This Luc based in vivo model system coupled with the in vitro screening system may facilitate the development of new therapies that may eradicate metastatic RCC. Also, our allograft tumor model in an immunocompetent animal may be advantageous for studying exact treatment efficacy and safety for immunogenic tumors such as RCC.

## ACKNOWLEDGMENTS

The Screening Committee of Anticancer Drugs provided the SCADS inhibitor kit.

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International Immunopharmacology 10 (2010) 985–989

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Preliminary report

# Chemopreventive anti-cancer agent acyclic retinoid suppresses allogeneic immune responses in rats

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ARTICLE INFO

*Article history:*
Received 3 December 2009
Received in revised form 15 April 2010
Accepted 22 April 2010

*Keywords:*
Acyclic retinoid
Anti-cancer drug
Chemoprevention
Immunosuppression
Rat

ABSTRACT

Acyclic retinoid NIK-333 (ACR) is a chemopreventive agent that acts by suppressing the recurrence of hepatocellular carcinoma (HCC) following initial treatment and is now being employed in clinical trials. The chemopreventive effects of ACR have been analyzed from various aspects, and it is well known that some retinoic acid (RA) derivatives affect host immunity. The objective of this study is to investigate the effects of ACR on host immunity. The results demonstrated that ACR prolonged heart and liver graft and recipient survival in rat allogeneic organ transplantation. The immunosuppressive effect of ACR administered at 100 mg/kg/day was almost equivalent to that of CsA administered at 1 mg/kg/day *in vivo*. In the mixed lymphocyte reaction (MLR), ACR suppressed lymphocyte proliferation non-specifically. Gene expression analysis of splenic lymphocytes from ACR-treated recipient rats revealed no distinct change in Interleukin (IL)-2 and increases in Interferon (IFN)-gamma. In conclusion, ACR possesses immunosuppressive potential *in vivo* and is a promising chemopreventive drug for long term use against HCC.



## 1. Introduction

It has been argued since the latter 1960s that retinoic acid (RA) and derivatives may have an effect on the immune system [1]. Most of these reports referred to the enhancement of host immunocompetency following the administration of RA [2–4]. However, it has recently become clear that some RA derivatives possess immunosuppressive or graft protection effects [5–8]. These findings suggest that each RA derivative possesses a unique character in terms of its effect on immuno-regulatory functions.

Acyclic retinoid NIK-333 (ACR), an analogue of RA, has been shown to suppress secondary hepatocellular carcinoma (HCC) following initial resection or ablation [9]. These chemopreventive effects of ACR have been analyzed from various aspects [10–15]. While many RA analogues have been considered as promising anti-cancer agents [16], they might also modulate host immune reactions [17]. In this study, we examined the effects of ACR on the host and cancer using an allogeneic cardiac transplantation model and a syngeneic tumor-implanted model in rats, respectively.

## 2. Materials and methods

### 2.1. Animals and cells

Inbred male DA ($RT1^a$), LEW/DuCrj ($RT1^l$) and F344/DuCrj ($RT1^{lvl}$) rats were purchased from Charles River Japan Inc. (Yokohama, Japan), and BN ($RT1^n$) and PVG ($RT1^c$) rats were purchased from SLC Inc. (Hamamatsu, Japan). Rats were used at 8–10 weeks of age, and all experiments in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Jichi Medical University and National Research Institute for Child Health and Development. The RCN-H4 cell line has been established as a highly liver-metastatic subline of the RCN9 parental cell line derived from

*Abbreviations:* ACR, acyclic retinoid; ConA, Concanavalin A; CsA, cyclosporin A; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HHT, heterotopic heart transplantation; MLR, mixed lymphocyte reaction; IFN, interferon; IL, interleukin; OLT, orthotopic liver transplantation; RA, retinoic acids; RAR, retinoic acid receptor; RXR, retinoid-X receptor; SI, stimulation index; ATRA, tretinoin.

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1567-5769/$ – see front matter. 
doi:10.1016/j.intimp.2010.04.017

F344/DuCrj rat (Riken Cell Bank, Tsukuba, Japan) [18]. Luciferase-transduced luc-RCN-H4 cells [19] were maintained in RPMI 1640 medium (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% fetal calf serum (FCS) (ICN-Flow, Aurora, OH) and penicillin–streptomycin (Gibco BRL, Grand Island, NY) at 37 °C in a humidified atmosphere of 5% $CO_2$.

*In vivo* implantation of luc-RCN-H4 cells and tumor progression monitoring using the noninvasive IVIS bio-imaging system (Xenogen, Alameda, CA) were as previously described [19]. Heterotopic heart transplantation (HHT) and orthotopic liver transplantation (OLT) were performed using a modified method of Olausson [20] and Kamada [21]. For the HHT, the graft beating was examined daily by palpitation, and functional graft rejection was defined as the day when the heart grafts ceased beating.

### 2.2. Reagents

ACR [(2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, $C_{20}H_{30}O_2$] (official name; NIK-333) was synthesized and kindly provided by Kowa Co., Ltd. (Tokyo, Japan). Cyclosporin A (CsA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ACR was dissolved in soybean oil and administered at 0.5 ml/100 g body weight for p.o. administration, and CsA was dissolved in olive oil and used at 0.15 ml/100 g body weight for p.o. administration or suspended in saline at a concentration of 1 mg/ml for s.c. injection. A stomach tube was used for the p.o. administration.

### 2.3. Mixed lymphocyte reaction (MLR)

Splenic lymphocytes were prepared by passing minced tissue through a cell strainer followed by density gradient centrifugation using Lympholyte-Rat (Ontario, Canada). Irradiated stimulator cells ($1\times10^5$) were cultured for 72 h with responder cells ($1\times10^5$) in 200 μl RPMI1640 medium supplemented with 10% FCS on a 96-well plate at 37 °C in a humidified atmosphere of 5% $CO_2$. Lymphocytes from BN rat were used as third-party, and Concanavalin A (ConA) was used as the control stimulant. Twenty-four hours prior to termination of the culture, 1 μCi of [$^3$H]-thymidine (Amersham Pharmacia Biotech, Amersham, UK) was added to each well. [$^3$H]-thymidine uptake was determined using a liquid scintillation counter (Packard Japan, Tokyo, Japan). Each [$^3$H]-thymidine uptake count (count per minute; cpm) was expressed in the form of a stimulation index (SI), which was calculated as follows:

*Stimulation Index*() = *Experimental group* (*cpm*) / *Syngeneic control*( *cpm*)

### 2.4. Reverse-transcriptase polymerase chain reaction

Total RNA was extracted from spleen cells using Isogen (Nippon Gene, Toyama, Japan) as previously described [19]. The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-GTA TCG TGG AAG GAC TCA TG-3′; GAPDH anti-sense, 5′-AGT GGG TGT CGC TGT TGA AG-3′; IFN-γ sense, 5′-CCC TCT CTG GCT GTT ACT GC-3′; IFN-γ anti-sense, 5′-CTC CTT TTC CGC TTC CTT AG-3′, IL-2 sense, 5′-GCG CAC CCA CTT CAA GCC CT-3′; and IL-2 anti-sense, 5′-CCA CCA CAG TTG CTG GCT CA-3. The PCR conditions for each set of primers comprised heating at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 s, 57 °C for 30 s and 68 °C for 2 min. The PCR products were analyzed electrophoretically using a 1% agarose gel.

### 2.5. Cell proliferation assay

luc-RCN-H4 cells ($4.0\times10^4$) were plated in triplicate onto 48-well plates and incubated in the presence of ACR at the indicated concentrations. Following 48 h incubation, luc-RCN-H4 cells were collected from each well and then counted using a hemacytometer.

### 2.6. Statistical analysis

Results are expressed as mean ± standard deviation (SD) and analyzed using a one-way analysis of variance (ANOVA) or unpaired Student's *t*-test for statistical evaluation. Graft and recipient survival data derived from the cardiac and liver transplantation experiments were analyzed using a Log-rank test. Differences among groups were considered significant if the *p* value was less than 0.05. The data are expressed as the means ± SD. All analyses were performed using the StatView ver. 5.0 software program (SAS Institute, Cary, NC).

## 3. Results

### 3.1. Immunologic effects in rat organ transplantation

In an effort to investigate the effect of ACR on host immunity, we initially conducted heterotopic heart transplantation (HHT) of rats (Table 1). In a preliminary toxic study in rats, ACR administration at a dose of 100 mg/kg/day for 4 weeks resulted in neutrophilia, low albumin, high alkaline phosphatase and high total cholesterol in blood, and caused no pathological change on rat spleen. Although administration of ACR at a dose of 200 mg/kg/day for the same duration did not result in any deaths, several serious adverse effects resulted such as anemia, body weight loss or bone fracture. Based on these observations, this study employed doses of 10, 25, 50 and 100 mg/kg/day. With HHT from a

**Table 1**
Graft/recipient survival under ACR or CsA administration in rat heterotopic heart/orthotopic liver transplantation.

| Graft | Combination | Drug | Dose (mg/kg/day) | *N* | Graft/recipient survival (days) | (Ave.) | *p* value |
|---|---|---|---|---|---|---|---|
| Heart | PVG–PVG | Vehicle | – | 8 | >15(8) | >15.0 | |
| | DA–PVG | Vehicle | – | 9 | 6, 6, 6, 7, 7, 7, 7, 8, 8 | 6.9 | <0.05 |
| | | ACR | 10 | 5 | 7, 7, 8, 8, 8 | 7.6 | |
| | | ACR | 25 | 5 | 7, 8, 8, 8, 9 | 8.0 | |
| | | ACR | 100 | 6 | 8, 9, 10, 10, 10, 13 | 10.0 | |
| | | CsA | 1 | 5 | 8, 8, 8, 12, 13 | 9.8 | |
| | | CsA | 10 | 4 | >15, >15, >15, >15 | >15.0 | |
| | DA–LEW | Vehicle | – | 5 | 4, 4, 5, 5a, 6a | 4.8 | <0.05 |
| | | ACR | 100 | 4 | 5, 7a, 7a, 9 | 7.0 | |
| Liver | LEW–LEW | Vehicle | – | 4 | >60, >60, >60, >60 | >60.0 | |
| | DA–LEW | Vehicle | – | 6 | 10, 11, 11, 11, 12, 12 | 11.2 | <0.05 |
| | | ACR | 10 | 3 | 18, 21, 51 | 30.0 | |
| | | ACR | 25 | 5 | 14, 18, 20, 25, 38 | 23.0 | |
| | | ACR | 50 | 2 | 26, 27 | 26.5 | |
| | | ACR | 100 | 2 | 17, 19 | 18.0 | |

ACR; p.o., CsA; i.m., a; splenectomy for MLR at day 7.

DA donor to a PVG recipient, ACR significantly prolonged cardiac graft survival at a dose of 25 mg/kg/day. This graft prolongation effect occurred in a dose-dependent manner, and ACR administration at 100 mg/kg/day showed a significant immunosuppressive effect almost equivalent to that resulting from CsA administration at 1 mg/kg/day. This immunosuppressive effect was also shown in the rat DA to LEW combination, which causes severe immunological rejection, with significant differences. We then evaluated the immunosuppressive effect of ACR in a rat orthotopic liver transplantation (OLT) model (Table 1). With OLT from a DA donor to a LEW recipient, ACR demonstrated a significant graft prolongation effect even at relatively low administration dose (10 mg/kg/day). Unlike the case with HHT, a dose-dependent effect was not observed in recipient rat survival with OLT. Interestingly, the mean survival with OLT was greatest with an administration dose of 10 mg/kg/day, although use of a liver transplantation model is not always the most appropriate method for the evaluation of immunosuppressive effects. Unlike the case with HHT, OLT in rats administered with ACR at 100 mg/kg/day resulted in short term diarrhea and more body weight loss following transplantation, with the possibility that the body weight loss influenced survival (data not shown). These results indicate that ACR can induce a graft prolongation effect on allogeneic organ transplantation, suggesting that ACR possesses immunosuppressive activity.

### 3.2. Effect of ACR on lymphocyte proliferation

We then set out to determine whether ACR can suppress allogeneic immune responses *in vitro*. The MLR assay was performed using lymphocytes isolated from the spleen of recipient LEW rats at 5 to 7 days post-transplantation. As shown in Fig. 1A, the stimulation index (SI) was significantly suppressed in lymphocytes isolated from ACR-administered LEW recipients (against DA donors). Furthermore, a similar suppression of MLR with ACR was observed against stimulant by third-party, indicating that ACR is able to suppress non-specific allogeneic immune responses. ACR was also able to suppress lymphocyte proliferation stimulated by ConA. These results demonstrate that ACR can non-specifically suppress lymphocyte proliferation. The observed immunosuppressive effect of ACR is not strong enough for it to be employed in the area of clinical immunosuppression.

Gene expression analysis of splenic lymphocytes from ACR-treated recipient rats revealed no distinct change in IL-2 and increases in IFN-γ (Fig. 1B). These results suggest that ACR does not affect IL-2 mRNA expression and can suppress lymphocyte proliferation. It was noted that the augmentation in IFN-γ expression was not inhibited by ACR, suggesting that ACR does not completely suppress host immune responses.

### 3.3. Effect of ACR on luc-RCN-H4 colon cancer cells

In an effort to determine the effect of ACR on cancer cell growth, ACR was administered to luc-RCN-H4 colon cancer cells *in vitro* (Fig. 2A). ACR was able to inhibit cell proliferation when present at a concentration greater than 50 μM. CsA administration showed marked inhibition at a concentration of 16 μM, which was presumed to involve a toxic effect (Fig. 2B). To examine the anti-tumor effect of ACR *in vivo*, luc-RCN-H4 cells were injected into the ileocolic vein of F344/DuCrj rats [19] and followed by daily administration of ACR sufficient to suppress HCC development by 3′-methyl-4-dimethylaminoazobenzene (80 mg/kg/day). Tumor progression was monitored by *in vivo* luminescent imaging [19]. In comparison with the control treatment (soybean oil), ACR exerted no significant influence on tumor-derived photons in the liver, indicating that ACR had no effect on tumor growth at this dosage (Fig. 2C). This observed *in vivo* anti-tumor effect was the same when lower doses of ACR were employed (data not shown). Although increasing the dosage of ACR might have resulted in a tumor-suppressing effect in this model, the use of doses over 100 mg/kg/day resulted in some adverse effects in the preliminary toxic test. On the other hand, although the use of CsA at 3 mg/kg/day showed no promotion effect, use at 15 mg/kg/day exerted promotion effects on tumor growth (Fig. 2D). We concluded that the use of ACR at the normal dose of 100 mg/kg/day does not show tumor promotion effects against luc-RCN-H4 colon cancer progression *in vivo*.

## 4. Discussion

RA is often recognized as an essential substance for homeostasis [16]. RA can facilitate passage of various immature cells, including malignant cells, to terminal differentiation through receptors that are widely distributed in the body [22,23]. These RA receptors fall into two groups comprising retinoic acid receptors (RARs) and retinoid-X receptor (RXRs), with each group being further classified into subtypes comprising RARα, β, γ and RXRα, β, γ, respectively. The distribution of these receptors on the cell surface and ligand affinities regulate the effectiveness of RA compounds, and it is thought that this determines

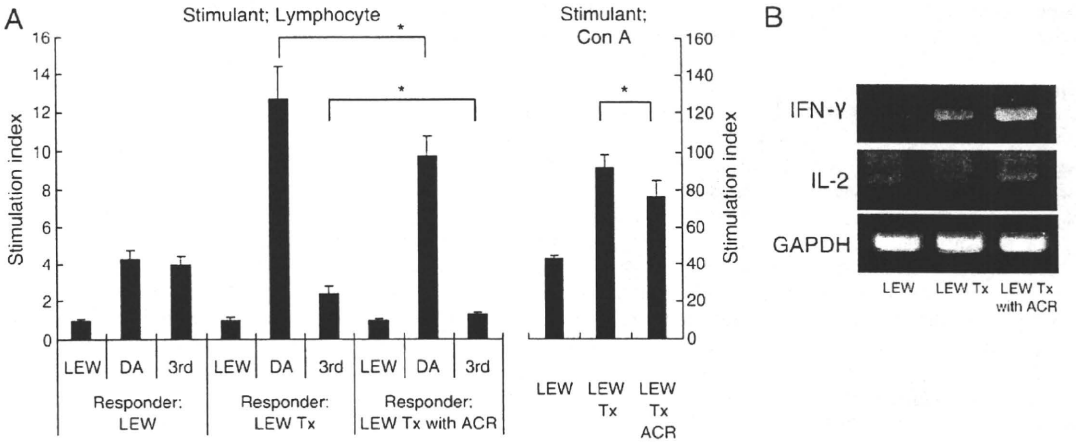


**Fig. 1.** ACR suppressed lymphocyte proliferation in the mixed lymphocyte reaction (MLR) (A, representative data). Responder cells were collected from removed spleen following graft rejection, as indicated in Table 1A. In experiments using naïve DA lymphocytes as stimulant, lymphocytes from graft-implanted rat showed intense proliferation compared with control LEW. This proliferation was suppressed by ACR administration. Suppression of proliferation by ACR was also observed in experiments using naïve BN lymphocytes as third-party stimulant and in experiments using Con A as stimulant. The original control cpm values were as follows; (Stimulant–Responder) LEW–LEW 3687.2, LEW–LEW Tx 1555.5, LEW–LEW Tx with ACR 1368.8. Gene expression analysis of spleen cells revealed that, unlike the case with IL-2, IFN-γ expression increased in ACR-administered recipients (B). *, $p<0.05$.

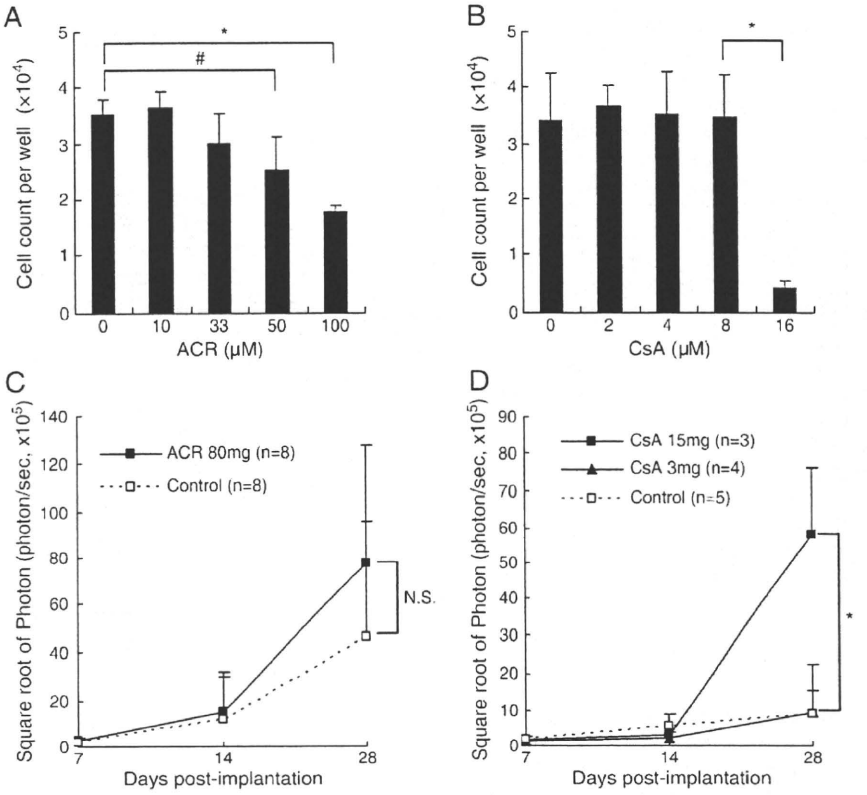


**Fig. 2.** Anti-tumor activity of ACR on luc-RCN-H4 cells *in vitro* and *in vivo*. In the cell growth assay, ACR showed growth inhibitory effects but only at levels above physiological concentrations (A). CsA administration showed marked inhibition at a concentration of 16 μM, which was presumed to involve a toxic effect (B). *In vivo* experiment, $5.0 \times 10^6$ luc-RCN-H4 cells in 0.5 ml PBS were injected into the ileocolic vein of rats. Luc-RCN-H4 cell-implanted rats were divided into two groups; ACR at 80 mg/kg/day and control (C), CsA at 3 mg/kg/day, 15 mg/kg/day and control (D). The metastatic fate of luciferase-positive tumor cells was followed using an *in vivo* imaging system on days 7, 14 and 28. The results showed that ACR administered at 80 mg/kg/day and CsA administered at 3 mg/kg/day had no effect on tumor growth. *, $p<0.05$, #, $p=0.06$.

the effects of RA at the single cell or organ levels, including cancer cells [22].

RA derivatives have been investigated as potential anti-cancer agents following the success of Tretinoin (ATRA) with promyelocytic leukemia [24]. Despite the emerging evidence, most RA derivatives do not always exert direct anti-cancer effects, and are often recognized as compounds useful in cancer chemoprevention [23]. To date, at least 7 RA analogues have been shown to possess tumor-suppressive activity clinically and experimentally [16]. ACR is one RA derivative which is presently being considered as a drug for the inhibition of HCC recurrence in clinical trials.

HHT is the most reliable model to evaluate allogeneic immune responses. Our HHT results demonstrated the substantial immunosuppressive effect of ACR (Table 1). Although the OLT results did not reveal a dose-dependent recipient survival trend, this is not surprising given that recipient survival following orthotopic liver transplantation was not always simply correlated with host immune conditions, especially in cases when the administered drug targeted the implanted graft. Our MLR experiments also demonstrated immunosuppression by ACR *in vitro*, supporting the *in vivo* immunosuppressive role of ACR in allogeneic immune responses. In this study, although no attempt was made to identify the mediator(s) or factor(s) involved in the ACR immunosuppressive effect, previous studies have reported the mechanism by which RA changes the leukocyte population and immunity of the host without IL-2 mediation [7,17]. Our results follow on from these reports in terms of outlining the immunosuppressive efficacy of RA derivatives.

Various reports have referred to the anti-tumor effect of RA derivatives in tumor implantation models [25,26]. Our *in vivo* experiments showed that although ACR administered at 80 mg/kg/day did not have any influence on tumor growth, the proliferation of luc-RCN-H4 cells *in vitro* was inhibited by ACR. This result should be interpreted by considering the following: (1) although the *in vitro* anti-cancer effect of ACR has been investigated in several studies as described above [10,11,13–15], these effects could not easily be analyzed *in vivo* since ACR is not a typical anti-cancer agent but a chemopreventive compound, and (2) the chemopreventive effect of ACR *in vivo* has already been proven clinically by Muto, et al. [9] and experimentally by Kagawa, et al. [12], and both studies included observations over a long time period. Therefore, we focus on our experimental results showing that the proliferation of implanted luc-RCN-H4 cells was not promoted by ACR. We supposed that a compound with immunosuppressive potential could be categorized into one of two groups according to its influence on cancer cell proliferation, being promotion [19,27–29] or suppression [30–33]. Needless to say, it is impossible to clearly make this assignment since the criteria employed to categorize a compound differ depending on the conditions of each study or experiment, although the fact that a compound does not possess the potential to promote cancer cell proliferation is the most important in a clinical situation. In this regard, we may suggest that ACR does not possess the potential to promote cancer cell proliferation in our experiments. Although we did not test ACR at doses greater than 100 mg/kg/day, clinical studies of ACR administered at 600 mg/body/day [9] and animal experiments involving administration of ACR at 80 mg/kg/day [12] should confirm the sufficient dose required to prevent secondary cancer. Additionally, there was very little toxic effect demonstrated in a previous study employing ACR at 80 mg/kg/day for 20 weeks [12]. The data from the preliminary study could support the fact that the immunosuppressive or anti-cancer

effect of ACR when administered at a dose of 100 mg/kg/day is not due to toxic effects but to bio-activity.

In addition to the non-promotive effect of ACR on cancer proliferation, the augmentation of IFN-γ expression in spleen lymphocytes by ACR would support its potential use in a clinical environment. It is well known that HCC is closely linked to viral hepatitis, such as Hepatitis B or C. The action of IFN-γ, a representative cytokine that possesses anti-viral activity through its effect on replication, may provide great advantages associated with the clinical use of ACR [34–36].

In this study, we showed that ACR administered orally at 100 mg/kg/day had an almost equivalent immunosuppressive effect as CsA administered subcutaneously by injection at 1 mg/kg/day. Although ACR is a promising chemopreventive drug for use in HCC, ACR exerts its effect only after long periods of administration. Therefore, we believe our new findings concerning host immunity could contribute significantly towards its potential clinical use.

## Acknowledgements

We would like to thank Mrs. Yasuko Sakuma, Ms. Megumi Hata and Ms. Harumi Kawana for their skillful technical assistance.

This study was supported by a grant to T.M. from the Health and Labor Science Research Grants from the Ministry of Health, Labor and Welfare (Research on Biological Resources) and by a grant from the "Strategic Research Platform" for Private Universities: matching fund subsidy from MEXT.

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GASTROENTEROLOGY 2010;139:1030–1040

# CD39/ENTPD1 Expression by CD4$^+$Foxp3$^+$ Regulatory T Cells Promotes Hepatic Metastatic Tumor Growth in Mice

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**BACKGROUND & AIMS:** Adenosine mediates immune suppression and is generated by the ectonucleotidases CD39 (ENTPD1) and CD73 that are expressed on vascular endothelial cells and regulatory T cells (Tregs). Although tumor-infiltrating immune cells include Foxp3$^+$ Tregs, it is not clear whether local adenosine generation by Tregs promotes tumor growth in a CD39-dependent manner. In this study, we have examined the effect of CD39 expression by Tregs on effector immune cell responses to hepatic metastases in vivo. **METHODS:** A model of hepatic metastatic cancer was developed with portal vein infusion of luciferase-expressing melanoma B16/F10 cells and MCA38 colon cancer cells in wild-type (wt) and mutant mice null for *Cd39*. Chimeric mice were generated by bone marrow transplantation (BMT) using *Cd39* null or wt C57BL6 donors and irradiated recipient mice. **RESULTS:** We demonstrate that hepatic growth of melanoma metastatic tumors was strongly inhibited in mice with *Cd39* null vasculature or in wt mice with circulating *Cd39* null bone marrow–derived cells. We show functional CD39 expression on CD4$^+$Foxp3$^+$ Tregs suppressed antitumor immunity mediated by natural killer (NK) cells in vitro and in vivo. Finally, inhibition of CD39 activity by polyoxometalate-1, a pharmacologic inhibitor of nucleoside triphosphate diphosphohydrolase activity, significantly inhibited tumor growth ($P < .001$). **CONCLUSIONS: CD39 expression on Tregs inhibits NK activity and is permissive for metastatic growth. Pharmacologic or targeted inhibition of CD39 enzymatic activity may find utility as an adjunct therapy for secondary hepatic malignancies.**

*Keywords:* Ectonucleoside Triphosphate Diphosphohydrolase-1; Regulatory T Cell (Treg); Cancer Therapy; Liver.

BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Although advances in therapies for hepatic metastatic disease have occurred,[1,2] limitations of vascular epithelial growth factor–based antiangiogenesis strategies and other regimens remain a major clinical problem. The tumor microenvironment may regulate local cellular responses by generating purinergic mediators that dampen antitumor immunity.[3] The effects of extracellular nucleosides and nucleotides are mediated by type 1 purinergic receptors for adenosine and type 2 purinergic receptors that bind extracellular nucleotides.[4] Adenosine may promote tumor growth by stimulating vascular endothelial cell proliferation and by inhibiting immune cell cytokine synthesis, trans-endothelium migration, and antitumor effector responses.[3,5,6]

CD39/ENTPD1 (ectonucleoside triphosphate diphosphohydrolase-1) is the dominant ectonucleotidase expressed by endothelial cells and immune regulatory T cells (Tregs). CD39 drives the sequential hydrolysis of both adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP).[7–10] AMP is further degraded by CD73/ecto-5′-nucleotidase to adenosine. CD39 expression by the vasculature is an absolute requirement for tumor angiogenesis.[11] We have noted that deletion of *Cd39* on Tregs results in heightened alloimmune responses.[9] Whether CD39 expression by tumor-infiltrating immune cells facilitates tumor growth has not been explored to date.

Melanoma and colon cancers are aggressive, lethal tumors that often target the liver. In this study, we note that in our model antitumor activity depends on natural killer (NK) cells. CD4$^+$Foxp3$^+$ Tregs inhibit NK cell–mediated antitumor functions, a pathway that depends on intrinsic CD39 expression. Pharmacologic inhibition of CD39, using POM-1 (polyoxometalate-1),[12] likewise inhibits tumor growth. These findings suggest that targeted inhibition of CD39 might find utility as an adjunct therapy for metastatic hepatic malignancy.

***Abbreviations used in this paper:*** **ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BMT, bone marrow transplantation; DNAM-1, DNAX accessory molecule-1; ENTPD1, ectonucleoside triphosphate diphosphohydrolase-1; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IFN-γ, interferon-γ; NK, natural killer; NTPDase, nucleoside triphosphate diphosphohydrolase; PE, phycoerythrin; POM-1, polyoxometalate-1; TCRβ, T-cell receptor β; TGF-β, transforming growth factor-β; TLC, thin-layer chromatography; Treg, regulatory T cell; wt, wild-type.**



0016-5085/$36.00
doi:10.1053/j.gastro.2010.05.007

## Materials and Methods

### *Animals*

Six- to 14-week-old male C57BL6 *Cd39* null mice were used.[8] Age-, sex-, and strain-matched wild-type (wt) mice and ($\gamma$c)/Rag2$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY). Rag1$^{-/-}$ mice were from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP (green fluorescent protein) knock-in mice were generated as described.[13]

Animal Experimentation Protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Beth Israel Deaconess Medical Center.

### *Antibodies and Reagents*

All chemicals were obtained from Sigma-Aldrich (St Louis, MO). Fluorescence-activated cell sorting (FACS) studies were performed with the use of fluorescein isothiocyanate-, phycoerythrin (PE)-, Cy-chrome-, or allophycocyanin-conjugated antibodies. The following antibodies used for FACS and analysis were from eBioscience (San Diego, CA): antimouse CD3 (clone: eBio500A2), CD4 (GK1.5), CD8 (53-6.7), CD28 (37.51), CD39 (24DMS1), T-cell receptor $\beta$ (TCR$\beta$; H57-597), and NK1.1 (PK136) (BD Bioscience, San Jose, CA). FACS data were analyzed with FlowJo software (TreeStar Inc, Ashland, OR). Antibodies used for immunohistochemistry were the following: CD31, CD4, CD8, CD11b, Gr-1 (LY6C and LY6G; BD Bioscience), F4/80, Thy1.2, Foxp3, NKp46 (R&D Systems, Minneapolis, MN), and polyclonal rabbit anti-mouse CD39 antibody.[14] Anti-PE MicroBeads and anti-Biotin MACSiBead Particles were from Miltenyi Biotec Inc (Auburn, CA). Nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitor POM-1 was obtained as described.[12]

### *Isolation of Spleen and Lymph Node Tregs, NK Cells, and Cytotoxicity Assay*

Lymphocytes were positively selected with the use of MOFLO or FACSAria cell sorter (BD Bioscience, San Jose, CA) producing >99% cell population. NK cells were

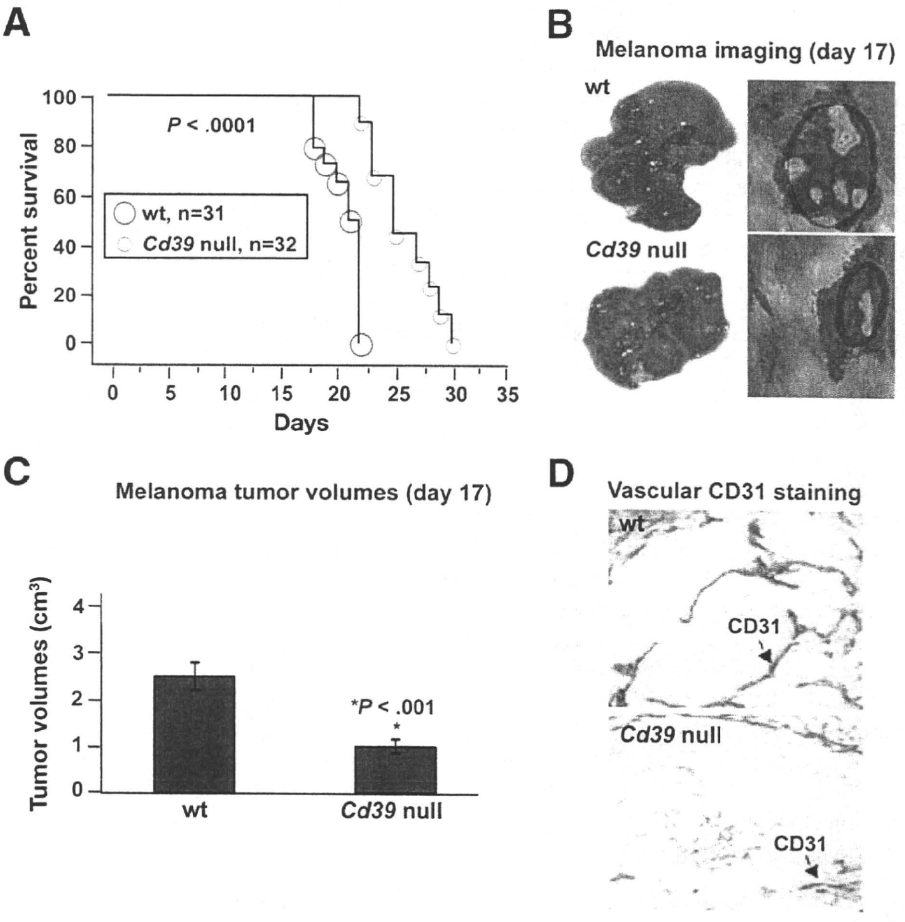



**Figure 1.** *Cd39* deletion inhibits metastatic melanoma growth in livers. (*A*) Survival and/or time to euthanasia in tumor-bearing mice after luc-B16/F10 cell infusion ($1.5 \times 10^5$ cells) through the portal vein ($P < .0001$). (*B*) Representative images of tumor-bearing livers on day 17 (*left panel*) and in vivo bioluminescent imaging of tumor metastasis (*right panel*). (*C*) Tumor volumes at day 17 (*$P < .001$). (*D*) Representative immunohistochemical staining on tissue sections obtained from (*B*) using anti-CD31 (a marker for endothelium) antibody (magnification ×400). Data are given as means ± SEMs.

sorted as NK1.1$^+$TCR$\beta^-$ cells, and Tregs were sorted as CD4$^+$GFP$^+$ cells with the use of Foxp3-GFP knock-in mice.[13] Cytolytic activity of NK cells was tested at a number of effector-to-target ratios against N YAC-1 cells with the use of the LIVE/DEAD Cell-Mediated Cytotoxicity Kit (Invitrogen Life Technologies, Carlsbad, CA).

### *Tumor Cell Lines*

Luciferase-expressing B16/F10 (luc-B16/F10 on BL6) cells were developed as described.[15] Syngeneic murine MCA38 colon cancer cells provided by Dr Nicholas P. Restifo (National Cancer Institute) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and glutamine. YAC-1 cells were purchased from American Type Culture Collection (Manassas, VA).

### *Tumor Cell Inoculation and In Vivo Bioluminescence Imaging*

Luc-B16/F10 and MCA38 cells were harvested by trypsinization and resuspended with Hanks' balanced salt solution/2% fetal bovine serum for injection. Luc-B16/F10 cells ($1.5 \times 10^5$ cells for standard and bone marrow transplantation [BMT] experiments, and $2 \times 10^5$ cells for adoptive transfer experiments) and MCA38 cells ($1.0 \times 10^5$ cells for all experiments except $2 \times 10^5$ cells for POM-1 treatments) were infused into liver through the portal vein. Tumor-bearing mice were killed and examined for tumor growth at indicated time points or if any distress or suffering was observed. In vivo tumor progression was examined with the use of the noninvasive bioimaging system IVIS (Xenogen, San Francisco, CA), as described previously,[15] at the Longwood Small Animal Imaging Facility. Perpendicular tumor diameters were also directly measured, and tumor volume was determined by integration with the following formula: $\Sigma t_1 + t_2 + \ldots . t_n$ ($t = a^2 \times b \times 0.52$; a = smaller tumor diameter, b = larger tumor diameter).[6,16]

### *Bone Marrow Transplantation*

Six-week-old male *Cd39* null mice and wt mice were exposed to 10 Gy (0.28 Gy/min, 200 kV, 4 mA) $\gamma$-ray total body irradiation, using an Andrex Smart 225 (Andrex Radiation Products AS, Copenhagen, Denmark) with a 4-mm aluminum filter. The marrow from the femur and tibia of matched *Cd39* null mice and wt mice was harvested, and cells were purified under sterile conditions. Irradiated recipient mice received $10 \times 10^6$ bone marrow cells intravenously. The success of BMT was validated by FACS analysis of immune cell populations (not shown). Mice that received a transplant were housed in autoclaved cages for 8 weeks before experimentation.[17]

### *Adoptive Transfer Experiments*

Freshly sorted CD4$^+$ T cells ($1 \times 10^6$), CD8$^+$ T cells ($0.5 \times 10^6$), Treg cells ($0.1 \times 10^6$), or Teff cells ($0.9 \times 10^6$) from *Cd39* null or wt mice or wt NK cells ($1.5 \times 10^6$) were injected into Rag1$^{-/-}$ mice. Wt NK cells ($1 \times 10^6$), alone or with wt Tregs ($1 \times 10^6$), or *Cd39* null Tregs ($1 \times 10^6$) were injected into ($\gamma$c)/Rag2$^{-/-}$ mice.

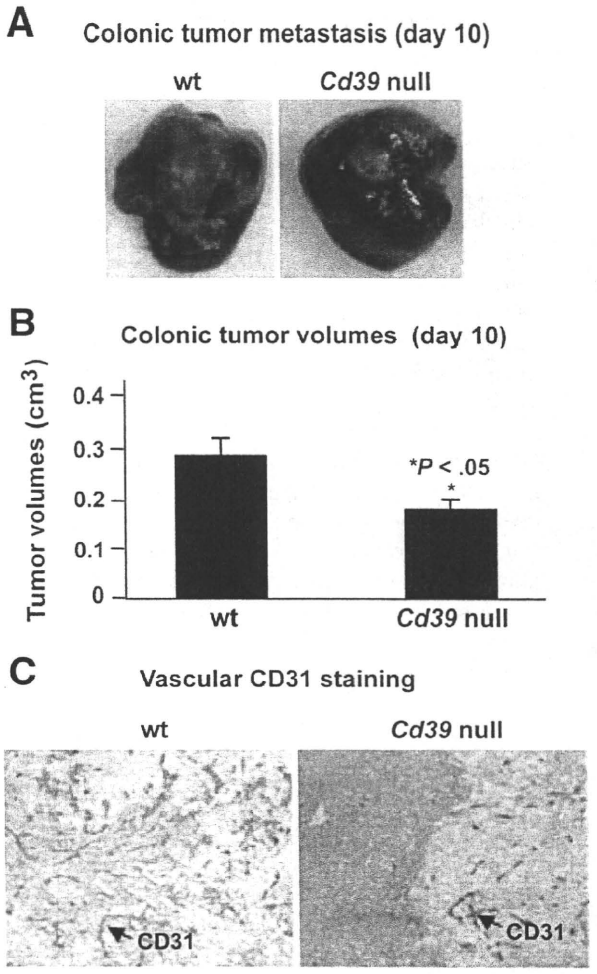



**Figure 2.** *Cd39* deletion inhibits metastatic colon tumor growth in livers. (*A*) Representative images of tumor-bearing livers at day 10 after portal vein infusion of $1.0 \times 10^5$ MCA38 cells. (*B*) Tumor volumes at day 10 (*$P < .05$). (*C*) Immunohistochemical staining on tissue sections obtained from (*A*) using anti-CD31 (a marker for endothelium) antibody (magnification ×400). Data are given as means ± SEMs.

### *Histology and Immunohistochemistry*

Paraffin-embedded or frozen sections of tumor-bearing livers were analyzed by immunohistochemical staining as described.[11,18] Apoptosis was determined with the use of TdT (terminal deoxynucleotidyl-transferase)-mediated nick-end labeling staining (Millipore, Temecula, CA).

### *Measurement of IFN-$\gamma$ Production by Enzyme-Linked Immunoabsorbent Assay*

Interferon-$\gamma$ (IFN-$\gamma$) plasma concentrations were measured with the use of the Mouse IFN-$\gamma$ (Femato-HS) ELISA Ready-To-Go kit from eBioscience.

### *Purification of Tumor- or Liver-Infiltrating Mononuclear Cells*

These cells were purified as described previously.[19]

### *Thin-Layer Chromatography Analysis*

NTPDase activity of CD39 on freshly isolated cells was analyzed with the use of thin-layer chromatography (TLC), as described.[9,19]

### *Statistical Analysis*

Results in this study are expressed as means ± SEMs of values (obtained from ≥4 mice per group and/or ≥3 independent in vitro experiments). All histology and immunohistochemical images are representative of ≥4 mice per group.

For statistical analyses, the 2-tailed Student *t* test was used. Animal time to euthanasia was analyzed with the Kaplan–Meier method and was compared with the log-rank test (StatView 5.0 analysis software; SAS Institute, Cary, NC). Significance was defined as $P < .05$.

## Results

### *CD39 Expression Facilitates Tumor Growth*

Portal vein infusion of luciferase-expressing melanoma B16/F10 (luc-B16/F10) cells and MCA38 colon cancer cells resulted in cells being retained in liver sinusoids; no tumors were found in lungs or other sites within 14 days (data not shown). Melanoma-bearing *Cd39* null mice had longer times to euthanasia and death than the wt mice ($P < .0001$; Figure 1*A*).

Tumor growth in liver was substantially inhibited in *Cd39* null mice (melanoma data in Figure 1*B* and *C* and colonic tumor studies in Figure 2*A* and *B*) and were associated with decreased angiogenesis (Figure 1*D* and Figure 2*C*). Luc-B16/F10 cells and MCA38 cells do not express CD39 per se in vitro or in vivo at any tumor progression stages (data not shown). Hence, endogenous CD39 expression is required for the development of metastatic tumors in the liver.

We next studied bone marrow reconstitution. Melanoma growth was significantly delayed in the livers of irradiated, reconstituted wt mice that had received *Cd39* null BM transplant (Figure 3*A* and *B*; $P < .05$), compared with wt mice that received wt BM transplant. Here, defective angiogenesis in tumors was associated with *Cd39* null vasculature. High levels of cell death were clearly demonstrated at the center of tumor nodules (Figure 3*C*).

Differences of hepatic melanoma growth among groups on days 14 and 21 were comparable to that seen on day 10 (Supplementary Figure 1; data not shown). Mortality was only seen in wt BM-wt mice, from day 17; all other groups survived for ≥21 days (data not shown). Taken together, these data indicate that CD39 expression by BM-derived cells is critical for tumor growth.

### *Characterization of Tumor-Infiltrating Cells*

Melanoma tumor tissues were infiltrated by $CD39^+$, $CD4^+$, $CD8^+$, and $Thy1.2^+$ cells; macrophages ($F4/80^+$); granulocytes and leukocytes ($Gr\text{-}1^+$); NK ($NK1.1^+TCR\beta^-$) and NKT ($NK1.1^+TCR\beta^+$) cells; as well as cells positive for CD11b, indicative of myeloid-derived suppressive cells (Supplementary Figure 2*A* and *B*).[20]

A proportion of the infiltrating $CD4^+$ cells was positive for both CD39 and Foxp3 (Supplementary Figure 2*C* and *D*), suggesting that Tregs infiltrate tumors.[13,21] Because Tregs express CD39 (Supplementary Figure 2*C* and *D*),[9] we measured ecto-ADPase activity of purified tumor-infiltrating mononuclear cells by TLC assay. *Cd39* null cells exhibited markedly decreased ecto-ADPase activity (Figure 3*D*).

### Cd39 *Null* $CD4^+$ *T Cells Inhibit Tumor Growth*

Immunity to melanoma could be mediated by a variety of immune cells (eg, NK, NKT, and $CD8^+$ T cells).[15,22-25] Tregs have been shown to suppress antimelanoma cytotoxic responses by $CD8^+$ cytotoxic T lymphocytes, NKT cells, and NK cells at the local tumor site.[15,22,23,26]

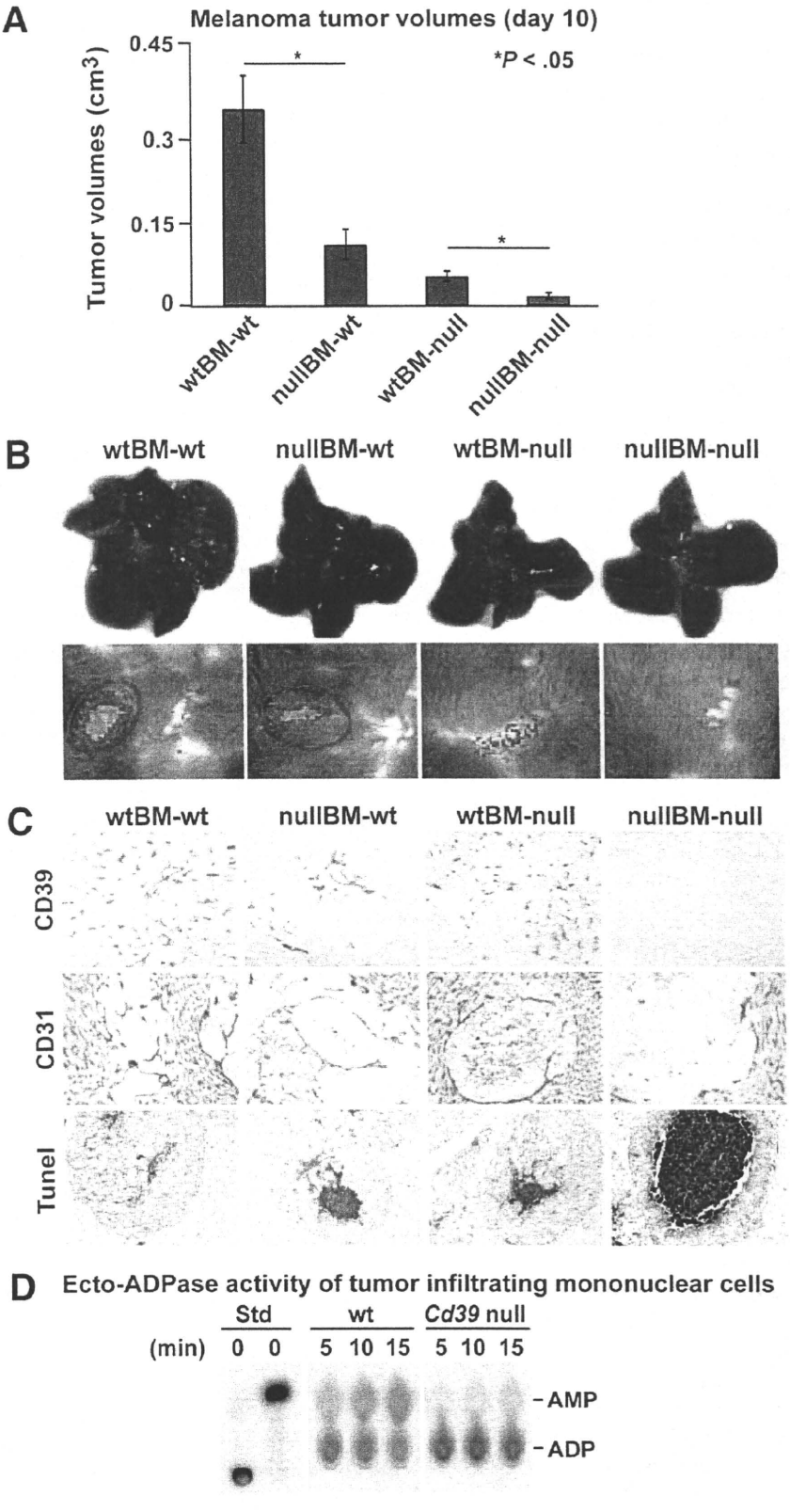
To examine first whether CD39 expression on $CD4^+$ T cells is involved in antitumor immunity, wt or *Cd39* null $CD4^+$ T cells in combination with effector wt $CD8^+$ T cells were adoptively transferred into $Rag1^{-/-}$ mice. $CD8^+$ T cells alone failed to control melanoma growth unless $CD4^+$ T cells were co-transferred (Figure 4*A*). Tumor growth was significantly inhibited when $Rag1^{-/-}$ mice were co-transferred with *Cd39* null $CD4^+$ T cells, compared with wt $CD4^+$ T cells ($P < .05$; Figure 4*A*).

Liver-infiltrating mononuclear cells of these mice were isolated, and the percentages of $CD4^+$, $CD8^+$, NK, and NKT cells were evaluated by FACS analysis (Figure 4*B*). Heightened levels of NK cells were observed in mice adoptively transferred with *Cd39* null $CD4^+$ T cells ($wtCD4^+wtCD8$, 23.25% ± 5.24% vs $nullCD4^+wtCD8$, 34.34% ± 8.18%; $P = .05$). In contrast, percentages of adoptively transferred $CD4^+$ or $CD8^+$ cells remained at the same level (Figure 4*B*).

We next transferred wt or *Cd39* null $CD4^+$ T cells into $Rag1^{-/-}$ mice followed by subcutaneous injection of 0.2 mL of Matrigel into the flanks 24 hours later. We did not observe any inhibition by $CD4^+$ T cells on the process of endothelial angiogenesis (Supplementary Figure 3).

### *NK Cells Mediate Antitumor Immunity*

We next performed similar adoptive transfer experiments with the use of NK cell–deficient $(\gamma c)/Rag2^{-/-}$ mice, instead of $Rag1^{-/-}$ mice. In $(\gamma c)/Rag2^{-/-}$ mice, no inhibition of melanoma growth was noted even after T-cell transfer (Figure 5*A*), suggesting that NK cells are required to inhibit melanoma growth. We next reconstituted $(\gamma c)/Rag2^{-/-}$ mice with wt NK cells followed by melanoma cell


A
Melanoma tumor volumes (day 10)
*P < .05
Tumor volumes (cm3)
0.45
0.3
0.15
0
wtBM-wt
nullBM-wt
wtBM-null
nullBM-null
B
wtBM-wt
nullBM-wt
wtBM-null
nullBM-null
C
wtBM-wt
nullBM-wt
wtBM-null
nullBM-null
CD39
CD31
Tunel
D
Ecto-ADPase activity of tumor infiltrating mononuclear cells
Std
wt
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AMP
ADP

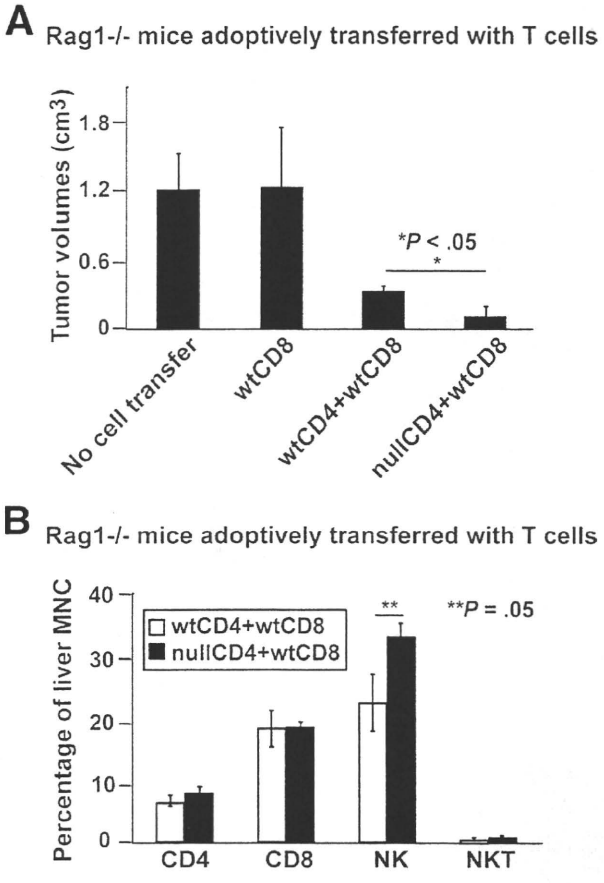


**Figure 4.** *Cd39* null CD4$^+$ T cells inhibit tumor growth. (*A*) Tumor volumes of tumor-bearing Rag1$^{-/-}$ mice adoptively transferred with lymphocytes (CD4$^+$ T cells, 1 × 10$^6$; CD8$^+$ T cells, 0.5 × 10$^6$), followed by portal vein infusion of 2 × 10$^5$ luc-B16/F10 cells, on day 14. (*B*) Percentage of CD4$^+$, CD8$^+$, NK1.1$^+$, and NKT lymphocytes of liver mononuclear cells isolated from tumor-bearing livers above. Data are given as means ± SEMs. *$P$ < .05, **$P$ = .05.

inoculation. NK-cell reconstitution greatly suppressed tumor growth in (γc)/Rag2$^{-/-}$ mice ($P$ < .001; Figure 5*B*), with migration of NK cells into the liver ($P$ < .001; Figure 5*C*) expressing NKp46 (Figure 5*D*). Antitumor activity of NK cells was noted to depend on cell number (data not shown). Colonic tumor growth was also inhibited in Rag1$^{-/-}$ mice, compared with (γc)/Rag2$^{-/-}$ mice ($P$ < 1E-06; Supplementary Figure 4*A* and *B*), associated with migration of endogenous NK cells into the liver ($P$ < 1E-06; Supplementary Figure 4*C*) and strong expression of NKp46 (Supplementary Figure 4*D*). These data indicate that NK cells play important roles in antitumor immunity.

### *Tregs Suppress NK Cell–Mediated Antitumor Responses in a CD39-Dependent Manner*

NK cell–mediated antitumor functions may be suppressed by Tregs in vitro and in vivo.[22,26] This regulation seems NKG2D mediated and transforming growth factor-β (TGF-β) dependent.[22,26,27] Extracellular purinergic mediators and related metabolites may also exert inhibitory effects on cytotoxic activity and cytokine production of activated NK cells.[28,29] Therefore, it is conceivable that CD39 expressed by Tregs regulates NK cell activity.

To test for this possibility, wt or *Cd39* null Tregs were activated with anti-CD3/CD28 and interleukin-2 (IL-2) for 72 hours before coculturing with wt NK cells in vitro. We demonstrated specific ability of in vitro–activated Tregs to suppress NK cell–mediated cytolysis of YAC-1 target cells ($P$ < .01, wtNK vs wtNK$^+$wtTreg; Figure 6*A*). *Cd39* null Tregs lacked inhibitory effects on NK cell–mediated cytotoxicity (Figure 6*A*).

Rag1$^{-/-}$ mice were then adoptively transferred with wt Tregs (CD4$^+$GFP$^+$Foxp3$^+$) from Foxp3-GFP knock-in mice or with wt Teff (CD4$^+$GFP$^-$Foxp3$^-$) or in combination, followed by melanoma cell inoculation. Decreased numbers of liver endogenous NK cells and lower plasma levels of IFN-γ were noted in mice transferred with Tregs alone, compared with mice transferred with Teff alone ($P$ < .05 and $P$ < .01, respectively) (Figure 6*B* and *C*). Effects of Tregs were dominant, as shown in Treg + Teff combinations (Figure 6*B* and *C*).

We reconstituted (γc)/Rag2$^{-/-}$ mice with wt NK cells alone or in combination with wt or *Cd39* null Treg, followed by melanoma cell inoculation. As expected, wt NK cells inhibited tumor growth. NK cells lost antitumor efficacy if co-transferred with wt Tregs. NK cell activity was preserved when *Cd39* null Tregs were co-transferred, with tumor growth abrogated ($P$ < .05) (Figure 6*D*). Antitumor activity correlated with intrahepatic enrichment of administered NK cells (Figure 6*E*). Hence, Tregs directly suppress NK cell–controlled tumor expansion in a manner dependent on Treg-specific CD39.

### *Pharmacologic Inhibition of CD39 Activity Impedes Tumor Growth*

A high proportion of tumor-infiltrating CD4$^+$ T cells are Foxp3$^+$ Tregs. Deleting these suppressors or



**Figure 3.** CD39 expression on bone marrow–derived cells facilitates tumor growth. Wild type (wt) and *Cd39* null (null) chimeric mice were generated by BMT (donor genotype designated first/recipients second). Eight weeks after BMT, 1.5 × 10$^5$ luc-B16/F10 cells were infused through the portal vein. (*A*) Tumor volumes on day 10 (n = 6–8 mice per group). (*B*) Representative images of tumor-bearing livers at day 10 (*top*) and in vivo bioluminescent imaging of tumor metastasis (*bottom*). (*C*) Representative immunohistochemical staining with the use of anti-CD39 and anti-CD31 antibodies and TdT (terminal deoxynucleotidyl-transferase)–mediated nick-end labeling staining for apoptosis (magnification ×400 for CD39; ×200 for CD31 and TUNEL staining). (*D*) Ecto-ADPase activity in tumor-infiltrating mononuclear cells. Tumor-infiltrating mononuclear cells were isolated from tumor tissues of wt and *Cd39* null tumor-bearing mice. Thin-layer chromatography (TLC) analysis of ADP hydrolysis to AMP with the use of 1.5 × 10$^5$ cells was analyzed. Data are given as means ± SEMs. *$P$ < .05.

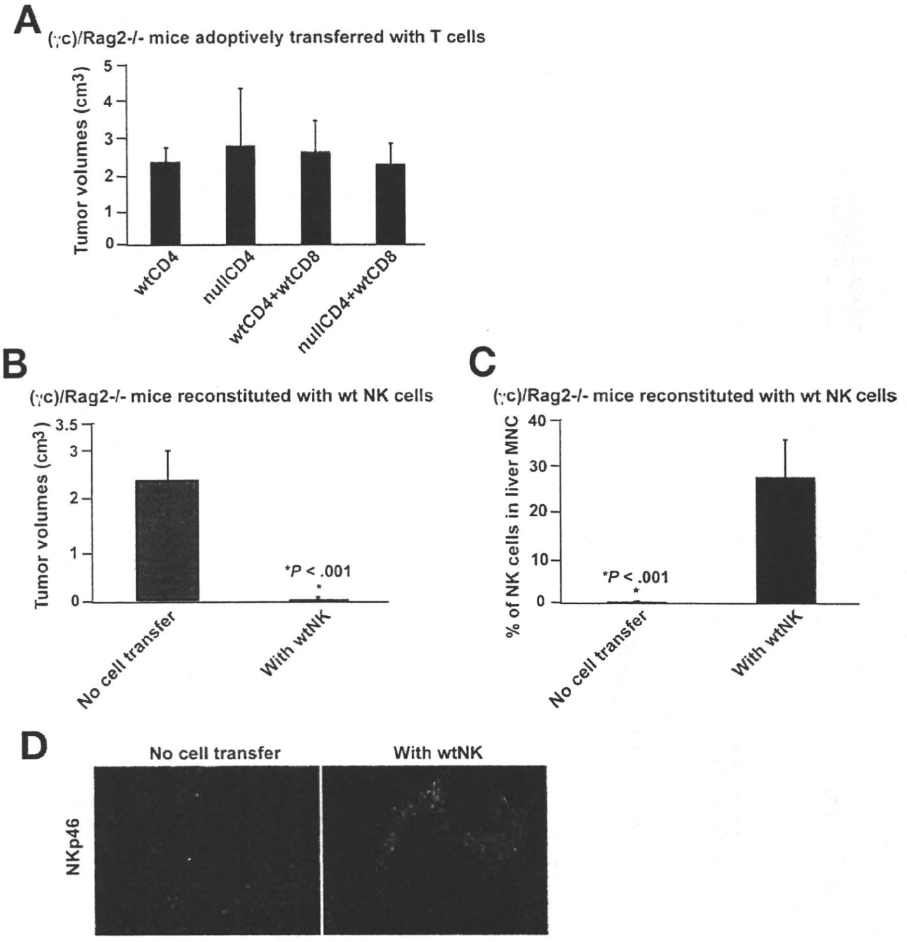


**Figure 5.** NK cells mediate antitumor immunity to melanoma. (*A*) Tumor volumes of tumor-bearing (γc)/Rag2$^{-/-}$ mice adoptively transferred with lymphocytes (CD4$^+$ T cells, 1 × 10$^6$; CD8$^+$ T cells, 0.5 × 10$^6$), followed by portal vein infusion of 2 × 10$^5$ luc-B16/F10 cells, on day 14. (*B*) Tumor volumes of tumor-bearing (γc)/Rag2$^{-/-}$ mice reconstituted with 1.5 × 10$^6$ of wt, spleen- and lymph node–derived NK cells (sorted as NK1.1$^+$TCRβ$^-$ using MOFLO), on day 14. (*C*) Percentage of NK cells among mononuclear cells isolated from tumor-bearing livers of *panel B*. (*D*) Immunofluorescence staining with the use of anti-NKp46 antibody on livers of *panel B*: DAPI staining nuclei in *blue* and NKp46 in *red* (magnification ×200). Data are given as means ± SEMs. *$P < .001$.



mitigating suppressive activity could boost antitumor immunity. Pharmacologic inhibition of CD39 enzymatic activity might be an anticancer treatment. POMs are anionic complexes that inhibit NTPDase activity[12] and have been proposed as chemotherapeutic agents against many human cancers, eg, colon cancer, lung cancer, and breast cancer.[30,31]

We observed that POM-1 inhibited ecto-NTPDase activity of Tregs in vitro (Figure 7*A*). POM-1 (5 or 10 mg/kg/d) was then injected intraperitoneally into wt mice for 10 days after melanoma cell challenge. Tumor growth on day 14 decreased in POM-1–treated wt mice, in contrast to saline-treated wt mice ($P < .001$; Figure 7*B*). No detectable liver and renal toxicity was observed at suppressive doses (Supplementary Figure 5). In parallel, POM-1 (5 mg/kg/d) did not affect melanoma tumor growth in *Cd39* null mice, indicating that inhibitory effects of POM-1 are mediated by CD39 ($P = .23$; Figure 7*C*). Furthermore, POM-1 (5 mg/kg/d) was shown to suppress hepatic metastatic colonic tumor growth in wt mice ($P < .05$; Figure 7*D*).

## Discussion

We have already shown that CD39 expression on endothelium is important for angiogenesis and is required for tumor growth in the lung.[11,18] We show now that infiltrating Tregs inhibit NK cell–mediated tumor cytotoxicity in a CD39-dependent manner in the liver. Tregs exhibit immunosuppressive effects on effector T cells, operational by adenosine-mediated pathways modulated by CD39 and CD73.[9] Our current findings strengthen the notion that the expression of CD39 and

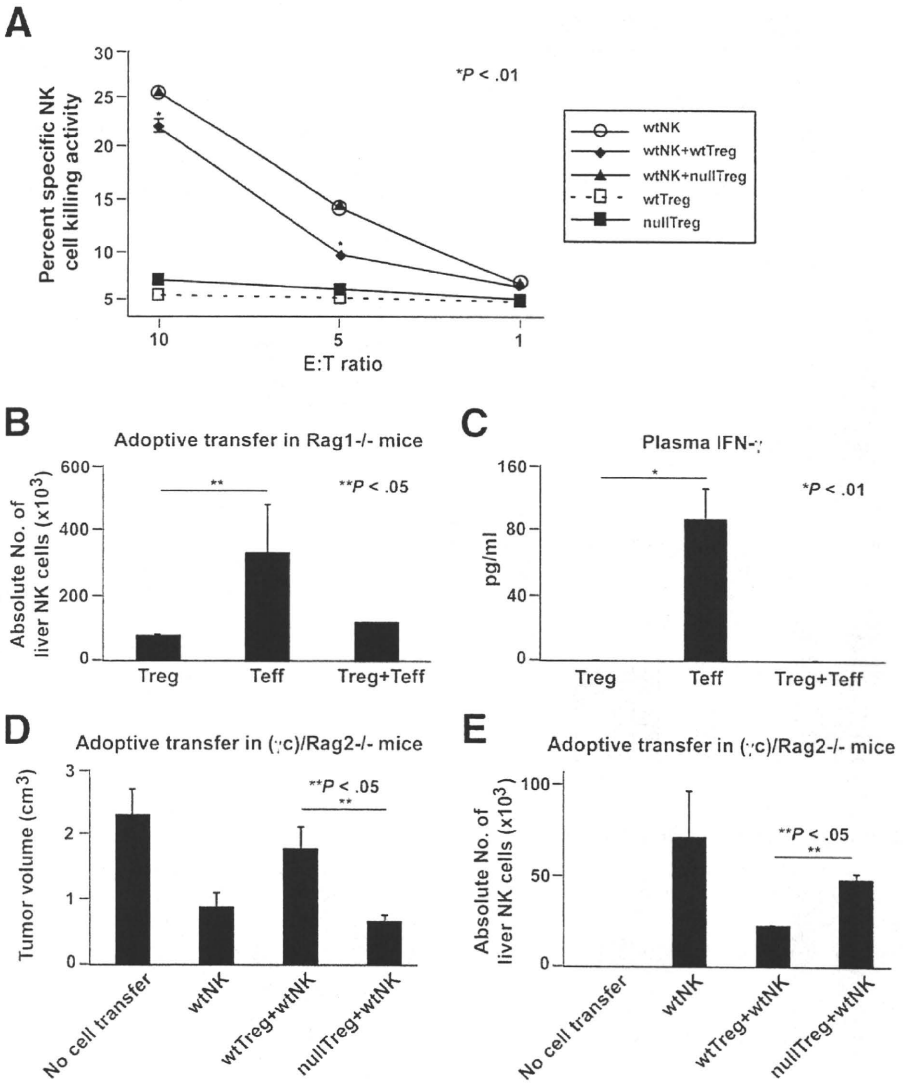


**Figure 6.** Tregs regulate NK cell–mediated antitumor immunity in a CD39-dependent manner. (*A*) *Cd39* null Tregs fail to suppress NK cell cytotoxicity in vitro. NK cells were cocultured at 1.5:1 and other ratios (not shown) with activated wt or *Cd39* null Tregs. The sorted $CD4^+GFP^+$ Tregs were stimulated with anti-CD3/CD28 bound MACSiBead Particles and interleukin-2 (100 ng/mL) for 72 hours before coculture. (*$P < .01$, wtNK vs wtNK+wtTreg). (*B*) Absolute liver NK cell numbers in tumor-bearing $Rag1^{-/-}$ mice adoptively transferred with sorted wt lymphocytes (Treg, $0.1 \times 10^6$; Teff, $0.9 \times 10^6$) as indicated, followed by portal vein infusion of $2 \times 10^5$ luc-B16/F10 cells, on day 14. (*C*) Levels of IFN-γ in plasma obtained from mice in *panel B*. (*D*) Tumor volumes of tumor-bearing $(\gamma c)/Rag2^{-/-}$ mice adoptively transferred with lymphocytes (Treg, $1.0 \times 10^6$; NK, $1.0 \times 10^6$), followed by portal vein infusion of $2 \times 10^5$ luc-B16/F10 cells, on day 14. Mice received phosphate-buffered saline as controls. (*E*) Absolute liver NK cell numbers in tumor-bearing mice of *panel D*. Data are given as means ± SEMs. *$P < .01$, **$P < .05$.

associated changes in nucleotide/nucleoside balance are integral components of Treg cell function.

NK cells are an important component of innate defenses against malignant cells. Tumor growth is accelerated in NK-deficient $(\gamma c)/Rag2^{-/-}$ mice. This phenotype could be corrected by NK cell reconstitution. NK cell-mediated antitumor functions are susceptible to direct Treg suppression in vitro and in vivo.[22,26,32] Suppression has been suggested to be, at least in part, NKG2D mediated and TGF-β dependent.[22,26,27] However, other mechanisms might also be involved.[33]

Adenosine interacts with 4 different adenosine receptor subtypes (A1, A2a, A2b, and A3 AR). A2a signaling has been shown to play a predominant role in inhibiting cytotoxicity of activated NK cells.[6,28,34,35] A2b receptors have low affinity and might promote tumor growth at higher concentrations of adenosine.[36] Whether A2a and/or A2b are responsible for suppressing NK function in our model is currently undetermined.[5,37]

NK cells express CD39, but lack CD73 expression.[19,38] Several studies have reported that purines (ATP = ADP > AMP = adenosine) inhibit cellular proliferation, cytokine

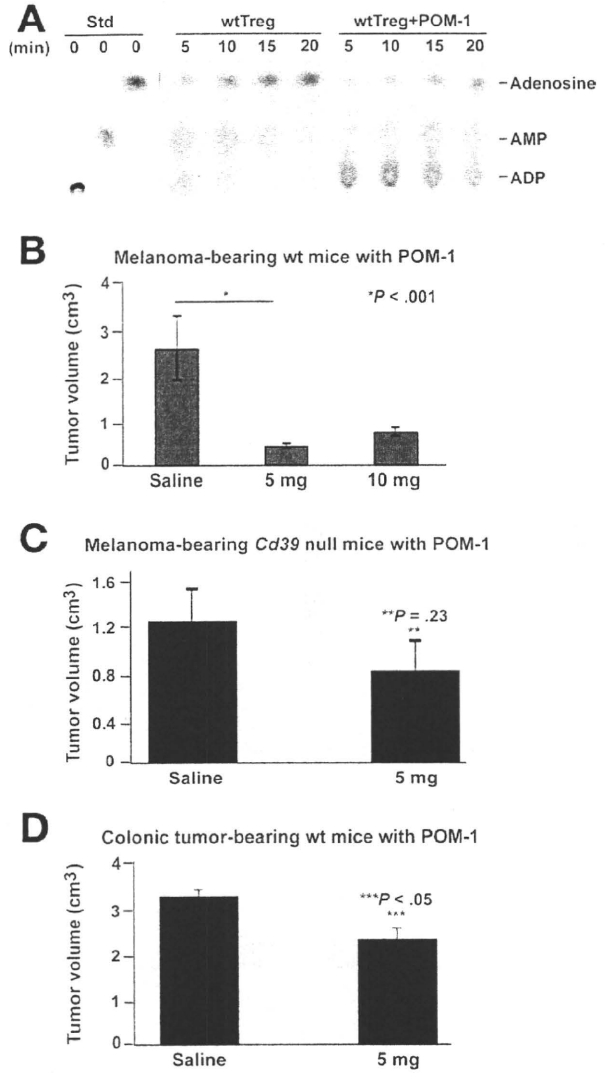


**Figure 7.** Pharmacologic inhibition of CD39 activity suppresses tumor growth. (*A*) Ecto-NTPDase activity of POM-1–treated wt Tregs in vitro. Wt Tregs ($CD39^+CD4^+Foxp3^+$, $3 \times 10^5$) were pretreated with 26 $\mu$m of POM-1, washed, and then subjected to TLC ADPase analysis. Tregs incubated in POM-1–free medium served as control. (*B*) Tumor volumes of melanoma-bearing wt mice (received $2 \times 10^5$ luc-B16/F10 cells) treated with POM-1 on a daily dosage of 5 or 10 mg/kg for 10 days, on day 14. Mice that received saline were used as controls. (*C*) Tumor volumes of melanoma-bearing *Cd39* null mice (received $2 \times 10^5$ luc-B16/F10 cells) treated with POM-1 on a daily dosage of 5 mg/kg for 10 days, on day 14. Mice that received saline served as controls. (*D*) Tumor volumes of colonic tumor-bearing wt mice (received $2 \times 10^5$ MCA38 cells) treated with POM-1 on a daily dosage of 5 mg/kg for 10 days, on day 14. Mice that received saline were used as controls. Data are given as means ± SEMs. $^*P < .001$, $^{**}P = .23$, $^{***}P < .05$.

production, and cytotoxic activity of activated NK cells.[29,34,39-41] Molecular mechanisms underlying purine-mediated suppression of NK cell activity are highly complex. Whether and how deletion of *Cd39* affects NK cell activity may depend on the types of the stimuli and the resulting balance between adenosine- and ATP/ADP-mediated signaling pathways and is being tested further.

Solid tumors are infiltrated by Tregs at a far higher frequency than seen in normal lymphoid organs. It is suggested that depleting Tregs with antibodies against surface markers, eg, CD25, would have a positive effect on the immunotherapy of cancer.[42] Nevertheless, CD25 is also expressed on many types of activated immune cells, and benefits of this strategy are questionable. We show here that blocking CD39 enzymatic activity with POM-1, a pharmacologic inhibitor of NTPDase activity,[12] could inhibit adenosine generation by Tregs in vitro and abrogate tumor growth in vivo.

CD39 is also expressed on a variety of immune cells, including B cells, dendritic cells, macrophages, NK cells, NKT cells,[9,19,35,43,44] and $CD4^+$ T memory cells ($CD4^+CD44^+CD62L^-Foxp3^-$).[45] Tregs are also not the only tumor-infiltrating suppressor cells. Myeloid-derived suppressor cells have been shown to be potent suppressors of various T-cell functions in tumor-bearing mice and in patients with cancer.[20] In mice, these cells express myeloid-cell lineage differentiation antigens GR-1 and CD11b.[20] Monocyte and neutrophil populations express CD39,[18,46,47] and it was shown here that tumor-infiltrating immune cells contain $GR\text{-}1^+$ and $CD11b^+$ cells (Supplementary Figure 2).

It was recently shown that B16/F10 melanoma cells express ligands for natural cytotoxicity receptors (NKp46 in mice) and DNAX accessory molecule-1 (DNAM-1), but not NKG2D.[48] Interference with both DNAM-1 and natural cytotoxicity receptor pathways results in dramatic decrease in killing of melanoma cells.[48] We have observed strong NKp46 staining on tumor-infiltrating NK cells, associated with decreased melanoma and colonic tumor growth (Figure 5 and Supplementary Figure 4).

NK cells may stimulate the maturation of dendritic cells,[49] produce cytokines that affect $CD4^+$ and $CD8^+$ effector T cells,[50] and regulate proliferation of T cells.[51] Our current data clearly show that NK and Teff cells interact, particularly in the context of IFN-$\gamma$ production, and that NK cells could directly influence adaptive immune responses.

We have noted effects of CD39 expression on pulmonary and subcutaneous metastases of melanoma, and colon and colorectal cancers (Beat Künzli and S.C.R., unpublished data, January 2008, and Jackson et al[11]). Taken together, our studies provide an intriguing mechanism for the purinergic suppression of NK cell–mediated antitumor immunity by CD39 expression on Treg. Other investigators have recently shown that CD39 may be expressed on infiltrating T cells to generate adenosine in lymphoproliferative disorders[52] or on Tregs associated with head and neck tumors.[53,54] Work has been done also to neutralize tumor cell–expressed CD73 in a model of breast cancer with salutary effects.[55] Our work strongly

suggests that it is CD39 expression on Tregs that modulates NK cell reactivity against tumor cells.

Detailed analyses of CD39 expression and Treg function in patients at different stages of hepatic metastatic disease may help improve our understanding of the molecular mechanisms resulting in tumor growth and metastasis. Inhibition of CD39 expression and/or activity might also find future utility as a component of anticancer immunotherapy.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.05.007.

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**Received November 10, 2009. Accepted May 13, 2010.**

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***Acknowledgments***

**The authors thank members of the Longwood Small Animal Imaging Facility of BIDMC for assistance with the in vivo bioluminescence imaging.**

**X.S. and Y.W. contributed equally to this work, and the surnames are arranged alphabetically.**

***Conflicts of interest***

**The authors disclose no conflicts.**

***Funding***

**This study has been supported by the National Institutes of Health (NHLBI P01-HL076540 and R01-HL094400).**

# Luminescence Imaging of Regenerating Free Bone Graft in Rats

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**Background:** Bone transplantation is an important procedure often used for bone defect reconstruction after trauma and malignancies. However, the kinetics of free bone graft–derived cells remains unclarified. The authors examined the kinetics of graft-derived cells using transgenic rats systemically expressing firefly luciferase.

**Methods:** Free iliac bone grafts ($5 \times 5 \times 2$ mm, $n = 10$) derived from luciferase transgenic rats were transplanted into the subcutaneous space of the back of wild-type Lewis rats, and the kinetics of graft-derived cells were evaluated over time by determining the level of luminescence emission.

**Results:** Although the luminescence level emitted by luciferase decreased after transplantation, a substantial luminescence level (mean, $1.6 \times 10^7$ photons/second) was emitted from donor-derived cells even at 180 days after transplantation, suggesting a long-term survival of graft-derived cells. In a computed tomographic image analysis of bone grafts retrieved 180 days after transplantation, high-luminescence grafts with a sufficient number of viable graft-derived cells (mean, $2.6 \times 10^7$ photons/second; $n = 4$) showed significantly higher bone graft volume (3.1-fold) and polar moment of inertia of area (7.2-fold) than low-luminescence grafts (mean, $1.0 \times 10^7$ photons/second; $n = 4$), indicating that high-luminescence grafts maintain better conditions.

**Conclusion:** These results suggest that bone graft–derived cells can survive for a long time and that the presence of a sufficient number of viable graft-derived cells is essential for graft engraftment and remodeling. (*Plast. Reconstr. Surg.* 127: 78, 2011.)

Bone transplantation is a basic surgical procedure used for reconstruction of bone defects from cancer or trauma and treatment of congenital anomalies of the extremity, such as brachydactyly. However, the procedure may not lead to substantial healing because of infection or resorption of the bone graft.

Various studies have been conducted on free bone transplantation. One of the concepts that has been supported for a long time is that of "creeping substitution," in that a large part of the cellular component of a free bone graft dies within a few days, followed by gradual absorption and replacement starting at the part in contact with a viable graft.[1] Although some studies have shown that cells in bone grafts eventually die,[2] others have demonstrated that cells located approximately 300 μm from the cortical bone surface of the bone graft survive.[3,4] Recent studies have also shown the survival of bone graft–derived cells.[5] It is clinically and experimentally known that fresh autologous bone grafts can achieve better engraftment than allogeneic or preserved bone grafts.[6] However, little is known regarding whether graft-derived cells can survive or not.

Luciferase-transgenic rats, in which the luciferase gene is transfected so that cells in almost all parts of the body have luciferase activity, have recently been developed.[7] The use of these genetically engineered animals has enabled us to examine the time course of the viability of transplanted cells in live animals using luciferase luminescence

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*Received for publication February 18, 2010; accepted June 22, 2010.*

DOI: 10.1097/PRS.0b013e3181f959b2

**Disclosure:** *The authors have no commercial, proprietary, or financial interests related to the information in this article.*

as a marker. Specifically, administration of the luminescence substrate luciferin to these animals results in the emission of luminescence intensity that is dependent on the amount of adenosine triphosphate produced in cells, enabling us to visually and quantitatively evaluate cell viability by measuring luminescence level.

In this study, we demonstrated that graft-derived cells are capable of long-term survival and determined the role of these cells in graft acceptance. These experimental results may have an implication to the long-term survival of free bone grafts, which are commonly applied in clinical practice.

## MATERIALS AND METHODS

### Experimental Design

All surgical interventions and animal care were performed in accordance with Jichi Medical University Guide for Laboratory Animals. Lewis rats were purchased from Charles River Japan (Yokohama, Japan). All surgical procedures were performed under anesthesia with intraperitoneal injection of Nembutal (Abbott, Chicago, Ill.). All rats were housed in cages and allowed normal cage activity without any restrictions. All animals were euthanized by means of an anesthetic overdose injection at final evaluation. All experiments were carried out using 10-week-old male rats.

### Surgical Procedure: Free Bone Transplantation and Vascularized Bone Transplantation

A vascularized tibial transplant model of the Lewis rat was established.[8] A 2.5-cm tibiofibular complex with cartilage removed from the knee joint surface on the tibial side and with a vascular pedicle of the femoral artery and vein was used as a vascularized bone graft. The vessels were anastomosed to the femoral artery and vein of a recipient rat with a 10-0 nylon suture under a microscope. The ischemic time during this procedure was approximately 30 minutes. The vascularized bone graft was implanted into a subcutaneous pocket formed in the right inguinal region, and another tibiofibular complex without a vascular pedicle collected from the other leg of the same donor rat was implanted as a free bone graft in the left inguinal region ($n = 3$). The wound was opened 14 days postoperatively, and the patency test of the anastomosed vessels was performed under a microscope. At the same time, the bone grafts were removed for histologic examination.

### In Vivo Bioluminescence Imaging

Luciferase-expressing Lewis transgenic rats were used.[7] To visualize luciferase expression ex vivo and in vivo, an in vivo bioimaging system (IVIS; Xenogen, Alameda, Calif.) was used. To detect photons from luciferase-expressing cells, D-luciferin (potassium salt; Biosynth, Postfach, Switzerland) was injected into the penile vein of anesthetized rats (30 mg/kg body weight) with isoflurane (Abbott). Lucimages were taken using IVIS and quantified using the IVIS Living Image software (Xenogen) package. Data were expressed as photons per second per square centimeter per steradian.

To examine whether the living bone derived from luciferase-expressing Lewis transgenic rats emitted luminescent light, fresh bones and bones devitalized by freezing twice for 5 minutes in liquid nitrogen were tested ($n = 3$). Macroscopic images were acquired in a drop of phosphate-buffered saline. Next, iliac free bone grafts ($5 \times 5 \times 2$ mm) from luciferase-expressing Lewis transgenic rats were transplanted into the subcutaneous space of recipient wild-type Lewis rats ($n = 10$). As a control, iliac bone grafts ($5 \times 5 \times 2$ mm) were also collected from wild-type Lewis rats and transplanted into the subcutaneous pockets of recipient rats using the same procedure ($n = 4$). Post-transplantation measurement consisted of 10 sequential sessions of 1-minute measurement, so that luminescence intensity could be recorded over time.

### Detection of LacZ Expression by Immunohistochemistry

LacZ-transgenic rats is derived from LEW (Rosa/LacZ-Lew) rats driven under the ROSA 26 promotor.[8] Free iliac bone transplantation ($5 \times 5 \times 2$ mm) from LacZ-transgenic rats to wild-type Lewis rats was performed ($n = 4$). Transplanted bone grafts were removed at various time points after transplantation for immunohistochemistry.

An iliac bone collected from a LacZ-transgenic rat was used as positive control, and an iliac bone collected from a wild-type Lewis rat was used as a negative control ($n = 4$). Bone tissues were fixed in 10% formalin at ambient temperature for 2 days, followed by decalcification in 10% ethylenediaminetetraacetic acid solution at ambient temperature for 14 days. Tissues were then paraffin-embedded and sliced into 5-$\mu$m sections.

LacZ expression was evaluated by immunohistochemistry using anti–$\beta$-galactosidase rabbit polyclonal antibody (1:1500; Biogenesis, Poole,