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*J Immunol* published online 25 January 2013
http://www.jimmunol.org/content/early/2013/01/25/jimmunol.1202282

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/25/jimmunol.1202282.DC1

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Multiple Tumor-Associated MicroRNAs Modulate the Survival and Longevity of Dendritic Cells by Targeting YWHAZ and Bcl2 Signaling Pathways

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Tumors use a wide array of immunosuppressive strategies, such as reducing the longevity and survival of dendritic cells (DCs), to diminish immune responses and limit the effect of immunotherapy. In this study, we found that tumors upregulate the expression of multiple microRNAs (miRNAs), such as miR-16-1, miR-22, miR-155, and miR-503. These tumor-associated miRNAs influenced the survival and longevity of DCs by affecting the expression of multiple molecules that are associated with apoptotic signaling pathways. Specifically, miR-22 targeted YWHAZ to interrupt the PI3K/Akt and MAPK signaling pathways, and miR-503 downregulated Bcl2 expression. The result of the increased expression of miR-22 and miR-503 in the tumor-associated DCs was their reduced survival and longevity. Thus, tumor-associated miRNAs can target multiple intracellular signaling molecules to cause the apoptosis of DCs in the tumor environment. Use of miR-22 and miR-503 as inhibitors may therefore represent a new strategy to improve DC-based immunotherapies against tumors.

The Journal of Immunology, 2013, 190: 000–000.

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dendritic (DCs), including conventional DCs and plasmacytoid DCs, are professional APCs that are critical for the induction of adaptive immunity and tolerance. However, mature DCs also undergo apoptosis in the different tissue and organs, especially the lymph node (1). DC apoptosis is an important event that regulates the balance between tolerance and immunity; many molecules participate in this process, including TNF-related activation-induced cytokine (TRANCE), CD154, FASL, amyloid peptide, TRAIL, LPS, type I IFN, leptin, and CCR7 (reviewed in Ref. 2). Defects in DC apoptosis can trigger autoimmunity, whereas environments in which the apoptosis of DCs occurs are immunosuppressive because they promote regulatory T cell generation and functional impairment of DCs. Several different death receptors have been identified on DCs, including Fas (CD95) and the TNF and TRAIL receptors. Although regulatory DCs have been shown to be induced by tumor-derived factors (3), DC apoptosis has also been observed in cancers, and the process parallels the induction of immunosuppression. A massive reduction in DC numbers has been observed in lymph node drainage from tumors, but not other lymph nodes, in multiple cancers (4–7). In tumor lymph nodes, tumor-derived TGF-β can induce DC apoptosis (8), and regulatory T cells can also directly interact with tumor Ag-bearing DCs to induce their apoptosis (9).

The apoptosis of DCs can be regulated by extrinsic and intrinsic pathways (10). The ratios between the antiapoptotic Bcl2/Bcl-xL molecules and the proapoptotic Bax/Bak molecules determine the lifespan of different DC subsets. A lower ratio of antiapoptotic Bcl2/Bcl-xL to proapoptotic Bax/Bak has been observed in shorter lived mDCs compared with longer lived plasmacytoid DCs (11). Transfection with Bcl2 or Bcl-xL prolongs the survival of mouse primary mDCs in vitro, and deletion of Bcl2 accelerates DC apoptosis in vivo (11). Because AKT downregulation correlates with Bcl2 downregulation and DC death, the PI3K/AKT pathway also plays an important role in the DC lifespan. Indeed, AKT deficiency leads to defective DC activation and survival (12), and inhibition of PI3K antagonizes DC survival mediated by CD40, CpG, LPS, TRANCE, TNF superfamily member 11, and PGE2 (2, 13). Cell survival has also been found to be dependent on the ERK pathway in several cellular models, whereas the activation of p38 and JNK promotes apoptosis (14). Interestingly, these apoptotic pathways may be regulated by microRNAs (miRNAs), including miR-16-1 (15), miR-155 (16), miR-21 (17), and miR-451 (18). miRNAs are noncoding small RNAs that regulate gene expression and cell growth and differentiation. It is thought that miRNAs have a central role in regulating the apoptosis of DCs. In this study, we demonstrate that the tumor-mediated miRNAs miR-22 and miR-503 affect the survival and longevity of DCs. Specifically, in tumor environments, these miRNAs target the YWHAZ and Bcl2 pathways and promote DC apoptosis.
Materials and Methods

Mice

Four- to six-week-old female C57BL/6 and BALB/c mice (Beijing Animal Center) were maintained in a pathogen-free animal facility for at least 1 wk prior to use. Experiments were performed in accordance with the institutional guidelines. Various s.c. tumor models, including Lewis lung carcinoma (American Type Culture Collection) and B16 melanoma in C57BL/6 mice and CT-26 colon carcinoma (American Type Culture Collection) in BALB/c mice, were used in this study. The number of tumor cells injected s.c. for each model was determined based on the ability of the cells to form a tumor 1.5 cm in diameter within 3–4 wk injection.

Cell lines, human monocytic-derived dendritic cells, and murine bone marrow–derived dendritic cells

The following cell lines were purchased from the American Type Culture Collection: murine monocyte/macrophage RAW264.7, murine melanoma B16, murine colon carcinoma CT-26, mouse fibroblast L, human lung carcinoma PG, human breast carcinoma MCF-7, human cervical carcinoma HeLa, human monocyte U937, human embryonic kidney 293T, and human lung fibroblast WI38. Ovarian carcinoma ID8 was purchased from K.F. Ruby (University of Kansas Medical Center). All cells were maintained in complete media, which consisted of RPMI 1640 supplemented with 10% FCS and 1% penicillin and streptomycin.

Human monocyte-derived DCs (moDCs) were prepared from monocytes (100,000 cells/ml) (19). Briefly, after Ficoll-Hypaque separation, peripheral blood cells were resuspended in complete media. The cells were incubated for 1 h at 37°C, and the nonadherent cells were removed by gentle pelting. The adherent cells were cultured in RPMI 1640 containing 10% FCS, 100 U/ml GM-CSF (R&D Systems), and 1000 U/ml IL-4 (R&D Systems) for 7 d.

Flow cytometric analysis

Murine BMDCs were collected in ice-cold PBS and incubated with the following anti-mouse Abs: FITC-, PE- or allophycocyanin-conjugated anti-mouse CD11c (N418), B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL1), CD40 (5C3), CD11b (M170), CD4 (L3T4), or CD8α (Ly-2) Abs. Human moDCs were collected in ice-cold PBS and incubated with the following anti-human Abs: FITC-, PE- or allophycocyanin-conjugated anti-human CD11c (3.9), CD86 (IT2.2), CD80 (2D10.4), CD40 (HB14), or CD11b (ICR-F44). All of the Abs used in the study were purchased from BD Biosciences. The cells were stained and resuspended in PBS with 1% paraformaldehyde and 1% FCS and kept at 4°C prior to flow cytometric analysis (FACScan; Becton Dickinson). Isotype-matched control mAbs were used as negative controls for all analyses.

For apoptotic analysis, the cells were stained with annexin V and propidium iodide according to the manufacturer’s instructions. Samples were examined by FACScan.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent, and reverse transcription was performed with the Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s protocol. For gene expression analysis, quantitative real-time PCR (qRT-PCR) was performed using the Quantitect SYBR PCR kit with a specific set of primers according to the manufacturer’s instructions (Qiagen). For mature miRNAs, qRT-PCR was performed using a standard TaqMan PCR kit and an Applied Biosystems 7900 HT sequence detection system. Amplification of U6 small RNA was performed to detect mature miRNAs, and amplification of GAPDH was performed on each experimental sample as an endogenous control. Fold changes were calculated using the ΔΔCT method according to the manufacturer’s protocol (Applied Biosystems). All reactions were run in triplicate. The primer sequences used are listed in Supplemental Table I.

miRNA, small interfering RNA, gene, and 3′ untranslated region expression constructs

miRNA mimics, miRNA inhibitors, and control oligonucleotides were purchased from Dharmacon. YWHAZ and Bcl2 small interfering RNAs (siRNAs) and the negative control siRNA were purchased from Santa Cruz Biotechnology. BLOCK-iT fluorescent oligonucleotides were purchased from Invitrogen. The 3′ untranslated region (UTR) of YWHAZ and Bcl2 was cloned from mouse spleen cell genomic DNA via PCR. Primers containing the restriction enzymes XhoI and NotI were used so that the sequences could be cloned into the siCheck-2 luciferase reporter vector (Promega). The YWHAZ and Bcl2 3′ UTR mutants were generated using four primers according to a previous method (21). Mutations were confirmed by DNA sequencing. YWHAZ and Bcl2 were directly cloned into pcDNA3.1 from total spleen cell RNA using the pcDNA3.1 TOPO kit. The sequences of the miRNAs were obtained from the miRbase (http://microrna.sanger.ac.uk/sequences/). The 3′ UTR sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez/). Primer 3 software was used for primer design (http://frodo.wi.mit.edu/). The primers used are listed in Supplemental Table I.

Transfection

For the miRNA mimics, inhibitors, siRNAs, and negative control oligonucleotides, cells were transfected with the indicated oligonucleotides (100 nM) using the Entransfer-R system (Engreen Biosystem) according to the manufacturer’s instructions. For some of the constructs, the cells were transfected using a nucleofection approach according to the manufacturer’s protocol (Amaxa Biosystems, Berlin, Germany). miRNA mimics, inhibitors, siRNAs, and negative control oligonucleotides were purchased from RiboBio (Guangzhou, China). Silencing of the target molecules was confirmed by RT-PCR after transfection for 24 h. The primers used in the siRNA experiments are listed in Supplemental Table I.

miRNA array

Expression levels of miRNAs in 1D8 mouse ovarian tumor– and CT-26 colon cancer–associated BMDCs or control BMDCs were analyzed using Exiqon miRNA arrays (Vedbaek, Denmark). Total RNA was isolated using the TRIzol reagent (Invitrogen Life Technologies). The concentration and purity of the RNA were determined using NanoDrop ND-1000. The miRNAs were labeled using the miRCURY array power labeling kit (Exiqon). The miRNA array hybridization was performed, and unbound miRNA labels were washed away using the miRCURY array wash buffer kit (Exiqon). The arrays were scanned on an Axon 4000B scanner (Molecular Devices), and the signal intensity was determined using GenePix Pro 6.0 software (Molecular Devices). miRNAs with a 1.5-fold increase or decrease in expression were regarded as differentially expressed.

Gene chip array

A gene chip analysis was performed on RNA from untreated BMDCs and BMDCs cocultured with 1D8 tumor cells using a transwell plate. Total RNA was isolated from DCs using TRIzol reagent (Invitrogen) followed by DNase I treatment by using DNA-free kit (Qiagen). Processing of the sample was performed following Affymetrix specifications. Fluorescence was detected using the Hewlett-Packard G2500 GeneArray scanner, and image analysis of each gene chip was performed with the Microarray Suite 5.0 software from Affymetrix using the standard default settings. Transcripts that were consistently significant in at least two of the four iterative comparisons were selected for the final candidate list. Any transcript that showed at least a 2-fold change in expression level (ratios are presented as log2) between the experimental sample and the control sample was considered significant.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed by the Teda School of Biological Science and Biotechnology, Nankai University. Total protein concentrations from RAW264.7 cells transfected with different miRNAs or control oligonucleotides were determined according to the Bradford method (Bio-Rad Laboratories, Hercules, CA) following the manufacturer’s protocol. Proteins on the two-dimensional gels were visualized by silver staining for pattern comparisons, and protein spots were selected for quantitative analysis when they showed a reduction ≥2-fold that of the control sample. Selected protein spots were digested, desalted with ZipTip (Millipore), and subjected to analysis by MALDI-TOF MS. The peptide mass fingerprints obtained for each protein of interest were searched against the National Center for Biotechnology Information nonredundant database using the MASCOT search engine (http://www.matrixscience.com).

Luciferase reporters

Cells were plated in a 48-well plate at a density of 4 × 104 cells/well in 250 μl culture medium 24 h prior to transfection; the cells were then
cotransfected with the indicated expression plasmids, the siCHECK-2 reporter plasmid, and control plasmids (Promega) using Lipofectamine reagent (Invitrogen) or Nucleofector technology (Amaxa Biosystems) according to the manufacturer’s recommendations. Renilla and firefly luciferase activities were measured 24 h after transfection using a dual luciferase kit (Promega).

Western blot analysis

Western blot analysis was performed according to our previous protocol (21). Hybridizations with primary Abs were performed for 1 h at room temperature in blocking buffer. The protein/Ab complexes were detected using peroxidase-conjugated secondary Abs (Boehringer Mannheim) and ECL (Amersham Biosciences). Rabbit anti–YWHAZ (Sigma-Aldrich), anti-Bcl2 (Cell Signaling Technology), anti-Bax (Sigma-Aldrich), anti-AKT (Cell Signaling Technology), anti-FOXO3 (Sigma-Aldrich), anti-phospho-AKT (Cell Signaling Technology), and anti–phospho-FOXO3 (Cell Signaling Technology) were used in addition to the following Abs purchased from Bios Biosynthesis Biotechnology (Beijing, China): anti-actin, anti-p38, anti-JNK, anti-ERK, anti–phospho-p38, anti–phospho-JNK, anti–phospho-ERK, and an HRP-labeled goat polyclonal Ab against rabbit IgG (H+L).

In vitro experiments

For in vitro preparation of tumor-associated DCs, 2 \times 10^5 murine or human tumor cells were cocultured with 2 \times 10^6 murine BMDCs or human moDCs in a 24-well transwell plate in RPMI 1640 medium supplemented with 3% FCS (Life Technologies) and 1% penicillin and streptomycin (Life Technologies) for 24 h. For in vitro apoptosis analysis, murine BMDCs or human moDCs were harvested, transfected, and then coincubated with the murine or human tumor cells in a transwell plate for 3 d. Specifically, 2 \times 10^6 DCs were placed into 24-well plates in 1 ml medium, and 2 \times 10^5 tumor cells were placed into the inserts on the top of each well. As controls, DCs were coincubated with murine or human fibroblasts or medium alone and placed in the inserts. The cells were also cultured with TGF-β, TNF-α, or under conditions of GM-CSF withdrawal. Apoptotic cells were assessed using a FITC-conjugated TUNEL reaction mixture with propidium iodide staining and an FITC-conjugated TUNEL reaction mixture that was provided with an in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN).

In vivo experiments

To generate the mouse tumor model, B16 cells (0.5 \times 10^6 in 100 μl PBS) were inoculated s.c. into the inguinal region backs of C57BL/6 mice. Tumors were allowed to develop for 9 d, and mice were then randomly divided into different experimental groups (six mice per group).

To analyze DC survival in vivo, BMDCs were transfected with different miRNAs, labeled with CFSE and injected s.c. (1 \times 10^6 cells/mouse) into the footpads of syngeneic C57BL/6 mice bearing B16 tumors. The draining popliteal lymph nodes were harvested at various time points after injection. The total numbers of lymph node cells were counted, and the percentage of CFSE<sup>+</sup> DCs was analyzed by flow cytometry. The proportion of CFSE<sup>+</sup> DCs in the draining lymph nodes of each mouse was calculated.

The effect of tumor-associated miRNAs on DC-based immunotherapy against tumors was assessed according to the previously reported protocol.

**FIGURE 1.** Tumor cells induce DC apoptosis. (A and B) Mouse tumor cells induced the apoptosis of murine BMDCs. BMDCs were cocultured with murine melanoma B16F10 cells, colon carcinoma C-26 cells, ovarian carcinoma 1D8 cells, RAW264.7 monocytes, or mouse L fibroblast cells (mFB) in a transwell for 72 h. B16F10 in FACS data indicated the BMDCs cocultured with B16F10. The cells were stained with annexin V and propidium iodide and analyzed by FACScan. The apoptosis of BMDCs was determined using an annexin V binding assay with propidium iodide staining and an FITC-conjugated TUNEL reaction mixture that was provided with an in situ cell death detection kit (Roche Diagnostics). (C) Human tumor cells induced the apoptosis of human moDCs. Human moDCs were harvested, transfected, and then coincubated with human breast carcinoma MCF-7 cells, lung carcinoma PG cells, cervical carcinoma HeLa cells, U937 monocytes, or WI38 human fibroblast cells (hFB) in a transwell for 72 h. Human tumor cells induced the apoptosis of human moDCs. Human moDCs were harvested, transfected, and then coincubated with human breast carcinoma MCF-7 cells, lung carcinoma PG cells, cervical carcinoma HeLa cells, U937 monocytes, or WI38 human fibroblast cells (hFB) in a transwell for 72 h. Original magnification \( \times 40 \). HeLa in FACS data indicated the moDCs cocultured with HeLa. The results shown are representative of three independent experiments. \( p < 0.05 \). Ctr., Cells from pooled draining lymph nodes from mice (six mice/group) injected with 1 \times 10^6 unlabeled BMDCs.
Mice bearing B16 tumors were randomly divided into different experimental groups (six mice per group) and treated via intratumoral injection with the transduced DCs. Groups treated with PBS only or untreated DCs were used as controls. The diameter of the perpendicular tumor was measured with a caliper to assess tumor size. Mice were sacrificed when they exhibited signs of distress or when the total tumor volume was >3000 mm³.

Statistical analysis
Data were presented as mean ± SD or mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by the post hoc Tukey–Kramer multiple comparison test and a Student t test. A two-sided p value <0.05 was considered statistically significant.

Results
Tumor-associated miRNAs regulate the survival and longevity of DCs
Cancers such as breast cancer (22, 23), hepatocellular carcinoma (24), and melanoma (25) are associated with DC apoptosis. In this study, when DCs were cocultured with different types of mouse or human tumors, the number of apoptotic and dead DCs significantly increased compared with DCs cultured with only medium or fibroblasts (p < 0.05); however, the tumor cells showed different abilities to induce apoptosis (Fig. 1A, 1B). In in vivo experiments, B16 tumors also promoted the apoptosis of BMDCs as compared with control (mean ± SEM, p < 0.01), consistent with other reports (26) (Fig. 1C). Similarly, human moDCs were sensitive to MCF-7 human breast carcinoma, PG lung carcinoma, and HeLa cervical carcinoma cell–mediated apoptosis (Fig. 1D, 1E), consistent with other reports (27).

To investigate the miRNAs that may be involved in regulating the survival and longevity of DCs in tumor microenvironments, we first analyzed the expression of miRNAs in tumor-associated BMDCs. GenoExplorer miRNA arrays (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42722, GPL16352, National Center for Biotechnology Information tracking system no. 16691611) showed that some miRNAs, such as miR-16-1, miR-

![FIGURE 2](http://www.jimmunol.org/) Expressions of miRNAs in tumor-associated murine BMDCs. (A) miRNA expression in tumor-associated BMDCs. BMDCs were generated and cocultured with the CT-26 mouse colon cancer or 1D8 mouse ovarian cancer cell lines in a 24-well transwell plate for 24 h. The expression of miRNAs in the tumor-associated BMDCs was analyzed using a GenoExplorer miRNA array chip according to the protocol described in Materials and Methods; 16, 21, 22, 142-5p, 146a, 155, and 503 are miR-16-1, miR-21, miR-22, miR-142-5p, miR-146a, miR-155, and miR-503 respectively. (B) Tumor-mediated miRNA expression was further confirmed in CT-26– and B16-associated BMDCs by qRT-PCR. BMDCs were cocultured with B16 or CT-26 cells, and miRNA expression was detected at the indicated times according to the protocol described in Materials and Methods. (C) Expression of miRNAs in the draining lymph nodes from the B16 and CT-26 mouse tumor models. The DCs were isolated from the draining lymph nodes of CT-26 and B16 or control (Ctr.) mice using Dynabeads (Invitrogen) coated with an anti-mouse CD11c Ab (MBL International, Woburn, MA) according to the manufacturers’ instructions. The CT-26 and B16 mouse tumor models were generated according to the protocol described in Materials and Methods. *p < 0.05, **p < 0.01. R.E., Relative expression.
miR-22, miR-142-5p, miR-146a, miR-155, and miR-503, were upregulated when the BMDCs were cultured with mouse CT-26 colon cancer cells or 1D8 ovarian cancer cells (Fig. 2A). These upregulated miRNAs were also detected by qRT-PCR in BMDCs cultured with mouse tumor B16 cells. Notably, except for miR-22 and miR-142-5p, the kinetics of the expression of these miRNAs was different from the BMDCs cultured with CT-26 cells (Fig. 2B), implying different mechanisms that are involved in the ID8- and CT-26-mediated modulation on miRNAs. Importantly, the upregulated miRNAs were also found in DCs isolated from the draining lymph nodes of mice bearing B16 and CT-26 tumors (Fig. 2C). Significant differences were apparent as compared with control (mean ± SD, p < 0.01). Thus, our results suggest that the expression of miR-16-1, miR-21, miR-22, miR-142-5p, miR-146a, miR-155, miR-503, and the previously reported miR-17-5p and miR-20 (21) may be upregulated by tumor-associated factors.

Although there exists a possibility for the roles of the downregulated miRNAs in DC apoptosis, we next only assessed effects of the tumor upregulated miRNAs on the tumor-induced apoptosis in vitro by coculturing murine B16 tumor cells with miRNAs or their inhibitor-transfected BMDCs with the demonstrated transfection (not shown). The results showed that tumor-associated miR-22 and miR-503 and the previously reported miR-16-1 (15) and miR-155 (16) significantly increased the number of apoptotic and dead DCs, whereas their inhibitors resisted tumor-induced apoptosis of DCs (Fig. 3A and not shown). Because others have shown the effect of miR-16-1 and miR-155 on mediating cellular apoptosis (15, 16), we next focused on the effects of miR-22 and miR-503 on modulating DC apoptosis. As shown in Fig. 3, after s.c. delivery of BMDCs transfected with miR-22 and miR-503 inhibitors, the percentage of live cells from the draining lymph node of murine B16 tumor model was significantly higher than that obtained from the lymph nodes of mice injected with control DCs (Fig. 3B, 3C). This disproportionate percentage of live cells was sustained for at least 5 d after delivery (Fig. 3B, 3C). These data suggest that the miR-22 and miR-503 inhibitors prolong DC lifespan.

We also employed apoptotic models that are mediated by TNF-α (28), TGF-β (29), or GM-CSF withdrawal (30) to further investigate the effects of the tumor-associated miRNAs on the apoptosis of DCs. miR-22 and miR-503 mimics promoted the apoptosis of DCs upon TNF-α, TGF-β, and GM-CSF withdrawal, whereas miR-155 promoted DC apoptosis only when exposed to TNF-α (not shown). miR-16 did not have an effect on DCs in these apoptotic models (not shown), implying that multiple signaling pathways are involved in the tumor-associated miRNA regulation of DC apoptosis.

The YWHAZ-based apoptotic signaling pathway may be directly or indirectly modulated by multiple tumor-associated miRNAs

Given the potential of miRNAs to regulate a large number of cellular transcripts, we decided to take a broad approach to identify targets and determine the mechanisms by which miRNAs act to regulate DC apoptosis. The expression of intracellular proteins in the monocyte cell line RAW264.7 transfected with tumor-associated miR-16-1, miR-20a, miR-22, miR-155, or miR-
503 was investigated using two-dimensional gels and MALDI-TOF MS. We found that these miRNAs could indeed downregulate the levels of multiple proteins, such as YWHAZ, ALB, HMox1, LDHA, ANXA2, CCT4, ANXA5, TCP1, CCT2, ANXA1, EEF2, VIM, PGK1, CCT7, TKT, Cofilin, CAP1, CFL1, P4HB, TP11, ALDOA, PGAM1, CS, GLUD1, PKM2, and ENO1 (Fig. 4, Supplemental Fig. 1). In addition to predictions of miRNA-targeted molecules, multiple potential targets of miR-16-1, miR-20a, miR-22, miR-155, and miR-503 were identified (Fig. 4, Supplemental Fig. 1, and not shown). For example, miR-22 markedly reduced the expression of YWHAZ (Fig. 4, Supplemental Fig. 1). Several molecules related to YWHAZ were also downregulated by tumor-associated miRNAs, including ALDOA, GLUD1, and ENO1 by miR-16, ANXA2 by miR-20a, CAP1 by miR-22, ANXA2 by miR-20a, and ENO1 by miR-503 (Supplemental Fig. 1). These data suggest that the tumor-associated miRNAs affected the YWHAZ-based apoptotic signaling pathway by reducing the expression of YWHAZ-associated molecules.

Tumor-associated miR-22 targets YWHAZ

YWHAZ plays an important role in the assembly of signaling complexes required for the activation of pathways downstream of growth receptors (31). Interestingly, miR-22 not only reduced the protein levels of YWHAZ but also its transcript levels (Figs. 4, 5A). Further analyses demonstrated that miR-22 targeted the 3′ UTR region of YWHAZ. We fused the 3′ UTR sequence of mouse YWHAZ to a luciferase reporter gene and found significant repression of luciferase activity by miR-22, suggesting a direct effect (Fig. 5B). We also performed experiments with mutated target mRNA sequences (Supplemental Table II) and miR-22 inhibitors. Both YWHAZ 3′ UTR mutation and miR-22 inhibitors abolished the interaction between miR-22 and the 3′ UTR of YWHAZ (Fig. 5B). When the expression of YWHAZ in tumor-associated DCs was assessed, we found that the DCs had reduced expression of YWHAZ (Fig. 5C). Thus, these data demonstrate that tumor-associated miR-22 can inhibit the expression of YWHAZ by binding to its 3′ UTR region.

Previous studies have shown that YWHAZ can interact with multiple molecules in different signaling pathways, such as AKT (32–36). Indeed, as shown in Fig. 5D, transfection with miR-22 reduced the phosphorylation of AKT, whereas miR-22 inhibitors promoted the phosphorylation of AKT. We also observed that miR-22 inhibitors induced the phosphorylation of FOXO3 after transfection for 5 min. Because these phosphorylated residues subsequently sequestered FOXO3 to YWHAZ to prevent apoptosis (37), miR-22 may promote the apoptosis of DCs by reducing the expression of YWHAZ and affecting the activity of AKT and FOXO3.

YWHAZ has been shown to promote ERK/MAPK activation but inhibit the activation of JNK and p38 MAPK (31). Indeed, as shown in Fig. 5D, administration of miR-22 mimics remarkably caused the downregulation of ERK phosphorylation and the upregulation of JNK and p38 phosphorylation; conversely, ERK was activated and the phosphorylation of JNK and p38 was inhibited by miR-22 inhibitors. The difference of p38 and ERK phosphorylation could be found after transfection for 5 min. JNK was also remarkable phosphorylated after miR-22 transfection as compared with control transfection. Because AKT and MAPK activity is associated with the expression of apoptotic molecules, such as Bcl2 and Bax, we next determined the effect of miR-22.

![FIGURE 4.](http://www.jimmunol.org/) Both miR-22 and miR-155 downregulate the expression of YWHAZ. (A) The levels of the YWHAZ protein (spot 1201) in miR-22– and miR-155–transfected RAW264.7 cells. miR-22–, miR-155–, or control oligonucleotide (Ctrl)–transfected RAW264.7 cells were analyzed by two-dimensional gel electrophoresis. 1201, protein spot number. (B and C) The selected spot 1201 is the YWHAZ protein. Spot 1201 was digested and subjected to analysis by MALDI-TOF MS.
on Bcl2 and Bax. Transfection with miR-22 reduced the expression of Bcl2 and enhanced the expression of Bax, whereas the miR-22 inhibitor inhibited the expression of Bax and enhanced the expression of Bcl2 (Fig. 5E). Thus, the ERK/MAPK signaling pathway is involved in the miR-22–mediated apoptosis of DCs.

Taken together, both the AKT/FOXO3 and ERK/MAPK signaling pathways may be involved in the miR-22–mediated apoptosis of BMDCs. Additionally, we found that miR-155, which induced DC apoptosis (Ref. 16 and Fig. 3), also downregulated the expression of YWHAZ and affected the expression of Bcl2 and Bax (Fig. 5E). Thus, the AKT/FOXO3 and ERK/MAPK signaling pathways may also play a role in the miR-155–mediated apoptosis of BMDCs.

The Bcl2-based apoptotic signaling pathway may be potentially modulated by multiple tumor-associated miRNAs

We analyzed the gene expression profile of BMDCs using a gene chip after exposure of the DCs to tumor cells. Approximately 2701 genes were significantly downregulated in the tumor-associated BMDCs compared with the control BMDCs (not shown). Combined with analyses using the TargetScan program, we predicted the potential target molecules of tumor-associated miR-16-1, miR-17-5p, miR-20a, miR-21, miR-142-5p, miR-146a, miR-155, and miR-503. As shown in Supplemental Fig. 2, some of these downregulated genes, such as BCL2, MTF1, DNAJC7, PRPF4B, FGD4, CDK6, MAP3K3, MAP3K9, KLF4, PPP3CA, FBKP1A, MAPK14, DPYSL2, TRIB2, RCN2, ITGA4, TRIB2, RGS2, PLXNA2, PLCB1, and SACS, may be potentially modulated.
by the tumor-associated miRNAs. These genes are directly or indirectly related to Bcl2 (Supplemental Fig. 2), which is a molecule that favors cell survival by inhibiting cell death (38). Thus, the Bcl2-based apoptotic signaling pathway may be potentially modulated by multiple tumor-associated miRNAs.

**Tumor-associated miR-503 targets Bcl2**

Next, we investigated the effect of the tumor-associated miRNAs on the predicted target molecules. We found that miR-503 and miR-16-1 could potentially target Bcl2 (Fig. 6A). Indeed, as shown in Fig. 6B and 6C, miR-503 downregulated Bcl2 at both the transcript and protein level. miR-16-1 also reduced the expression of Bcl2 (Fig. 6B), consistent with a previous report (39). We then fused the 3' UTR sequence of mouse Bcl2 to a luciferase reporter gene and found a significant repression of luciferase activity by miR-503 compared with control vectors, suggesting a direct effect (Fig. 6D). We performed further experiments with mutated target mRNA sequences (Supplemental Table II) and miR-503 inhibitors; as expected, both the Bcl2 3' UTR mutation and miR-503 inhibitors abolished the interaction between miR-503 and the 3' UTR of Bcl2. When the expression of Bcl2 was assessed in tumor-associated DCs, these tumor-associated DCs had reduced expression of Bcl2 (Fig. 6E). These data suggest that in addition to what has been previously reported for miR16-1 (39), tumor-associated miR-503 may interact directly with the 3' UTR of Bcl2 to downregulate the expression of Bcl2. Thus, tumor-associated miR-503 could regulate the apoptosis of DCs by affecting Bcl2 expression.

**Inhibitors of miR-22 and miR-503 promote the DC-based immunotherapy against tumors**

Both Bcl2 and YWHAZ play a critical role in regulating the apoptosis of different types of cells (30, 40–42). Because miR-22 and miR-503 could affect the survival and longevity of DCs in the tumor environment by targeting YWHAZ and Bcl2, these miRNAs might be promising targets for DC-based immunotherapies against tumors. To test the effects of miR-22 or miR-503 on an ex vivo DC vaccine against tumors, we tested the efficacy of DCs transfected with miR-22 and miR-503 inhibitors on an established tumor. As shown in Fig. 7, tumor growth was slower in mice vaccinated with DCs transfected with miR-22 and miR-503 inhibitors, whereas mice vaccinated with DCs transfected with miR-22 and miR-503 mimics exhibited faster tumor growth. Significant differences in tumor growth between control and miR-22 or miR-503 inhibitor transfected groups or between control and miR-22 or miR-503 mimics transfected group were apparent after 12 d ($p < 0.05$). There were significantly differences in tumor weight as compared with control (mean ± SEM, $p < 0.01$). Consistent with other reports (43), siRNAs targeting Bcl2 and YWHAZ in DCs also suppressed the DC-based immunotherapy against tumors; conversely, Bcl2 and YWHAZ improved the DC-based immunotherapy against tumors (Fig. 7).

**FIGURE 6.** miR-503 targets Bcl2. (A) Bcl2 is a potential target of miR-503. In addition to the downregulated genes in tumor-associated BMDCs shown in Supplemental Table II, miR-503 and miR-16-1 potentially target Bcl2 based on the prediction of TargetScan 5.1 (http://www.targetscan.org/cgi-bin/vert_50/targetscan.cgi?mirg=mmu-miR-223) PICTAR (http://pictar.bio.nyu.edu/), and MIRANDA (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl). Cycle number 2071 indicates the 2071 genes that are downregulated in tumor-associated BMDCs. Cycle numbers 243 and 821 indicate the number of predicted target molecules by miR-16-1 and miR-503, respectively. (B and C) Both miR-503 and miR-16-1 downregulate the expression of Bcl2. BMDCs were transfected with different concentrations of miR-503 or miR-16-1. The transcript and protein levels of Bcl2 were detected by qRT-PCR and Western blot, respectively. (D) miR-503 can target the 3' UTR of Bcl2. The Bcl2 luciferase reporter vector (Wt.UTR) or mutant Bcl2 luciferase reporter vector (Mut1.UTR) was cotransfected with miR-503 mimics (miR503), miR-503 inhibitors, or control oligonucleotides (Oligos.ctr.) into 293T cells. Luciferase activity was measured after 48 h according to the protocol described in Materials and Methods. The data are expressed as the relative luciferase activity of control samples that were cotransfected with an equal concentration of negative control vectors and are presented as the means ± SD from triplicate tests. The luciferase activity in 293T cells cotransfected with Bcl2 Wt. UTR and control oligonucleotides was arbitrarily set at 1. Error bars represent the SD. *$p < 0.05$, **$p < 0.01$. (E) The expression of Bcl2 was regulated by tumor cells. Murine BMDCs were cocultured with murine melanoma B16 cells, colon carcinoma CT-26 cells, ovarian carcinoma ID8 cells, and murine fibroblast cells (mFB). The transcript and protein levels of Bcl2 were detected by qRT-PCR and Western blot. R.E., relative expression.
Tumors have been found to be remarkably reduced in multiple DCs (8, 24, 25). DC numbers in the sentinel lymph nodes of mice that ingested tumor cells and statistically analyzed weights were measured at day 28 after infection. The tumor sizes were measured at the indicated times and statistically analyzed by ANOVA followed by the post hoc Tukey–Kramer multiple-comparison Test. Tumor weights were measured at day 28 after injecting tumor cells and statistically analyzed using a Student t test.

Discussion

In this study, we identified murine tumor-associated miRNAs that affected the survival and longevity of DCs. First, tumor-associated miR-22 reduced the survival of DCs by targeting YWHAZ, which diminished the activity of AKT and FOXO3 to promote apoptosis. Second, the targeting of miR-22 to YWHAZ inhibited ERK activity, promoted the activation of JNK/p38, and affected the expression of Bcl2 and Bax. Third, tumor-associated miR-503 directly targeted Bcl2 to promote the apoptosis of DCs. Finally, tumor-associated miR-16, miR-22, miR-155, and miR-503 could also affect the expression of multiple molecules related to apoptotic signaling pathways. Thus, tumor-associated miRNAs can target multiple intracellular signaling molecules to reduce the survival of DCs in the tumor environment.

Tumor cells may not only promote the survival of DCs and induce regulatory DCs to mediate immune tolerance (3) but also cause the apoptosis of DCs. Several types of solid and blood cancers, such as pancreatic, breast, prostate, hepatocellular, lung, head and neck carcinomas, and leukemia, are accompanied by impaired function and reduced numbers of DCs (23, 44, 45). DCs are often depleted from the tumor site itself and also from the circulation. The reduced numbers of DCs may be from the enhanced apoptosis. Indeed, our data showed that tumor-mediated miRNAs such as miR-22 and miR-503 promoted the apoptosis of DCs by targeting YWHAZ and Bcl2. Others also found that tumor-derived TGF-β may induce DC apoptosis (8). This may be an important mechanism for tumor immune tolerance and escape.

Apoptotic pathways may be regulated by miRNAs, including miR-16 (15), miR-155 (16), miR-21 (17), and miR-451 (18). The various mediators released from tumor cells (e.g., TGF-β) flow into sentinel lymph nodes and affect the survival and longevity of DCs (8, 24, 25). DC numbers in the sentinel lymph nodes of tumors have been found to be remarkably reduced in multiple cancers (4). However, the mechanism of tumor-mediated apoptosis of DCs is unclear. Our results demonstrate that tumor cells induce the upregulation of multiple miRNAs, including miR-22, miR-503, miR-16-1, and miR-155. A recent study by Cubillos-Ruiz et al. (46) reported that tumor-associated DCs had reduced miR-155 levels. This may be caused from different time points and/or different models. Indeed, miR-155 and also miR-503 are immediately upregulated, but their expression is remarkably downregulated with time extension upon exposure to tumors. However, these tumor-associated miRNAs, particularly miR-22 and miR-503, reduce the survival and longevity of DCs by promoting their apoptosis. Other studies have also shown that miR-16 and miR-155 are involved in the apoptosis of tumor cells (15, 18) and DCs (16) by targeting YWHAZ and Bcl2.

DC-based immunotherapies have been extensively explored for cancer treatments, but they have had limited success. A major problem is that DCs undergo apoptosis after injection. Thus, delivery of apoptosis-resistant DCs may improve the efficacy of DC immunotherapies. The decreased viability of the DCs could be explained by the reduced expression of the antiapoptotic proteins Bcl2 and YWHAZ, which have previously been shown to control the longevity of murine DCs (30, 40–42). The administration of siRNAs targeting these proapoptotic molecules in DCs increases DC survival. Treatment of DCs with TRANCE, a prosurvival factor, results in better adjuvant properties. Our data show that DCs transfected with miR-22 and miR-503 inhibitors may improve antitumor response. However, the role of overexpressed miRNAs on DC survival/apoptosis might only be a part of the DC conditioned by tumor cells. Others such as soluble and membrane-bound factors are likely involved, and the deleterious effects on DCs is not limited to their survival.

Disclosures

The authors have no financial conflicts of interest.
References


