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PRINCIPAL INVESTIGATOR: Jose R Conejo-Garcia

CONTRACTING ORGANIZATION: Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104-4265

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Jose R. Conejo-Garcia

E-Mail: jrconejo@wistar.org

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13. SUPPLEMENTARY NOTES

During the tenure of this pilot project, we identified that miR-181a is universally up-regulated in ovarian cancer-infiltrating lymphocytes. Unexpectedly, overexpression of miR-181a in anti-tumor (protective) T cells results in impaired effector functions in the tumor microenvironment, rather than in enhanced TCR recognition of tumor antigens. Genomic analysis of the genes silenced upon miR-181a up-regulation revealed a ~2-fold decrease in the expression of the enzyme Tryptophan 2,3-dioxygenase (TDO2), suggesting that impaired tryptophan metabolism may be the cause of defective responses by tumor-reactive T cells overexpressing miR-181a. No differences in immunological readouts were found between ovarian cancer-bearing hosts treated with cisplatin *vs.* oxiliplatin. Our results indicate that miR-181a impairs, rather than augmenting, T cell protection in ovarian cancer, and point to miR-181a as a novel target for down-regulating interventions.

15. SUBJECT TERMS
Tumor immunology; immunotherapy; tumor microenvironment; miRNA

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Introduction

The original goals of this pilot project were to demonstrate the feasibility of modulating the expression of miR-181a in immune cells, to boost T cell-mediated immune protection. The original statement of work was as follows:

Task 1. Up-regulate miR-181a levels to boost the therapeutic effectiveness of transferred anti-tumor T cells. (Months 1-12):

a. Expression of miR-181a in properly conditioned anti-tumor T cells and therapeutic interventions (Months 1-9).

b. Activation and memory differentiation of T cells expressing miR-181a vs. control lymphocytes (Months 9-12).

c. Milestone: Definition of the therapeutic potential of expressing miR-181a in tumor-reactive T cells (Month 12).

Task 2. Define the synergy between chemotherapies and anti-tumor T cells expressing miR-181a. (Months 13-24):

a. Comparison of the immunogenic effect of oxaliplatin vs. cisplatin (Months 12-20). Expected outcome: Superior therapeutic effects and enhanced anti-tumor immunity elicited by oxaliplatin, compared to cisplatin.

b. Use of oxaliplatin as an adjuvant to support transferred T cells in cisplatin-treated hosts (Months 15-24). Expected outcome: Enhanced survival and anti-tumor immunity in oxaliplatin-treated mice, compared to controls.

c. Milestone: Identification of the administration of oxaliplatin as an immunogenic host conditioning intervention that synergizes with the adoptive transfer of anti-tumor T cells (Month 24).

Body

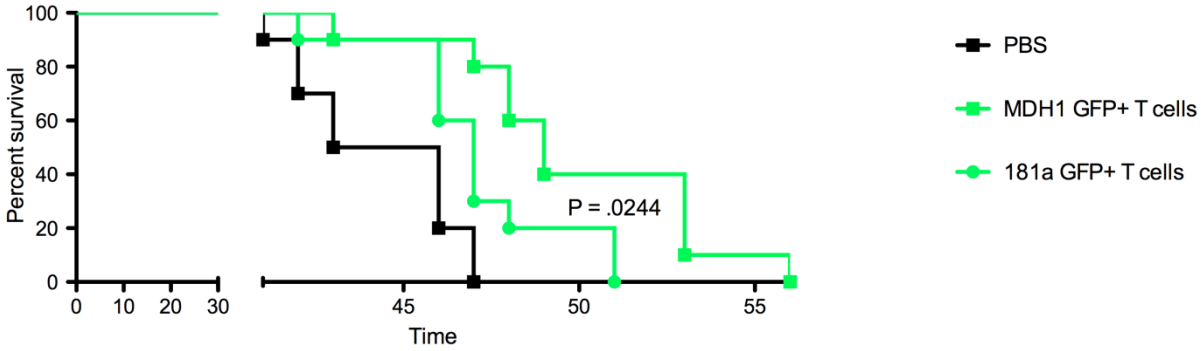
We have addressed both tasks, as follows:

Task 1. Up-regulate miR-181a levels to boost the therapeutic effectiveness of transferred anti-tumor T cells.

a) Expression of miR-181a in properly conditioned anti-tumor T cells and therapeutic interventions.

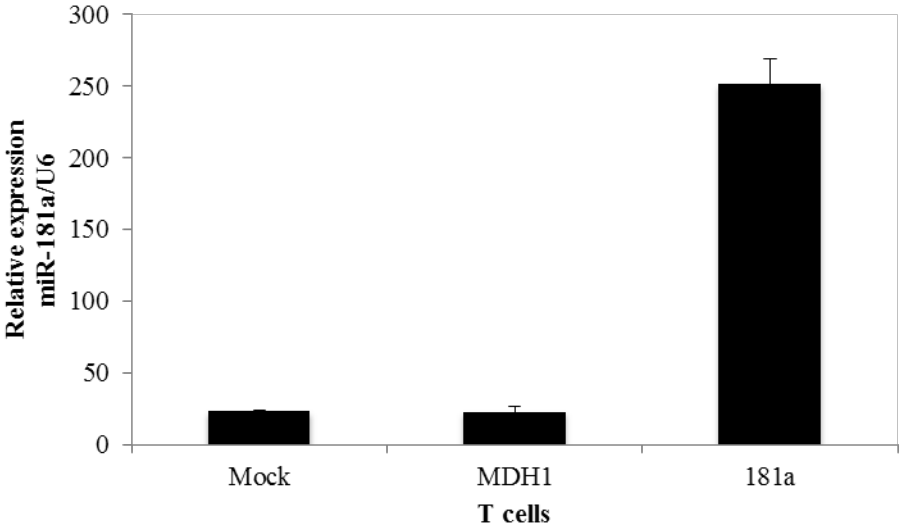
Previous reports indicate that miR-181a, by silencing critical phosphatases, decreases the threshold of activation of the TCR. Our original hypothesis therefore was that tumor-reactive T cells overexpressing miR-181a would be sensitized to recognize MHC-I-restricted antigens presented by tumor cells more effectively, becoming more resistant to PD-1-mediated exhaustion and other mechanisms driving T cell unresponsiveness in the tumor microenvironment. We therefore generated tumor-reactive (T cells primed for 7 days against APCs pulsed with tumor antigens), which were transduced with miR-181a encoding retroviruses or the empty vector. Unexpectedly, miR-181a-overexpressiong anti-tumor T cells were significantly less protective than mocked transduced, identically primed lymphocytes, after adoptive transfer into ovarian (ID8-*Defb29/Vegf-a*) cancer-bearing mice (**Figure 1**).

Figure 1. Ectopic expression of miR-181a impairs the protective activity of tumor-reactive T cells adoptively transferred in ovarian cancer-bearing hosts. One million miR-181a (181a) or mocked-transduced (MDH1) T cells were transferred into tumor-bearing mice (n=6 group; 2 independent experiments), at days 7 and 14 after tumor challenge, and survival (tensional ascites) was monitored.



To rule out unexpected technical issues with ectopic expression of miR-181a, we confirmed that T cells transduced with miR-181a encoding vectors indeed expressed higher levels of this miRNA (**Figure 2**).

Figure 2. Tumor-reactive T cells transduced with miR-181a show significantly higher levels of miR-181a. The expression of miR-181a in positively or mocked-transduced T cells was quantified in FASC-sorted (GFP^+) lymphocytes by Real-Time Q-PCR (normalized by U6 expression).

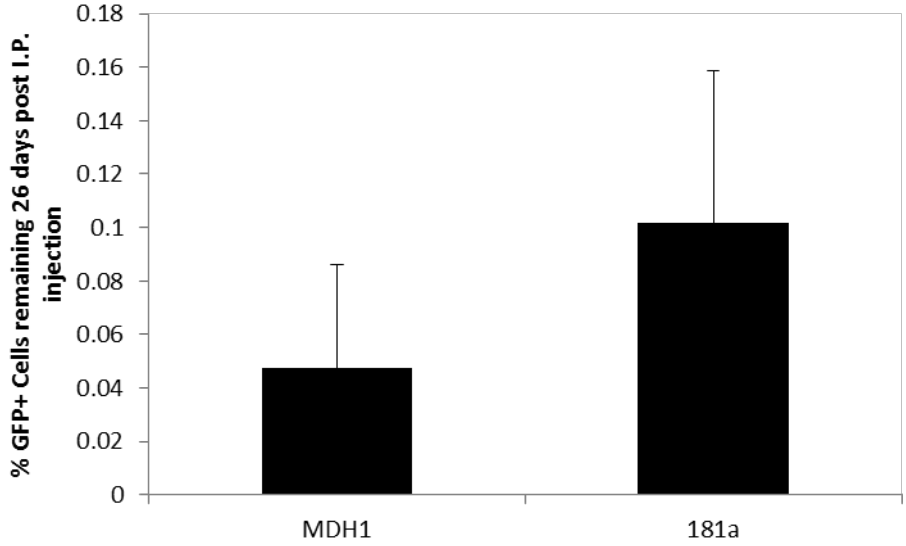


<u>Outcome</u>: Together, these results suggest that the presence of **miR-181a promotes, rather than impairing, T cell unresponsiveness in the tumor microenvironment**. Correspondingly, we found that ovarian cancer-associated (ID8-*Defb29/Vegf-a*) T cells express higher levels of miR-181a, compared to both naïve or tumor antigen primed T cells. In addition, adoptively tumor-reactive T cells progressively up-regulate miR-181a in the ovarian cancer microenvironment (not shown).

These results do not support the original hypothesis of this pilot project, but provide new information about the role of miR-181a (and miRNAs in general) in the activity of anti-tumor T cells. The goal of future studies therefore should down-regulating, rather than overexpressing, mIR-181a in anti-tumor lymphocytes.

b) <u>Activation and memory differentiation of T cells expressing miR-181a *vs.* control lymphocytes</u>.

Figure 3. Tumor-reactive T cells transduced with miR-181a persist in the ovarian cancer microenvironment. Tumor-reactive T cells overexpressing miR-181a or control (mocked-transduced) anti-tumor T cells were transferred into tumor-bearing mice (day 7 after tumor challenge). Twenty six days later, the proportions (and absolute numbers) of (GFP+) originally transferred lymphocytes were determined by FACS analysis. No significant differences were observed.



We also found that ectopic overexpression of miR-181a does not impair the persistence of tumor-reactive T cells *in vivo* in the ovarian cancer microenvironment, because the proportion of miR-181a-overexpressing, adoptively transferred T cells, was not significantly different from the proportion (or absolute numbers) of control (mocked transduced) lymphocytes (**Figure 3**).

<u>Outcome</u>: These results therefore indicate that **overexpression of mIR-181a, which impairs T cell effector functions, does not significantly affect the persistence or memory attributes of anti-tumor T cells**.

Task 2. Define the synergy between chemotherapies and anti-tumor T cells expressing miR-181a:

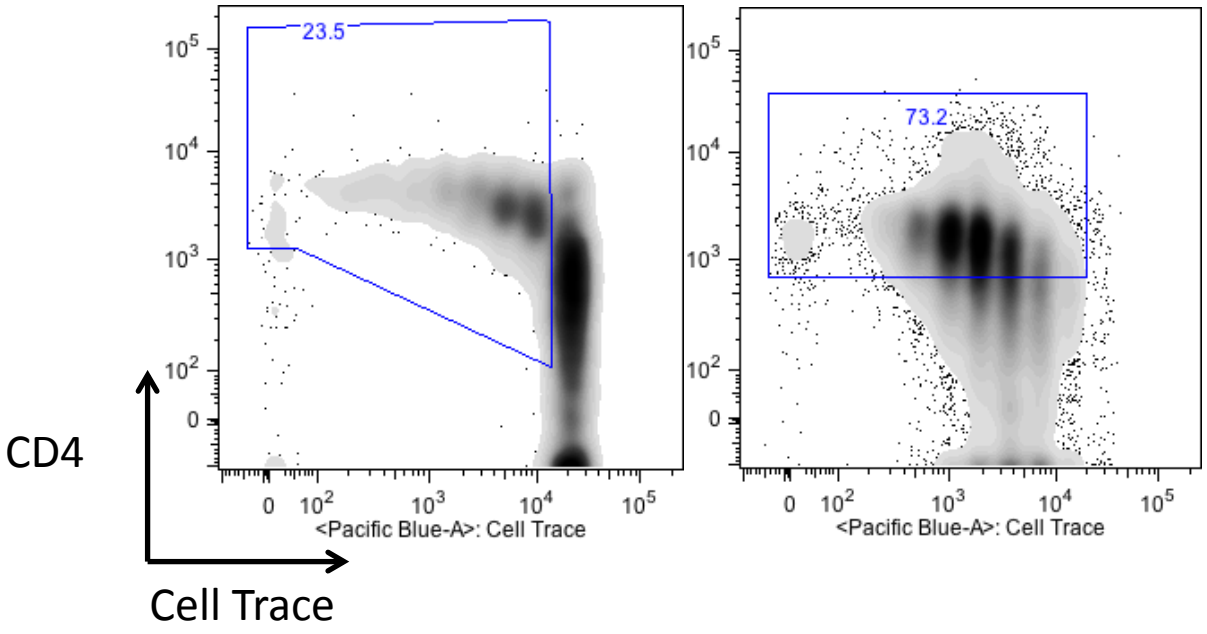
a. Comparison of the immunogenic effect of oxaliplatin vs. cisplatin

We conducted experiments treating ID8-*Defb29/Vegf-a* ovarian cancer-bearing mice with these chemotherapeutic drugs. However, we did not observe significant differences in the magnitude of anti-tumor immunity. **Outcome**: **No differences in anti-tumor immunity elicited by oxaliplatin, compared to cisplatin in ID8-*Defb29/Vegf-a* tumor-bearing individuals**.

b. Use of oxaliplatin as an adjuvant to support transferred T cells in cisplatin-treated hosts

Because we did not observe an immunogenic effect of oxaliplatin, and because ectopic expression of mIR-181a resulted in impaired effector activity, we investigated alternative interventions that could boost the therapeutic effect of adoptively transferred anti-tumor T cells not transduced with mIR-181a. In our hands, multiple emerging targeted therapies, including several PI3K or MEK inhibitors, impaired the activity of TCR-stimulated T cells in vitro and in vivo. However, the administration of the IL-15 superagonist ALT-803 (active in humans and mice; Altor Bioscience) boosted the expansion of tumor–reactive lymphocytes activated with CD3/CD28 Ab-coated beads. To determine the effect of ALT-803 in vivo specifically on transferred anti-tumor T cells, we challenged mice (n=6/group) with intraperitoneal ID8 ovarian cancer cells engineered to express a membrane-bound (truncated) variant of Ovalbumin (OVA). Ten days after tumor challenge, mice received Cell Trace Violet-labelled OVA-specific transgenic OT-I T cells, along with a single i.p. injection of ALT-803 or control vehicle. As shown in **Figure 4**, tumor antigen (OVA)-reactive T cells underwent a dramatic expansion *in vivo* in the ovarian cancer microenvironment upon ALT-803 administration, as determined by Cell Trace Violet dilution. No noticeable toxicity was detected, suggesting that this drug could become a powerful adjuvant in patients treated with anti-tumor T cells.

Figure 4. ALT-803 induces the expansion of adoptively tran sferred tumor reactive T cells in the ovarian cancer microenvironment. OVA expressing ID8 tumor cells were inoculated into Ly5.2 mice. On day 10, T cells from a naive OT-I mouse were harvested, stained with Cell Trace Violet, and injected into the mice. ALT-803 (0.2 mg/Kg body weight) was administered i.p. on day 10. On day 14, peritoneal washes were collected and analyzed by FACS for CD45.2, CD8, OT-I T cells.



Outcome: **ALT-803, but not chemotherapeutic drugs or kinase inhibitors, support the expansion of adoptively transferred anti-tumor T cells in ovarian cancer-bearing hosts**.

Key research accomplishments

a. miR-181a is up-regulated in tumor-reactive T cells in the ovarian cancer microenvironment.

b. Expression of miR-181a in tumor-reactive T cells induces a significant defect in their effector activity. Activation and memory differentiation are not affected.

c. Milestone 1: We identified that anti-tumor T cells overexpressing miR-181a exert defective protection against malignant progression, compared to control anti-tumor lymphocytes. Correspondingly, miR-181a emerges as a new immunotherapeutic target for down-regulation, rather than overexpresion.

d. Milestone 2: ALT-803, but not chemotherapy, PI3K or MEK inhibitors, support the expansion of adoptively transferred anti-tumor T cells in ovarian cancer-bearing hosts

Reportable outcomes

a. Manuscripts published so far:

1-Cubillos-Ruiz JR, Baird J, **Tesone AJ**, Rutkowski M, Scarlett UK, Camposeco-Jacobs AL, Anadon-Arnillas J, Harwood N, Korc M, Fiering S, Sempere L, **Conejo-Garcia JR** (2012). Reprogramming tumor-associated dendritic cells in vivo using microRNA mimetics triggers protective immunity against ovarian cancer. *Cancer Res*., 72: 1683-1693.

2-Scarlett U, **Conejo-Garcia JR** (2012). Modulating the tumor immune microenvironment as an ovarian cancer treatment strategy. *Expert Rev Obstet Gynecol.*, 7: 413-19 (Review).

3-Rutkowski MR, Stephen T, **Conejo-Garcia JR** (2012). Anti-tumor immunity: Myeloid leukocytes control the immune landscape. *Cell Immunol*., 278: 21-6 (Review).

b. Career developments:

1-Amelia J Tesone, the nested Teal Scholar in this award, co-authored one of the aforementioned manuscripts.

Conclusions

In summary, our results identify a negative role for miR-181a in the effector function of protective (anti-tumor) T cells. Although the initial hypothesis of this high-risk, high-reward pilot project was that miR-181a could boost the activity of anti-tumor T cells, our results still underscore the importance of the immune system's modulation of tumor progression. Most importantly, our results provide a mechanistic rationale for the modulation of miRNA activity in tumor-associated leukocytes as a novel cancer intervention

In addition, we have tested the adjuvant effect of a variety of clinically available drugs to support the activity of adoptively transferred anti-tumor T cells. Our study identifies ALT-803, a novel IL-15 agonist in clinical trials, as a drug that effectively boosts the in vivo proliferation of administered anti-tumor T cells in the tumor microenvironment. In contrast, chemotherapeutic drugs, PI3K or MEK inhibitors, have a negative or neutral effect.

Appendices

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Reprogramming Tumor-Associated Dendritic Cells *In Vivo* Using miRNA Mimetics Triggers Protective Immunity against Ovarian Cancer

Juan R. Cubillos-Ruiz[1], Jason R. Baird[1,2], Amelia J. Tesone[5], Melanie R. Rutkowski[5], Uciane K. Scarlett[5], Ana L. Camposeco-Jacobs[1], Jorge Anadon-Arnillas[1], Noah M. Harwood[1], Murray Korc[3,4], Steven N. Fiering[1,2], Lorenzo F. Sempere[3], and Jose R. Conejo-Garcia[5]

Abstract

Modulating the activity of miRNAs provides opportunities for novel cancer interventions. However, low bioavailability and poor cellular uptake are major challenges for delivering miRNA mimetics specifically to tumor cells. Here, we took advantage of the spontaneous enhanced endocytic activity of ovarian cancer-associated dendritic cells (DC) to selectively supplement the immunostimulatory miRNA miR-155. *In vivo* processing of nanoparticles carrying oligonucleotide duplexes mimicking the bulged structure of endogenous pre-miRNA (but not siRNA-like oligonucleotides) dramatically augmented miR-155 activity without saturating the RNA-induced silencing complex. Endogenous processing of synthetic miR-155 favored Ago2 and, to a lesser extent, Ago4 loading, resulting in genome-wide transcriptional changes that included silencing of multiple immunosuppressive mediators. Correspondingly, tumor-infiltrating DCs were transformed from immunosuppressive to highly immunostimulatory cells capable of triggering potent antitumor responses that abrogated the progression of established ovarian cancers. Our results show both the feasibility and therapeutic potential of supplementing/replenishing miRNAs *in vivo* using nonviral approaches to boost protective immunity against lethal tumors. Thus, we provide a platform, an optimized design, and a mechanistic rationale for the clinical testing of nonviral miRNA mimetics. *Cancer Res; 72(7); 1683–93. ©2012 AACR.*

Introduction

miRNAs are small endogenous noncoding RNAs implicated in the posttranscriptional control of gene expression in developmental, physiologic, and pathologic processes. Biologically active/mature miRNAs bind to partially complementary sequences [miRNA recognition element (MRE)] in hundreds of mRNAs, which diminish protein production via mRNA degradation and/or translational repression. miRNA-mediated regulation therefore constitutes a major mechanism to control global gene expression patterns (1–3).

miRNAs are quickly challenging our understanding of genetic regulation in health and disease, including cancer etiology (4) and the generation and inhibition of antitumor immune responses (5–9). Biologically active miRNAs bind to MREs on multiple mRNAs and simultaneously silence multiple target genes. This process can directly or indirectly modulate global gene expression and eventually determines transcriptional programs associated with a specific phenotype.

Because immune responses–including those against tumor antigens–depend on rapid phenotypic changes, it is not surprising that miRNAs have emerged as critical regulators of virtually all immune cell types (5, 7). miR-155 epitomizes the role of miRNAs in the immune system. miR-155 is basally expressed at low levels in B cells (7, 8), T cells (10), macrophages (11), dendritic cells (DC; ref. 7), and progenitor/stem cell populations (10). Activation signals such as antigen, toll-like receptor (TLR) stimulation and inflammatory cytokines, rapidly increase miR-155 expression in various leukocytic subsets, including bone marrow DCs (BMDC) and macrophages (7, 8, 11). Interestingly, BMDCs matured in the absence of miR-155 upregulate MHC-II and costimulatory molecules but are incapable of effectively activating T cells (7).

We previously showed that leukocytes with predominant phenotypic attributes of regulatory DCs (including the expression of CD11c and DEC205) home to perivascular locations in the ovarian cancer microenvironment, where they express multiple immunosuppressive mediators (12–14). From their position around blood vessels, these regulatory DCs inhibit the protective function of antitumor T cells infiltrating the tumor from the blood. Although specific delivery of RNA oligonucleotides to cancer cells is challenging because of low

Authors' Affiliations: Departments of [1]Microbiology and Immunology, [2]Genetics, [3]Medicine and [4]Pharmacology and Toxicology, Dartmouth Medical School, Lebanon, New Hampshire; and [5]Tumor Microenvironment and Metastasis Program, The Wistar Institute, Philadelphia, Pennsylvania

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for M. Korc, IU Simon Cancer Center, 980 W. Walnut Street, Walther Hall, R3 C528E, Indianapolis, IN 46202.

Corresponding Author: Jose R. Conejo-Garcia, Tumor Microenvironment and Metastasis Program, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. Phone: 215-495-6825; Fax: 215-495-6817; E-mail: jrconejo@Wistar.org

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bioavailability, poor cellular uptake, and abundant phagocytic activity of other cell types in the tumor microenvironment (15), the enhanced endocytic pathways and relative accessibility of ovarian cancer–associated myeloid leukocytes makes them ideal targets for nanocomplex-mediated delivery. Thus, we previously showed that polyethylenimine (PEI)-based nanocomplexes are selectively and avidly taken up by DCs at ovarian cancer locations, in the absence of any targeting motif (12). Using this optimized system, we now show that activity of mature miR-155 can be augmented in tumor-associated DCs by delivering novel Dicer substrate RNA duplexes that mimic the structure of endogenous precursor miR-155 hairpin (Dmi155) and that are efficiently processed *in vivo*. Replenishing miR-155 levels in tumor-associated DCs reprogrammed their immunosuppressive phenotype by modulating the expression of nearly half of the mRNAs in their transcriptome. Synthetic enhancement of miR-155 signaling in DCs boosted potent antitumor immune responses that abrogated the progression of established ovarian cancers. Our results show the feasibility of supplementing/replenishing miRNAs *in vivo* to boost antitumor immunity against aggressive, advanced, and frequently lethal tumors.

Materials and Methods

Production of PEI-based nanoparticles encapsulating DS RNA duplexes

Endotoxin-free PEI for *in vivo* experiments "*in vivo*-jetPEI" was purchased from PolyPlus Transfection. Dicer substrates (Dsi) were synthesized at Integrated DNA Technologies (IDT) using the following chimeric sequences:

Control GFP-specific Dicer substrate (GFP Dsi):
Plus: 5′ rUrGrCrArGrArUrGrArArCrUrUrCrArGrGrGrUrCrArGrCTT 3′
Minus: 5′ rArArGrCrU rGrArC rCrCrU rGrArA rGrUrU rCrArUrCrUrG rCrArUrU 3′

Control GFP-specific "bulged" Dicer substrate:
Plus: 5′ rUrGrCrArGrArUrGrArArCrUrUrCrArGrGrGrUrCrArGrCTT 3′
Minus: 5′ rArArGrCrU rGrArC rCrCrU rG rGrUrU rCrArU rCrUrGrCrArUrU 3′

siRNA-like miR-155 Dicer substrate (Dsi155):
Plus: 5′ rUrUrA rArUrG rCrUrA rArUrU rGrUrG rArUrA rGrGrGrGrUT T 3′
Minus: 5′ rArArA rCrCrC rCrUrA rUrCrA rCrArArUrUrArGrCrArUrUrA rArUrU 3′

miRNA-like bulged miR-155 Dicer substrate (Dmi155):
Plus: 5′ rUrUrA rArUrG rCrUrA rArUrU rGrUrG rArUrA rGrGrGrGrUT T 3′
Minus: 5′ rArArA rCrCrC rCrUrA rUrCrA rA rUrUrA rGrCrArUrUrA rArUrU 3′

In all cases, "r" represents a ribonucleotide and the absence of an "r" indicates a deoxynucleotide. The "plus" strand contains 2 terminal deoxynucleotides that resemble the loop of endogenous pre-miRNA and that function as cleavage signal for Dicer. The "plus" strand refers to the strand that will give rise to the mature miRNA after Dicer processing and preferential incorporation into the RNA-induced silencing complex (RISC).

To generate PEI-based nanoparticles encapsulating Dsi, 50 to 100 μg of each annealed duplex were complexed with "*in vivo*-jetPEI" at an N/P ratio of 6, following the recommendations of the manufacturer (PolyPlus Transfection). For biodistribution experiments, Dsi were fluorescently labeled in the 3′ end of the plus strand using Cy3 (IDT). Biotinylated Dsi were also chemically synthesized at IDT and include a Biotin group in the 5′ end of the "plus" strand. Thus, after intracellular processing of the Dsi, the mature form of the miRNA remains biotinylated *in vivo*.

Transfection and *in vivo* delivery of Dsi

Lipofectamine 2000 (Invitrogen) was used for *in vitro* transfection of Dsi into HEK293 cells in 96-well plates, following the recommendations of the manufacturer. For *in vivo* biodistribution, phenotypic and gene silencing experiments, mice bearing ID8-*Defb29/Vegf-A* tumors (12) for 3 to 4 weeks were intraperitoneally injected with PEI-Dsi nanoparticles (50 μg of Dsi complexed with "*in vivo*-jetPEI" at N/P 6, per mouse). In all phenotypic and functional experiments, tumor-associated DCs from mice injected with nanoparticles were sorted from ascites or peritoneal wash samples by flow cytometry on the basis of CD45, CD11c, and MHC-II positive expression.

Tumor progression experiments

Wild-type C57BL/6 mice were intraperitoneally injected with 2×10^6 parental ID8 (kindly provided by K. Robby, University of Kansas Medical Center, Kansas City, KS; ref. 16) and treatments started 15 days posttumor injection. A total of 2×10^6 aggressive ID8-*Defb29/Vegf-A* ovarian carcinoma cells were injected intraperitoneally and treatments started after 8 days. In all cases mice received 50 μg of Dsi complexed with "*in vivo*-jetPEI" at N/P 6 in glucose 5% at the indicated time points. Some experimental groups were also intraperitoneally injected with 50 μg anti-CD40 antibody (clone FGK4.5) 3 hours before administration of PEI-based nanoparticles containing Dsi.

For tumor rechallenge protection experiments, 3×10^6 $CD3^+$ T cells negatively immunopurified from the spleens of tumor-bearing mice treated with PBS (day 32 after tumor challenge) or αCD40 Ab plus Dmi155-PEI nanoparticles (day 61 after tumor challenge; treatments at days 8, 13, 18, 23, 27, and 60) were intravenously transferred into naive C57BL/6 mice previously irradiated with 300γ (5 mice per group). Twenty-four hours later mice were challenged in the flank with ID8-*Defb29/Vegf-A* ovarian carcinoma cells, as described (14). Tumor pictures were taken 25 days later. Tumor volumes were calculated by the formula $V = 0.5\,(L \times W^2)$, in which L is length and W is width.

Results

Dicer substrate RNA duplexes generate functionally active mature miR-155

miR-155 plays an important role in oncogenesis (9) but is also required for optimal antigen presentation and T-cell

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activation by mature DCs (7). We found that immunosuppressive $CD45^+CD11c^+MHC\text{-}II^+$ DCs (12, 13, 17–19) sorted from advanced orthotopic ID8-*Defb29/Vegf-A* tumors, an aggressive model of ovarian cancer that recapitulates the inflammatory microenvironment of human ovarian carcinomas (13, 14, 20, 21), showed significantly reduced levels of mature miR-155 (Fig. 1A). However, *in vivo* administration of CD40 plus TLR3 agonists, which synergistically transform tumor-associated DCs from immunosuppressive to immunostimulatory (13), induced a dramatic upregulation of mature miR-155 (Fig. 1B). We therefore hypothesized that miR-155 upregulation in DCs *in vivo* at tumor locations could be the crucial event promoting their capacity to elicit therapeutic antitumor immunity.

To augment miR-155 activity, we generated novel synthetic Dicer substrate (Dsi) RNA duplexes. To become functionally active, Dsi require processing by Dicer, the same RNAse type III enzyme that processes endogenous miRNA precursors and exogenous siRNAs. In addition, Dsi exhibit markedly enhanced silencing efficiency compared with conventional 21-mer siRNA oligonucleotides (22, 23). In all cases, we designed a forward (sense) RNA strand containing the sequence of endogenous mature miR-155 followed by 2 terminal deoxynucleotides in the 3′ end. We then generated 2 structural versions for miR-155 mimetic compounds by using different passenger (antisense) strands: An internally bulged complementary strand that recapitulates the precursor miRNA hairpin (Dmi155) and a perfectly matching, siRNA-like, complementary strand (Dsi155; Fig. 1C). Control irrelevant bulged or siRNA-like Dsi designed to target GFP were also produced in parallel.

Transfection of HEK293 cells with either Dsi155 or Dmi155 led to a dramatic dose-dependent increase in the intracellular

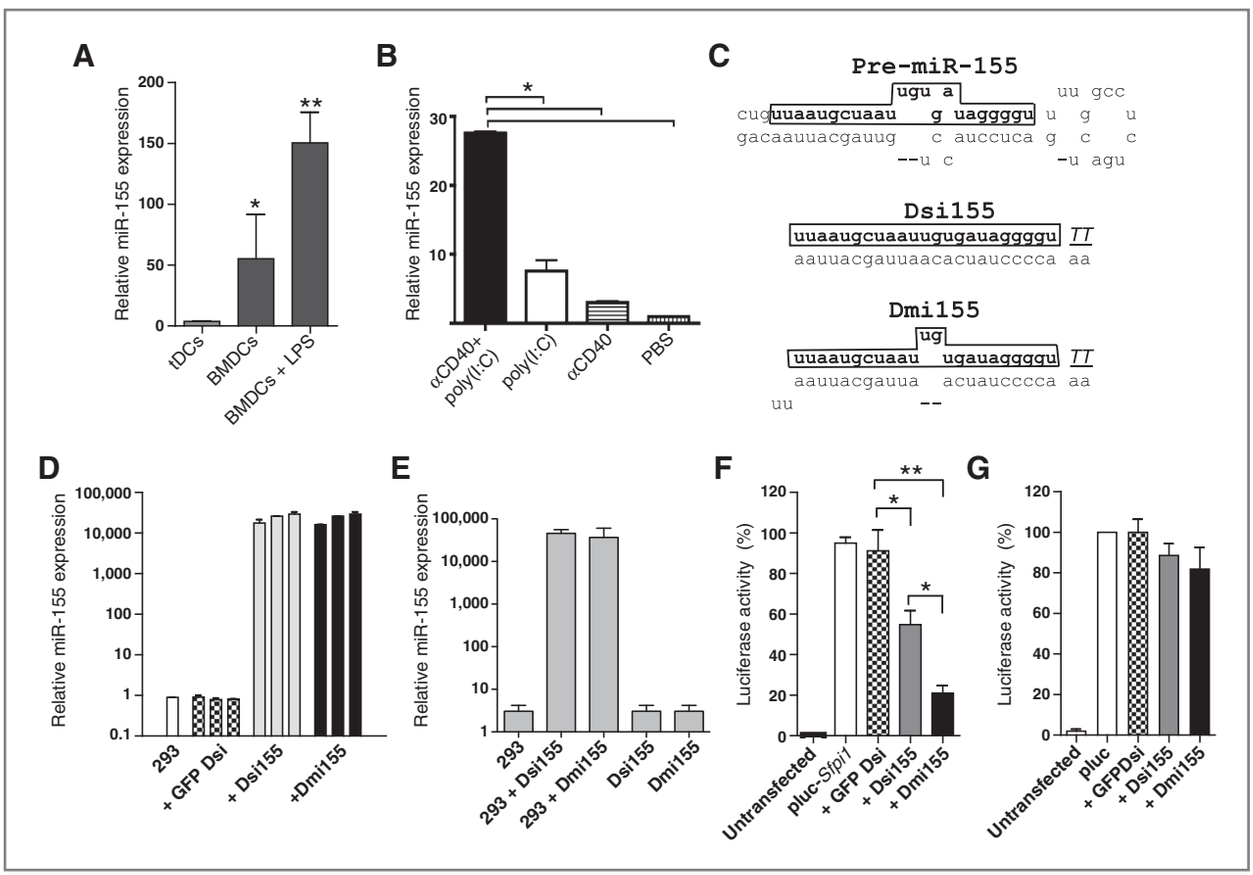

Figure 1. miR-155 expression by tumor-associated DCs and activity of RNA mimicking pre-miR-155. A, $CD45^+CD11c^+MHC\text{-}II^+$ tumor-associated DCs (tDCs) were sorted from ID8-*Defb29/Vegf-A* tumor ascites. BMDCs, generated as described (19), were stimulated with 1 μg/mL of LPS for 6 hours. Representative of 3 independent experiments. B, ID8-*Defb29/Vegf-A* tumor-bearing mice ($n = 3$) received intraperitoneal PBS, αCD40 (50 μg), poly(I:C) (100 μg), or αCD40 in combination with poly(I:C). $CD11c^+MHCII^+$ DCs were sorted from peritoneal wash after 48 hours. Representative of 2 independent experiments. C, top, endogenous pre–miR-155; middle, siRNA-like Dsi155; bottom, bulged miRNA-like Dmi155. Underlined, deoxynucleotides. Framed, mature miR-155. D, HEK293 cells in 96-well plates were transfected with 5, 10, or 25 pmol of miR-155 mimicking or control GFP-specific Dsi and 18 hours later mature miR-155 was quantified. Representative of 5 independent experiments. E, HEK293 cells in 96-well plates were transfected with 50 pmol of Dsi155 or Dmi155 and RNA was isolated 18 hours later. Fifty pmol of Dsi155 or Dmi155 were directly used as template as control. Representative of 3 independent experiments. In all cases, mature miR-155 was quantified by stem-loop qRT-PCR and data were normalized to U6 snRNA. F, a luciferase reporter vector harboring the MRE of miR-155 on *Sfpi1* was cotransfected into HEK293 cells together with different RNA duplexes. Luciferase activity in whole cell lysates was measured 24 hours later. Representative of 4 independent experiments. G, experiments were conducted as in E but using a luciferase-expressing construct without *Sfpi1* MRE. Representative of 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$ (Mann–Whitney in all cases).

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levels of processed miR-155, as detected by mature miRNA-specific stem-loop quantitative reverse transcriptase PCR (qRT-PCR; Fig. 1D). Confirming the selective detection of processed miRNAs by the cellular machinery, negligible signal was detected when synthetic Dsi155 or Dmi155 were directly reversed transcribed and amplified before transfection (Fig. 1E). To determine the functionality of processed miR-155 RNA generated from synthetic RNA, we cotransfected HEK293 cells with a luciferase reporter construct containing the miR-155 MRE of *Sfpi1*, an experimentally validated target gene of miR-155 (24). As expected, Dmi155 and, to a significantly lesser extent, Dsi155, rapidly silenced luciferase protein expression, whereas control (GFP specific) Dsi had no effect (Fig. 1F). Importantly, duplexes did not alter luciferase expression when the reporter constructs lacked the cognate miR-155 MRE (Fig. 1G). Together, these data showed that synthetic Dsi RNA duplexes can be used to effectively generate functionally active mature miR-155 in the cell, and suggest that a bulged structure may be important for the functionality of the miRNA generated.

Functional miR-155 delivered to tumor-associated DCs via PEI-Dsi nanocomplexes is preferentially loaded onto Ago2

We have shown that intraperitoneally injected nanocomplexes of PEI and siRNA are avidly and selectively taken up by tolerogenic DCs residing at ovarian cancer locations (12). As expected, PEI-based nanoparticles encapsulating Cy3-labeled Dmi155 were also preferentially engulfed by $CD45^+CD11c^+$ DCs in the tumor (peritoneal) microenvironment (Fig. 2A and B). Less than 1% of tumor cells incorporated the nanoparticles and only 3% of other leukocytes (primarily myeloid-derived suppressor cells and canonical macrophages) showed rhodamine fluorescence (Fig. 2B). Synthetic miR-155 was rapidly compartmentalized in the perinuclear region, typical of endosome–lysosome vesicle formation (ref. 25; Supplementary Fig. S1A). Most importantly, tumor-associated DCs endocytozing Dsi155 or Dmi155 nanocomplexes *in vivo* showed a marked increase in the intracellular levels of mature miR-155, as detected by stem-loop qRT-PCR, compared with tumor-associated DCs in mice untreated or receiving control GFP-specific Dsi (Fig. 2C). Ectopic mature miR-155 did not saturate the cellular silencing machinery because other endogenous mature miRNAs were found at comparable levels in DCs incorporating various RNA duplexes (Fig. 2D and Supplementary Fig. S1B).

To confirm the functional activity of miR-155 generated *in vivo* upon synthetic RNA processing, we analyzed the expression of 3 different experimentally validated targets of miR-155. Strikingly, the expression of *C/ebpβ* (10, 26, 27) and *Socs1* (28) was rapidly and potently silenced only in tumor-associated DCs engulfing nanoparticles of bulged Dmi155, but not perfectly matching Dsi155 or irrelevant Dsi (Fig. 2E and F). In addition, although Dsi155 induced a significant decrease in the expression of *Sfpi1* (10, 24), the silencing effect elicited by bulged Dmi155 was significantly greater (Fig. 2G). Therefore, although both Dsi155 and Dmi155 are biologically processed into mature miR-155, these data suggested that the structural features of the RNA duplex more closely mimicking the endogenous pre-miRNA hairpin are important for optimal silencing of target genes.

After Dicer processing, mature miRNAs are loaded by various Argonaute proteins (Ago1-4) into the RISC, a process that guides this multiprotein system to silence target mRNAs via cleavage, translational repression, or deadenylation (29). However, only Ago2 has slicer activity (30). Notably, we detected significantly greater amounts of mature miR-155 generated from both Dsi155 and Dmi155 by stem-loop qRT-PCR in Ago2 immunoprecipitates of peritoneal microenvironmental cells, compared with precipitation using Ago4 or, to an even lesser extent, Ago1 antibodies (Fig. 3B). Most importantly, significantly higher amounts of mature miR-155 processed from Dmi155 versus Dsi155 were found in slicer activity-endowed Ago2 pull-downs (Fig. 3B). Correspondingly, superior recovery of various known miR-155 targets was evidenced in Ago2-immunoprecipitated RNA only upon *in vivo* delivery of PEI-Dmi155, compared with administration of PEI-Dsi155 (Fig. 3C and D). Together, these results indicated that a bulged structure, similar to that of endogenous pre-miR-155, facilitates the efficient incorporation of mature miR-155 into the RISC via optimal loading onto Ago2 and, to a lesser extent, Ago4 and Ago1 proteins. There is no reliable Ago3 antibody for immunoprecipitation experiments and consequently Ago3 association studies could not be realized at this time.

Bulged Dmi155 reverts the tolerogenic activity of ovarian cancer-associated DCs and promotes their capacity to boost antitumor immunity

Because miR-155 is critical for DC-mediated antigen presentation (7) and its expression increases in response to CD40/TLR agonists, we hypothesized that delivery of miR-155 to CD40/TLR-stimulated tumor-associated DCs could further improve their antigen-presenting capacity at tumor locations. As expected, the proliferation of CFSE-labeled OT-1 T cells *in situ* in the ovarian cancer microenvironment was significantly enhanced in mice pulsed with full-length OVA when anti-CD40 plus (irrelevant) GFP Dsi-PEI nanocomplexes were administered (Fig. 4A and B). However, delivery of bulged Dmi155 induced a stronger antigen-specific T-cell proliferation at tumor locations (Fig. 4A and B), indicating that ectopic supplementation of miR-155 robustly enhances the immunostimulatory capacity of tumor-associated DCs beyond TLR activation.

Consistent with improved antigen presentation, higher proportions of antigen-experienced ($CD44^+$) $CD4^+$ and $CD8^+$ T cells were found in the tumor microenvironment of mice treated 4 times with CD40 agonists plus GFP Dsi-PEI nanocomplexes, compared with untreated mice (Fig. 4C and D and Supplementary Fig. S1C). The proportion of antigen-experienced T cells at tumor (peritoneal) locations was again further increased by treatment with PEI-complexed (bulged) Dmi155. Consistent with ineffective loading onto Ago2, the effect of perfectly matching Dsi155 was not superior to control GFP Dsi formulations (Fig. 4C and D and Supplementary Fig. S1C). Correspondingly, the number of tumor (peritoneal) T cells

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secreting Granzyme B in ELISPOT analyses in response to tumor antigens was also significantly increased upon administration of bulged Dmi155, whereas treatment with siRNA-like Dsi155 did not enhance tumor antigen–specific T-cell responses more than control GFP Dsi (Fig. 4E). Likewise, mice treated with Dmi155 also showed a marked increase in the numbers of splenic T cells secreting Granzyme B upon restimulation with tumor antigens (Fig. 4F). These responses were tumor specific because they were significantly diminished when antigen-presenting cells (APC) were pulsed with irrelevant (3T3) cells in independent experiments (Supplementary Fig. S1D). Furthermore, Dmi155 treatment resulted in a significant increase in the proportions of total splenic $CD8^+$ T cells exhibiting central memory attributes ($CD44^+$ $CD62L^+$; Fig. 4G and Supplementary Fig. S1E). Finally, *in vivo* production of Th1 cytokines with antitumor potential such as TNFα, IL-12, IFNγ, and CCL5 (19, 31) was significantly enhanced at tumor locations in mice receiving Dmi155, compared with Dsi155 or control RNA (Fig. 4H). Together, these results indicated that delivery of bulged pre-miR-155 mimetic RNA, but not siRNA-like reagents, enhances the capacity of otherwise regulatory DCs at tumor locations to effectively present antigen, boost T-cell–dependent antitumor immunity, and induce the secretion of immunostimulatory cytokines

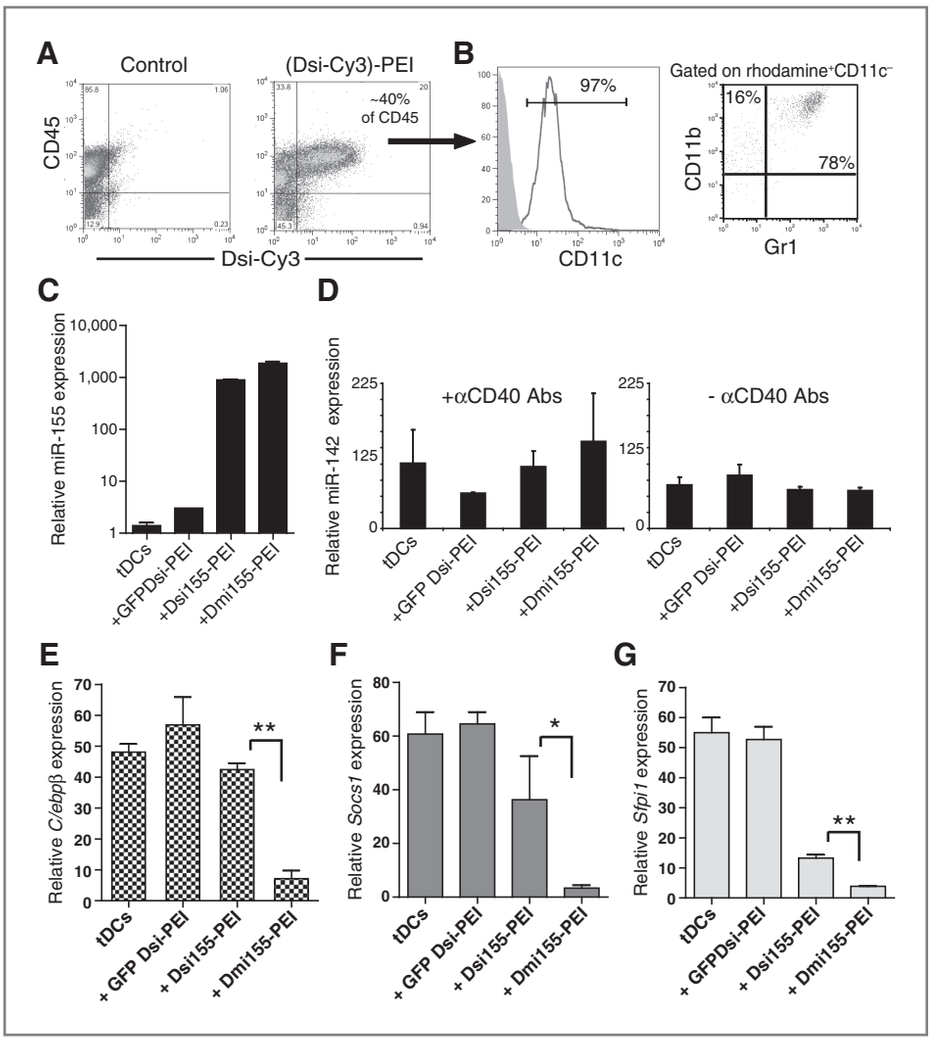

Figure 2. PEI-based nanocomplexes encapsulating functional Dmi155 are preferentially engulfed by tumor-associated DCs *in vivo*. A, Cy3-labeled Dmi155 nanocomplexes were intraperitoneally injected into mice bearing advanced ID8-*Defb29/Vegf-A* tumors. Fluorescence-activated cell sorting (FACS) analysis was done after 18 hours. Data are representative of 5 independent experiments. B, detailed analysis of leukocytes incorporating rhodamine-labeled nanocomplexes. Shaded histogram, isotype control staining. C, mice bearing advanced ID8-*Defb29/Vegf-A* ovarian tumors were left untreated or injected intraperitoneally with PEI complexed with different RNA duplexes. Eighteen hours later, $CD11c^+$MHC-II^+ tumor-associated DCs (tDCs) were sorted from peritoneal wash samples. Mature miR-155 was quantified by stem-loop qRT-PCR and the expression normalized to U6 snRNA. Data are representative of 2 independent experiments. D, mice were treated as in C or additionally injected with 50 μg of αCD40 3 hours before nanoparticle administration. miR-142-p5 expression in sorted tumor-associated DCs was quantified and normalized to U6 snRNA. E to G, expression of miR-155 mRNA targets in tumor-associated DCs engulfing PEI-Dsi nanocomplexes for 18 hours. Data were determined by qRT-PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are representative of 2 independent experiments with similar results. *, $P < 0.05$; **, $P < 0.01$ (Mann–Whitney).

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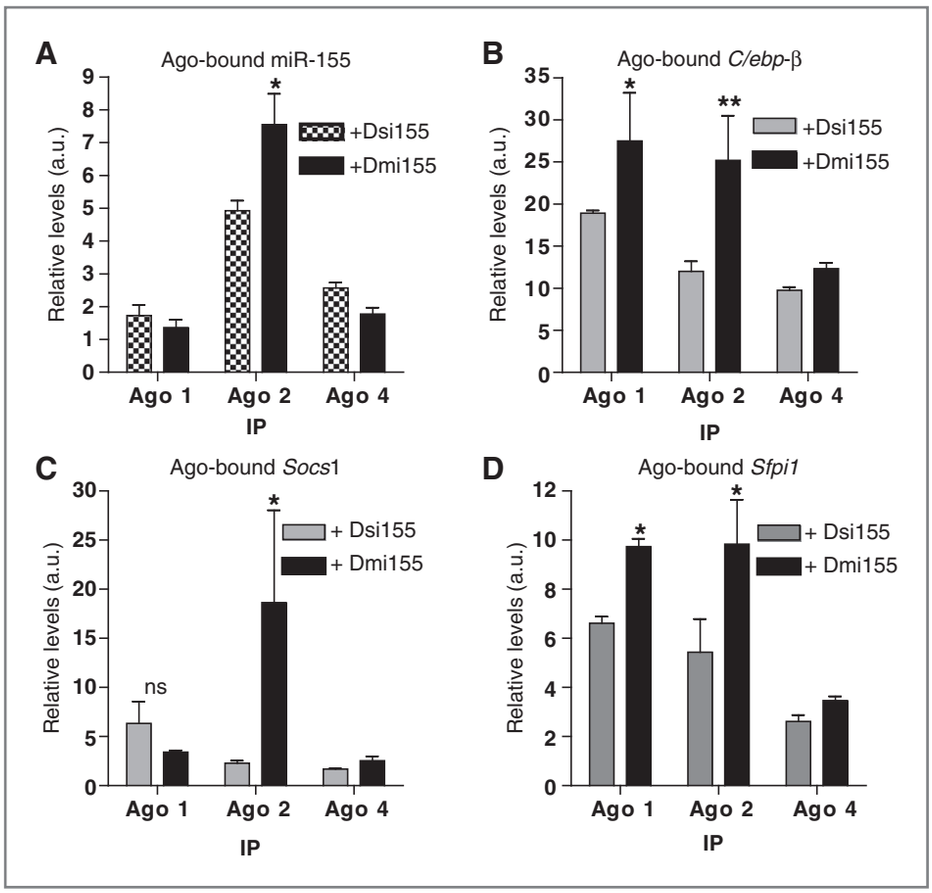

Figure 3. Loading of ectopic miR-155 onto different Ago variants. Mice bearing advanced ID8-*Defb29/Vegf-A* ovarian tumors were intraperitoneally injected with αCD40 and PEI-based nanocomplexes carrying either Dsi155, Dmi155, or control GFP-specific Dsi. Eighteen-hour postinjection, total peritoneal ascites were lysed and immunoprecipitated using monoclonal antibodies specific for Ago1, Ago2, or Ago4. Immunoprecipitated RNA was reversed transcribed and qRT-PCR was used to determine the levels of miR-155, normalized to background levels of immunoprecipitated U6 snRNA in each sample (A), and 3 known targets genes, normalized to background levels of GAPDH in each sample (B to D). *, $P < 0.05$; **, $P < 0.01$ (Mann–Whitney). IP, immunoprecipitation. a.u., arbitrary units.

beyond the sequence-independent, nonspecific activation of CD40 and TLRs (12, 13).

miR-155 delivery to tumor-associated DCs abrogates the progression of established ovarian cancer

Because tumor-associated DCs harboring increased levels of mature miR-155 exhibited functional properties of highly immunostimulatory APCs, we next determined the immunotherapeutic potential of delivering miR-155 mimetic RNA to ovarian cancer DCs, along with synergistic CD40 agonists (13). Mice growing orthotopic established ID8 ovarian tumors (16) were treated with agonistic anti-CD40 antibodies plus PEI-complexed control Dsi RNA (GFP specific), Dsi155, or Dmi155. Importantly, no obvious toxicity or secondary tumor growth in distant organs derived from the uptake of miR-155 mimetic RNA by cancer cells was observed in any case. As we previously reported (12), the intrinsic immunostimulatory activity of PEI-complexed RNA induced a significant increase of approximately 50% in the median survival of tumor-bearing mice treated with irrelevant (GFP specific) Dsi, compared with untreated mice (Fig. 5A). Consistent with the limited immunostimulatory effects on DCs, survival of mice treated with PEI-complexed Dsi155 was not superior to that elicited by the TLR5 agonist PEI alone (12), or by CD40 agonists plus the TLR5 agonist PEI, and was similar to that in mice treated with anti-CD40 antibodies plus irrelevant GFP Dsi (Fig. 5A). In contrast, treatment with bulged Dmi155 induced significantly superior effects and even abrogated disease progression in 33% of mice, which remained alive 80 days after controls succumbed to the disease (Fig. 5A). These results showed the therapeutic potential of supplementing miR-155 to tumor-infiltrating DCs *in vivo* using bulged RNA that mimics the structure of endogenous miR-155 and are consistent with the deficient silencing activity of miR-155 processed from perfectly matching oligonucleotides that merely include the sequence of mature miRNAs.

In addition, when treatments were administered to mice growing more aggressive ID8-*Defb29/Vegf-A* ovarian tumors, CD40 agonists synergized with PEI-complexed irrelevant double-stranded RNA oligonucleotides to induce a marked increase of approximately 43% in the median survival, and the effect of Dsi155 was again indistinguishable (Fig. 5B). Confirming our previous observations (13), agonistic anti-CD40 antibodies alone induce no therapeutic benefit against these tumors (Fig. 5B), unless they were combined with TLR agonists such as PEI (a TLR5 agonist; ref. 12) or double-stranded RNA. Notably, irrelevant bulged and siRNA-like Dsi targeting GFP induced identical effects (Supplementary Fig. S1F) and were, therefore, indistinctively used in subsequent experiments. Most importantly, mice receiving only 4 additional injections (days 13, 18, 23, and 27) of CD40 agonists plus PEI-complexed bulged Dmi155 exhibited a significant overall survival increase of approximately 65% compared with

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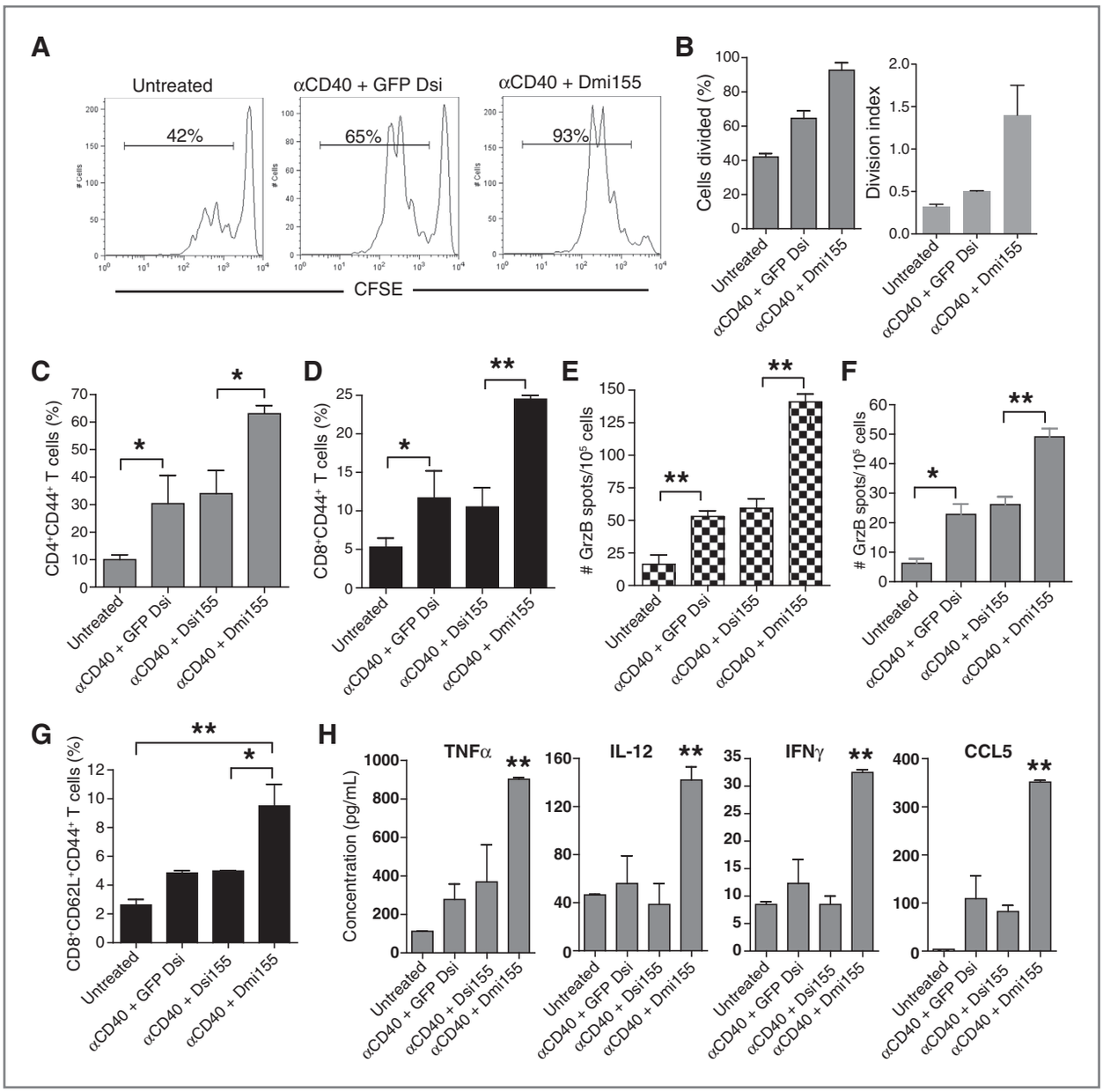

Figure 4. miR-155 delivery to tumor-associated DCs enhances antigen presentation and triggers antitumor immunity. A, mice growing ID8-*Defb29/Vegf-A* ovarian tumors for 3 weeks received 0.6 mg full-length endotoxin-free OVA (SIGMA, grade VII) intraperitoneally. Three hours later, mice were left untreated or injected with 50 μg anti-CD40 followed by 50 μg PEI-Dsi (N/P 6). Eighteen hours later, mice received 2×10^6 CFSE-labeled OVA-specific $CD3^+$ T cells negatively purified from OT-1 transgenic mice (intraperitoneally). Peritoneal wash samples (10 mL) were collected 48 hours later and T-cell proliferation was analyzed by FACS on the basis of CFSE dilution. B, left, percentage of cells divided in duplicate for each sample; right, division index of proliferating cells (FlowJo). Data are representative of 2 different mice per group. C to H, enhanced antitumor immune responses in mice treated with αCD40 plus Dmi155-PEI nanocomplexes. ID8-*Defb29/Vegf-A* tumor-bearing mice ($n = 3$ per group, 2 independent experiments) were treated at days 8, 13, 18, and 23 post tumor injection and peritoneal wash samples were analyzed at day 27. The proportion of antigen-experienced $CD4^+$ (C) and $CD8^+$ (D) T cells infiltrating tumor locations was determined by FACS (gated on $CD3^+$ cells). E and F, representative ELISPOT analysis showing increased numbers of tumor-reactive, Granzyme B–secreting T cells in the peritoneal cavity (E) or spleens (F) of mice treated with α-CD40 and Dmi155-PEI nanoparticles. GrzB, Granzyme B. G, proportion of $CD8^+$ T cells exhibiting central memory-like markers in the spleen of treated mice. H, total ascites supernatants were collected 18 hours after the administration of each indicated treatment and cytokines were measured by Bio-plex. Data are representative of 2 experiments. *, $P < 0.05$; **, $P < 0.01$ (Mann–Whitney in all cases).

untreated mice. This therapeutic effect was significantly stronger than that induced by an identical schedule of GFP Dsi-PEI or Dsi155-PEI treatments (Fig. 5B).

To confirm the antitumor effects of miR-155 mimetics in the absence of the mRNAs upregulated by CD40 activation, we finally treated aggressive tumor-bearing mice with an identical

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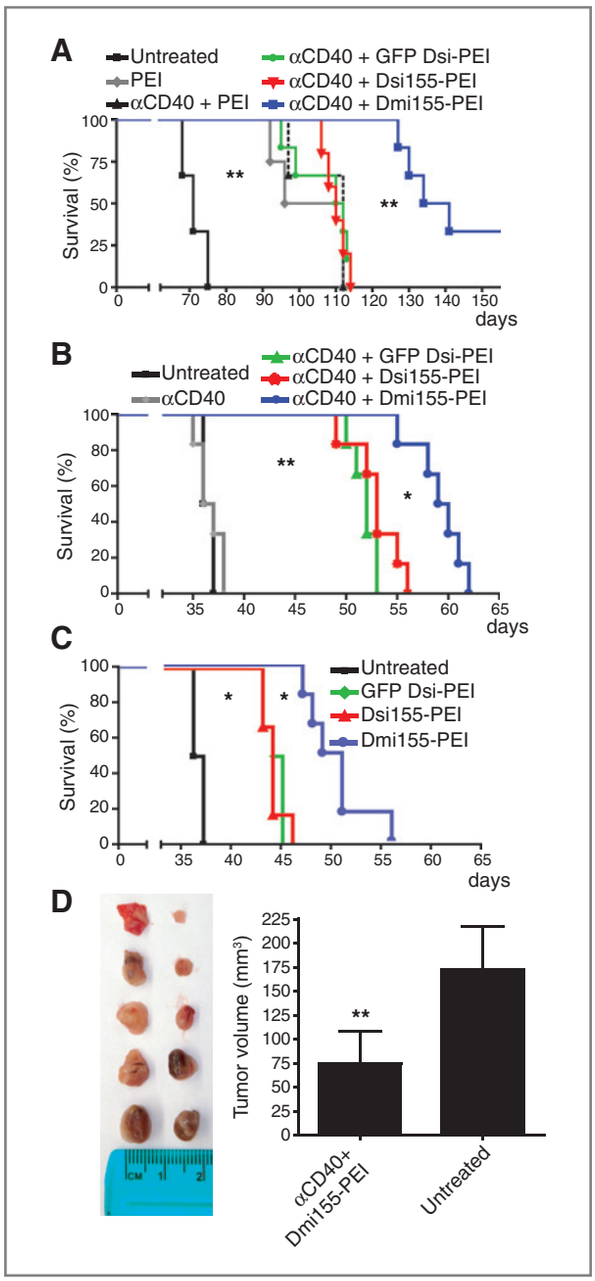

Figure 5. miR-155 delivery to tumor-associated DCs abrogates progression of established ovarian cancers. A, mice growing parental ID8 tumors (6 per group) received αCD40 antibodies and PEI-complexed Dsi at days 15, 21, 27, 28, 33, 48, and 63. Dmi155-treated mice received 2 more injections at days 114 and 129, after Dsi155-treated mice have died. ID8-*Defb29/Vegf-A* tumor-bearing mice (6 per group) were treated after 8 days with PEI–Dsi nanocomplexes in the presence (B) or absence (C) of α-CD40 agonistic antibodies. Additional treatments were given at days 13, 18, 23, and 27. D, sublethally irradiated healthy C57BL/6 mice received T cells negatively purified from the spleens of ID8-*Defb29/Vegf-A* tumor-bearing mice treated with PBS or α-CD40 agonistic antibodies plus Dmi155-PEI nanoparticles and were then challenged in the flank with the same ovarian carcinoma cells. Tumor growth in both groups was monitored 26 days later. Left, side-by-side comparison of resected tumors. Right, average tumor size in both groups.*, $P < 0.05$; **, $P < 0.01$ (log-rank or Student *t* test).

regimen of only control or miR-155 mimicking compounds. As shown in Fig. 5C, corresponding, although obviously weaker effects were observed. Notably, survival increases resulting from miR-155 supplementation were associated with T-cell–dependent protection because T cells from CD40/Dmi155-treated mice restrained tumor growth upon rechallenge, compared with T cells from untreated mice (Fig. 5D). Together, these results showed that only Dmi155 mimicking the bulged structure of endogenous pre-miR-155 is able to induce therapeutic benefits and synergize with the *in situ* activation of CD40 to extend survival in hosts bearing established aggressive ovarian carcinomas.

In vivo delivery of miR-155 mimetic RNA reprograms the transcriptome of tumor-associated DCs

To understand how mature miR-155 processed from delivered Dmi155 promotes the immunostimulatory phenotype of tumor-associated DCs in such striking manner, we next analyzed transcriptional changes in treated mice. Strikingly, deep sequencing analysis of the transcriptome of tumor-associated $CD45^+CD11c^+MHC\text{-}II^+$ DCs revealed that Dmi155, directly or indirectly, induced the silencing of thousands of transcripts, including multiple genes associated with an immunosuppressive phenotype (Supplementary File S1). Overall, 48% of total genes detected in tumor-associated DCs were downregulated 2-fold or more at the mRNA level by Dmi155 treatment. Those included known immunosuppressive targets of miR-155 such as *C/epbβ*, recently described as critical regulator of the immunosuppressive environment created by growing cancers (32); multiple mediators of Tgf-β signaling pathway, including *Tgfβ1, Smad1, Smad6*, and *Smad7*; and *Ccl22*, which recruits regulatory T cells to the tumor microenvironment (18). Unexpectedly, we also found that *Satb1*, a master genomic organizer (33), is expressed in tumor-associated DCs and silenced by miR-155.

In addition, we found downregulation of multiple transcripts not previously associated with miR-155. We focused on *Cd200*, a know mediator of DC-induced tolerance (34). Supporting that *Cd200* is indeed a *bona fide* immunosuppressive target of miR-155, luciferase activity was silenced by Dmi155, but not by irrelevant Dsi, in the presence of the 3′-untranslated region (3′-UTR) of *Cd200* (Fig. 6). The specificity of the analysis is supported by the parallel silencing of *Satb1*, recently confirmed as a target of miR-155, but not of *Pdcd4*, the expression of which is not significantly altered *in vivo* (Fig. 6).

Interestingly, *Cd200* is not a predicted target of miR-155 in any major databases. This is not surprising because 56% of published targets of miR-155 are also not contained in any major databases, including Miranda, Targetscan, DianaMT, miRDB, Mirwalk, PITA, RNA22, and PicTar.

Together, these data indicated that the transformation of plastic DCs at tumor locations into immunostimulatory cells by synthetic miR-155 is the result of complex genome-wide transcriptional changes rather than the silencing of a limited set of targets. In addition, our optimization of miRNA mimetics and delivery system provides multiple experimental hints for new targets of individual miRNAs, which should help to

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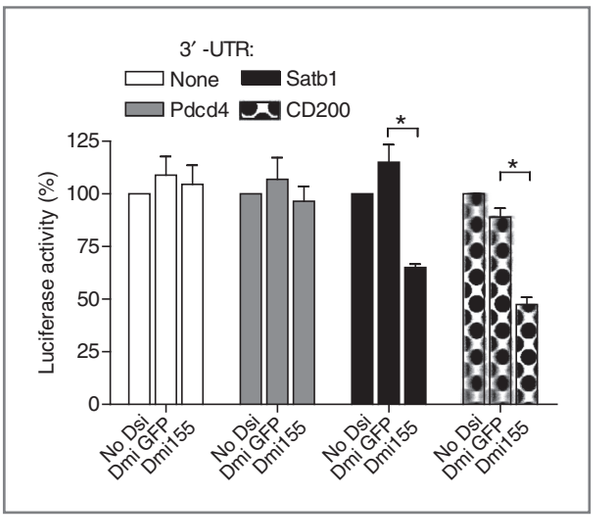

Figure 6. CD200 is a novel target of miR-155. HEK293 cells were independently cotransfected with different Dsi and reporter plasmids harboring the complete 3′-UTR region of the indicated genes (see Methods). Luciferase activity was measured 24 hours posttransfection. Data are normalized to the internal *Renilla* control in each reporter plasmid and are representative of 2 independent experiments. *, $P < 0.05$ (Mann–Whitney).

improve bioinformatical predictions by providing new clues for the design of more reliable algorithms.

Discussion

Here we show for the first time the feasibility of modulating miRNA activity selectively in ovarian cancer microenvironmental leukocytes using a nonviral approach, which promotes their capacity to elicit protective immunity.

Although expression of noncoding RNA in cancer cells can be achieved with viral vectors, the therapeutic use of viruses remains a clinical challenge. In addition, low bioavailability, poor cellular uptake, and preferential uptake by abundant phagocytic cells (15) are still major hurdles for specific delivery of genetic materials stabilized in nano- or microparticles to tumor cells. In contrast, the enhanced endocytic pathways and massive infiltration of the myeloid leukocytes that systematically accumulate in solid tumors make them ideal targets for nanocomplex-mediated delivery. Because of its relative accessibility, ovarian cancer–associated leukocytes are an ideal target for this approach.

We selected supplementing miR-155 because silencing of a nonredundant set of targets by this miRNA seems to be required for proper antigen presentation (7). However, miR-155 expression is frequently detected at high levels human cancer, both in solid tumors including breast, colorectal, lung, pancreatic, and thyroid carcinomas and in liquid tumors including lymphomas and some acute myeloid leukemias (9, 35). The association between oncogenesis and effective immunity is not surprising as robust adaptive immune responses entail rapid expansion of leukocytes. Furthermore, artificial upregulation of mir-155 leading to oncogenic conditions involves sustained overexpression in hematopoietic progenitors. In our study, the transient increase of mir-155 in lineage-committed myeloid cells such as tumor-associated DCs did not enhance ovarian cancer progression and did not result in generation of any secondary tumors. Instead, miR-155 delivery elicited robust antitumor immune responses that prolonged survival in mice bearing aggressive established ovarian cancer. Thus, miR-155 processed from endocytosed Dmi155 induced genome-wide transcriptional changes in DCs *in situ* at tumor locations, which significantly enhanced their immunostimulatory capacity. Because the expression of nearly half of the transcriptome was affected by synthetic miR-155 delivery, this significant phenotypic transformation was the result of complex coordinated transcriptional changes, rather than the silencing of a limited set of targets. Consequently, many genes known to promote tolerance were downregulated, including *C/ebpβ*, crucial immunosuppressive factor in cancer microenvironmental cells (32), and multiple mediators of the Tgfβ signaling pathway. In addition, we confirmed that other unpredicted targets of miR-155 such as *Cd200* were indeed silenced in standard luciferase assays.

Although PEI-siRNA nanocomplexes stimulate multiple TLRs on tumor-associated DCs (12), the robust enhancement in antigen presentation, production of Th1 cytokines, and expansion of tumor-reactive T cells selectively elicited by Dmi155 was significantly superior, compared with the nonspecific activation of DCs elicited by control sequences. Importantly, phenotypic transformation of DCs at tumor locations was not the result of the saturation of the RISC complex, because other mature miRNAs were clearly detected in Dmi155-treated cells. It is therefore very unlikely that the significant immunostimulatory effects, which abrogate the progression of established tumors, are the result of the sequestration of all Ago variants.

Most importantly for the clinical testing miRNA mimetics, we found that perfectly matching (siRNA-like) and bulged (miRNA-like) duplexes were both processed by tumor-associated DCs to generate mature miR-155. However, miR-155 generated from multiple batches of siRNA duplexes exhibited deficient silencing activity toward target genes *in vivo*, compared with Dmi155. Correspondingly, significantly higher amounts of mature miR-155 processed from Dmi155 versus Dsi155 were found in pull-downs of Ago2, the only Ago variant with slicer activity. miRNAs first associate with Agos as RNA duplexes that require activation, defined as conversion of the RNA duplex into a single-stranded miRNA. This activation process is the rate-limiting step in Ago loading and crucially depends on the thermodynamic instability of RNA duplexes (36). However, the cleavage activation pathway specific to Ago2 seems to be the only one insensitive to RNA thermostability in embryonic fibroblasts (36). It is possible that immune cells behave differently, so that Dmi155 and Dsi155 bind to Ago2 variants with similar affinity, but bulged duplexes with weaker thermodynamic stability are more efficiently processed and activated. Not mutually exclusive, it is also possible that chaperone proteins regulating the upload of small hairpin RNAs onto the RISC complex recognize the difference between

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a bulged versus a matching structure in DCs, so that different compositions are incorporated with distinct efficiency.

In summary, our results show the feasibility of delivering synthetic miRNAs to tumor microenvironmental cells as a novel cancer intervention and provide fundamental clues for the optimization of this approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.R. Cubillos-Ruiz, J.R. Baird, S.N. Fiering, L.F. Sempere, and J.R. Conejo-Garcia.
Development of methodology: J.R. Cubillos-Ruiz, J.R. Baird, S.N. Fiering, L.F. Sempere, and J.R. Conejo-Garcia.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Cubillos-Ruiz, J.R. Baird, A.J. Tesone, M.R. Rutkowski, A.L. Camposeco-Jacobs, J. Anadon-Arnillas, N.M. Harwood, M. Korc, and J.R. Conejo-Garcia.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.R. Cubillos-Ruiz, J.R. Baird, A.J. Tesone, M.R. Rutkowski, J. Anadon-Arnillas, N.M. Harwood, M. Korc, S.N. Fiering, and J.R. Conejo-Garcia.
Writing, review, and/or revision of the manuscript: J.R. Cubillos-Ruiz, A.J. Tesone, M.R. Rutkowski, U.K. Scarlett, S.N. Fiering, L.F. Sempere, and J.R. Conejo-Garcia.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.R. Baird.
Generated data: U.K. Scarlett.
Study supervision: S.N. Fiering and J.R. Conejo-Garcia.

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EXPERT REVIEWS

Modulating the tumor immune microenvironment as an ovarian cancer treatment strategy

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Uciane K Scarlett and Jose R Conejo-Garcia*

Tumor Microenvironment and Metastasis Program Leader, The Wistar Institute, 3601 Spruce St, Philadelphia, PA 19104, USA
**Author for correspondence:*
Tel.: +1 215 495 6825
Fax: +1 215 495 6817
jrconejo@wistar.org

After more than 30 years of iterations of surgical debulking plus chemotherapy, the need for complementary ovarian cancer treatments has become clear. In the ovarian cancer microenvironment, myeloid immunosuppressive leukocytes, lymphocytes, fibroblasts and endothelial cells, as well as their secreted products, surface molecules and paracrine survival factors, all provide opportunities for novel interventions. The potential of targeting microenvironmental elements in ovarian cancer patients is underscored by recently successful antiangiogenic therapies. The compartmentalized nature of ovarian cancer, its immunogenicity and its accessibility make it an ideal disease for targeting nontumor host cells. This review discusses the 'state-of-the-art' of the field, with an emphasis on the potential of modulating the activity of abundant microenvironmental immune cells, which govern both angiogenesis and immunosuppression.

Keywords: angiogenesis • dendritic cell • fibroblast • immunotherapy • ovarian cancer • tumor immunology • tumor microenvironment

Ovarian cancer is the fifth most common cancer among women. With over 120,000 women worldwide dying each year from the disease, it has the highest fatality-to-incidence ratio of all gynecologic cancers [1]. In the USA, ovarian cancer causes even more deaths than any other type of female reproductive cancer, melanoma or brain tumors [2]. The major clinical challenge for this disease is that patients are typically presented with symptoms only after the cancer has metastasized, leading most diagnoses to take place at advanced stages [3].

Based on converging genomic and clinical information, the divergent hypothesis emerging now in the field is that only a small fraction of ovarian cancers (designated type I) persist as stable masses for extended periods [4]. By contrast, the type of ovarian carcinomas that are responsible for 90% of deaths (designated type II) [5] could evolve aggressively without an identifiable localized precursor macroscopic mass [4]. In addition, the lack of effective treatments demands urgent alternative interventions against advanced tumors.

Treatments have evolved very little, and are still primarily restricted to surgical debulking plus cyclic iterations (i.e., intravenous vs intraperitoneal) of untargeted chemotherapies, focused on the tumor cell cycle. Only very recently, complementary interventions targeting elements of the tumor microenvironment (TME) have started undergoing clinical testing. Currently, the only drug in clinical use that targets the TME is bevacizumab, which blocks vascular EGF to inhibit angiogenesis [6]. Last December, results from two positive Phase III trials illustrated for the first time the potential of targeting the TME through this drug [7,8]. Thus, to increase the number of targets within the TME, a rigorous understanding of the ovarian cancer microenvironment is necessary and will open both new avenues for effective therapies.

This review will emphasize the areas that the authors consider to be the most promising targets for the design of new clinical interventions in the near future, which also represent the most critical aspects that are currently moving the field forward; namely, the immunobiology of ovarian tumors and their vascularization, which utilize closely related mechanisms. A detailed overview of all recent papers in the broad area of

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the ovarian cancer microenvironment is beyond the scope of this review. The authors acknowledge that the view of other authors could be different, and also that new developments in the field could change this focus.

The ovarian cancer microenvironment

Ovarian cancer is a peculiar disease in that multiple cellular types and molecules become accessible to both primary and metastatic masses typically, as they disseminate throughout the peritoneal cavity. For tumor cells, ascites provides an ideal milieu to detach and seed distally. Furthermore, crucial microenvironmental differences that define metastatic spreading in other tumors are not necessarily found in ovarian carcinoma. For instance, the authors found identical leukocyte infiltrates in matching solid metastatic and primary tumors in virtually every patient analyzed, as well as comparable extracellular structures and cell types [9–11]. By contrast, metastatic masses in most cancers are very different from the primary tumor. However, the inflammatory component of matching ascites and dissociated solid tumors is typically very different in patient samples. Ascites contains a predominant population of canonical $CD45^+CD14^+CD11b^+$macrophages, as well as $CD31^-CD45^-FAP^-$ tumor and $CD31^+CD45^-$ endothelial cells. It also includes an abundant mix of T cells, including Th1, Treg, $CD8^+$ and, to a lesser extent, Th17 lymphocytes. However, unlike other tumors [12], very few Th2 cells are found in the ovarian cancer microenvironment. The same cell types are also represented in corresponding solid tumors, but FAP^+ fibroblasts are obviously much more abundant, and the inflammatory microenvironment is much more complex. Thus, solid tumors mobilize a heterogeneous population of myeloid cells different from classical macrophages [13]. Over the last years, the authors have demonstrated that the most abundant leukocyte subset in solid tumors express determinants of *bona fide* dendritic cells, including CD11c, DEC205, CD86 and relatively high levels of MHC class II [9,10,14–18]. In at least a third of specimens, these cells lack the expression of the macrophage marker CD11b. In other patients, conclusive categorization is more complicated by phenotypic overlap with macrophages and myeloid-derived suppressor cells [19]. However, irrespective of nomenclature, these cells respond to immunostimulatory signals by upregulating costimulatory molecules and, at least in mouse models, by uptaking, processing and presenting antigens [10,11,16]. Most importantly, these leukocytes are crucial for both the generation and maintenance of tumor vasculature in ovarian tumors [9]. In addition, they secrete growth factors and proteases that promote tumor growth. As will be discussed later, they are also critical promoters of immunosuppression [9–11,16–18,20–24]. Correspondingly, their depletion in preclinical models delays tumor progression and boosts antitumor immunity [9], underscoring the potential of targeting this major microenvironmental compartment.

Accumulating evidence suggests that chronic inflammation in ovarian cancer plays a role in the development of the disease [25–27]. Ovulation induces insult to the ovarian surface, which triggers an influx of leukocytes to facilitate repair. Over time, the process of continuous damage and repair of the epithelial cells ('the incessant ovulation hypothesis') increases the chances of genetic error [28]. The initial inflammatory trigger, in addition to other environmental and genetic cues, is thought to create the foundation for chronic inflammation in ovarian cancer. This has been demonstrated in ovarian epithelial cells, where induced inflammation controlled the production of keratinocyte chemoattractants (KC/IL-8) and growth-regulated oncogenes (*GRO1/2*) with a slightly lower induction of CCL20, IP-10 and CCL7, which were collectively involved in the recruitment of inflammatory neutrophils, lymphocytes and dendritic cells (DCs) [25]. Furthermore, the expression of IL-6, TNF and CXCR4 has been found in high-grade serous tumors, where they play a crucial role in promoting angiogenesis and the recruitment of myeloid infiltrates [29].

Exploiting spontaneous antitumor immunity in the TME as a therapeutic goal

Not all microenvironmental leukocytes, however, promote tumor progression. Among all nontumor cells in the ovarian cancer microenvironment, T lymphocytes represent the only element that spontaneously exert confirmed clinically relevant, although obviously noncurative, immune pressure against disease advancement [30–32]. Direct recognition of specific tumor antigens by these cells has now been conclusively demonstrated by independent groups, and their infiltration patterns clearly predict the patient's outcome. For instance, in multiple independent studies, the narrow set of long-term (>10 years) survivors consistently show significantly stronger T-cell infiltrates in their tumor samples [30,33,34]. Whether their protective effect can be attributed to both $CD4^+$ and $CD8^+$ subsets [30,35], or only to the latter [33,34], is debatable, because $CD4^+$ T cells include a subset of Tregs that clearly promotes immunosuppression and is associated with accelerated cancer progression [36]. Nevertheless, it is clear that boosting T-cell-mediated protective activity represents a major opportunity for the design of novel therapeutic interventions against this devastating disease. DC-based vaccines, however, have shown limited clinical success due to the difficulty to overcome tumor-induced immunosuppression. Nevertheless, enhanced immunogenicity was demonstrated in two independent studies upon vaccination with either NY-ESO-1b or a heptavalent keyhole limpet hemocyanin construct [37,38], highlighting the potential for an ovarian cancer vaccine.

Currently, the most promising immunotherapy is based on engineering T cells to express chimeric receptors targeting specific tumor surface markers. This approach has produced impressive clinical results against other tumors. Among multiple candidates for targeting on the surface of ovarian cancer cells, mesothelin appears to be a relatively safe goal, for which clinical reagents are available [39–41]. Other obvious possibilities include Her2/neu and MUC1, which are shared by other tumors. A limitation of this powerful approach, however, is that engineering chimeric T cells involves a high degree of sophisticated expertise, patient-specific preparations and technological resources. It is therefore unlikely to generate broad pharmaceutical interest. Consequently, it is probably that these promising (although also complicated and

expensive) procedures will be limited to select institutions in the future. On the basis of previous T-cell-based approaches, it is also possible that adoptive transfer of chimeric T cells will work in some patients and not in others.

Immunosuppressive elements of the TME as novel therapeutic targets

In contrast to T cells, virtually any other leukocyte in the TME contributes to tumor growth and, paradoxically, immunosuppression. The most abundant hematopoietic subset myeloid leukocytes with attributes of DCs (Figure 1) are particularly immunosuppressive in solid tumors. From their perivascular location, these cells abrogate the activity of antitumor T cells extravasating from blood vessels. Their suppressive activity involves multiple complementary mechanisms that include the production of Arginase (Figure 1) and the expression of various surface immunosuppressive ligands [9,10,16,21]. Therefore, any vaccination or T-cell-based strategy will probably require concurrent targeting of this abrasive compartment for success.

The crucial role of myeloid leukocytes in ovarian cancer progression has been recently illustrated by the authors' studies in a preclinical model of sarcomatoid carcinoma in immunocompetent and previously healthy hosts [11]. As in patients, measurable antitumor immunity, which was initiated by DCs at very early stages, was detected. Notably, these responses were able to put tumors in check for relatively prolonged periods. However, after this latency period, tumors started to grow very rapidly. It was found that the key to this switch in progression is a gradual phenotypic and numerical change in tumor-infiltrating DCs, whereby they are transformed from an immunostimulatory to an immunosuppressive cell type. Correspondingly, depleting DCs at early stages accelerates tumor initiation, but at later stages prevents exponential growth, in the absence of any direct targeting of tumor cells. Promotion of immunostimulatory DCs at the macroscopic stage may therefore improve patient outcomes. The results thus support that microenvironmental myeloid leukocytes drive both inhibition of tumor growth (first) and aggressive malignant expansion (later). These data also support that the time ovarian tumors take to progress from macroscopically detectable tumors to terminal disease is very short, which may hinder the implementation of effective early diagnostic strategies. Of course, the applicability of this model to the human disease can be only assumed; but it is important to emphasize that the inflammatory microenvironment of advanced tumors in the system faithfully recapitulated the molecular and cellular components of leukocytes in human solid tumors [11].

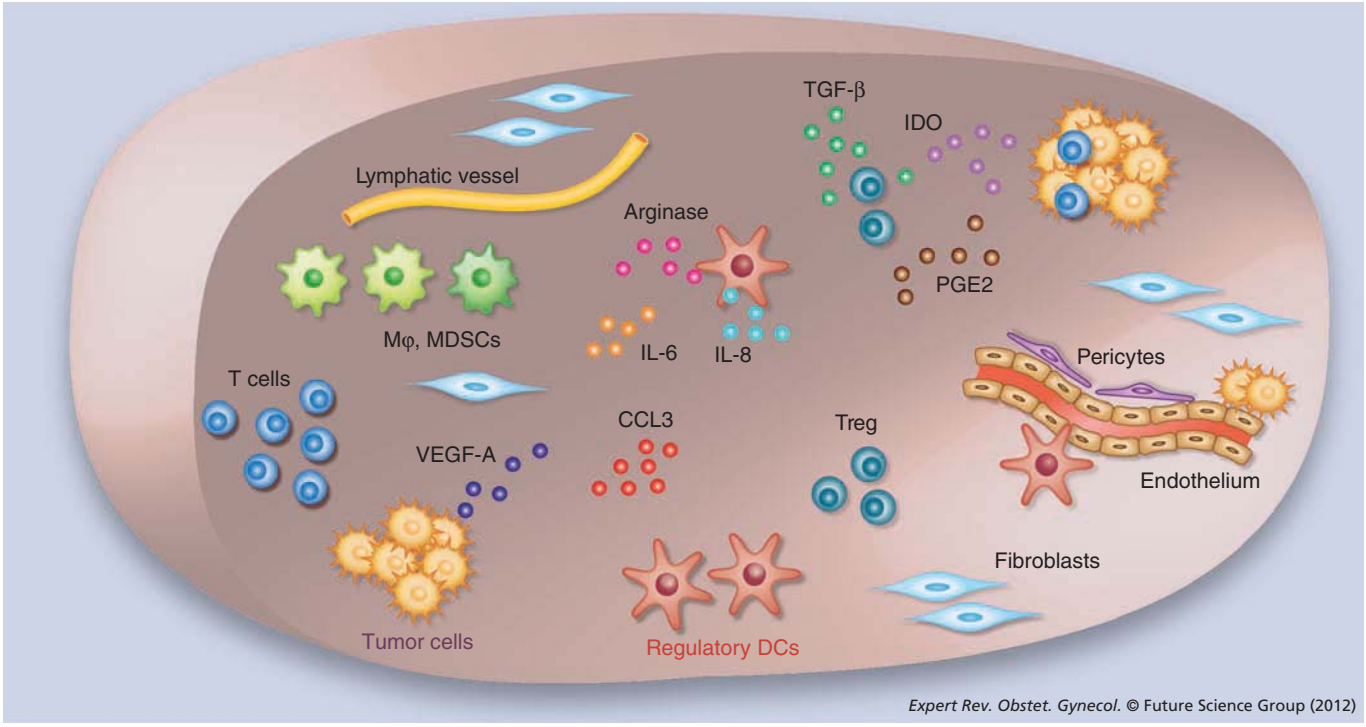

Figure 1. Targetable elements in the ovarian cancer microenvironment. In solid ovarian tumors, infiltration of tumor islets by antitumor T cells is associated with improved outcomes. The protective function of T cells is eventually abrogated, among other factors, by signals produced by tumor cells (e.g., TGF-β, PGE2 and IDO); by Treg; and by a very abundant and heterogeneous population of immunosuppressive/proangiogenic myeloid leukocytes with predominant attributes of DCs (through Arginase production and upregulation of a variety of inhibitory surface molecules). Fibroblasts and collagen constitute the main elements of the stroma, in which anarchically distributed vessels, covered by few pericytes, provide nutrients for tumor growth. Abundant inflammatory cytokines (e.g., IL-6, IL-8 or CCL3), primarily produced by infiltrating leukocytes, also impact the functions of other microenvironmental compartments. Finally, fibroblasts inhibit antitumor immunity through unknown mechanisms.
DC: Dendritic cell; MDSC: Myeloid-derived suppressor cell Mϕ: Macrophages.

Although the effectiveness of this approach needs to be tested in ovarian cancer patients, the potential of targeting immunosuppression in cancer patients is best illustrated by the recent success of antibodies blocking common checkpoints in T cells, such as CTLA4 and, especially, PD-1 [42]. Regulatory myeloid leukocytes in ovarian cancer utilize both mechanisms, and many tumor antigens have been identified in advanced ovarian tumor cells. Tumor-associated myeloid leukocytes therefore emerge as promising direct or indirect targets to unleash the spontaneous activity of antitumor T cells and induce the regression of established tumors.

In situ reprogramming tumor-associated leukocytes

Despite its grim prognosis, ovarian cancer offers significant advantages for the design of interventions targeting the TME and, in particular, tumor-associated leukocytes. First, even at a metastatic stage, ovarian cancer is most frequently restricted to the peritoneal cavity, so that treatments do not need to be systemically administered. Second, its peritoneal nature also makes ovarian tumors accessible, so that therapies can be directly administered where the targets are. Third, proangiogenic/immunosuppressive myeloid leukocytes are extremely abundant and predominantly accumulate at the growing edge of tumor masses. They also show enhanced endocytic activity [9,16], therefore they are ideal targets for selective take-up of particulate nanomaterials delivered intraperitoneally. Taking advantage of these peculiarities, DCs from ovarian cancer locations have been successfully depleted, which results in significant therapeutic effects in the absence of any direct targeting of tumor cells [9]. This could be clinically achieved using immunotoxins or various nanoparticles that are spontaneously and rabidly engulfed by these cells *in vivo*, resulting in significant immunogenic effects [16,17].

Most importantly, as tumor-associated myeloid leukocytes spontaneously take up tumor materials [10], promoting their capacity to present these antigens *in vivo* [14] represents a major opportunity for complementary interventions. The authors have demonstrated both the feasibility and the potential of this strategy through multiple approaches in preclinical models. For instance, the authors have shown that CD40 and Toll-like receptor (TLR) agonists synergize to transform tumor-associated DCs from an immunosuppressive to an immunostimulatory cell type when administered intraperitoneally. Because both agonists have been tested in clinical trials against different tumors [43,44], and synergistic activity has been demonstrated in various settings [45,46], combinatorial testing in ovarian cancer patients is only a matter of industrial interest.

Alternatively, the enhanced endocytic activity of these phagocytes can be exploited to selectively deliver, *in vivo* and *in situ*, polyplexes in the nanometer range carrying functional oligonucleotides [16,47]. Although excellent groups are trying to optimize the delivery of functional siRNA to ovarian cancer cells [48], preventing phagocytic uptake *in vivo* in the peritoneal cavity has been extremely challenging in our hands. By contrast, nanocomplexes made of biocompatible polymers and stabilized dsRNA are avidly engulfed by immunosuppressive phagocytes at tumor locations without any targeting motif. These nanocomplexes, besides silencing their targeted mRNAs, activate multiple TLRs, thus promoting the immunostimulatory potential of otherwise tolerogenic myeloid leukocytes. In addition, longer, Dicer-mediated cleavage-dependent oligonucleotides mimicking the sequence and the structure of endogenous miRNAs can be used to recapitulate the broad range of silencing activities of endogenous immunoactivating miRNAs [47].

Finally, the pathways driving the tolerization of initially immunocompetent DCs in the TME can be locally blocked with neutralizing antibodies or antagonists. Two signals that appear to be critical for the phenotypic switch in leukocytes that drives aggressive malignant expansion in ovarian cancer are TGF-β and PGE2 [11], for which reagents (e.g., COX2 inhibitors) are available.

Targeting the crosstalk between immune & nonimmune host cells in the TME

Besides immune cells and angiogenic cytokines, the TME obviously provides many other compartments for therapeutic interventions. Importantly, intervening on a particular element may dramatically affect other microenvironmental events. For instance, overexpression of endothelin-B in the tumor endothelium has been reported to prevent T-cell adhesion and subsequent homing to tumors [49]. Targeting tumor vasculature, therefore, could also have a profound impact on antitumor immunity. Not mutually exclusive, targeting immune cells that are crucial for neovascularization [14] could in turn abrogate angiogenesis, which is intimately associated with immunosuppression [50].

Finally, another prime opportunity to interrupt synergistic interactions between different cellular compartments in the TME is by targeting (as an individual or combinatorial intervention) cancer-associated fibroblasts (CAFs). CAFs provide structural and secretory support for tumor growth and dissemination (Figure 1) and therefore are targets on their own right. However, recent evidence indicates that depletion of FAP^+ cells, primarily expressed by CAFs, results in immunological control of established nonovarian tumors of different histological origins [51]. Tumor regression appears to be mediated by TNF-α and IFN-γ, which are primarily produced by immune cells. Therefore, CAFs also dampen antitumor immunity by abrogating the activity of IFN-γ-producing immune cells (primarily lymphocytes). The precise pathways whereby CAFs impair the protective activity of microenvironmental T cells remain completely unknown, but are the subject of intense investigation in various laboratories. Most importantly, FAP is a relatively specific surface marker in CAFs, as normal fibroblasts only express marginal levels. Therefore, FAP can be targeted with antibodies, vaccines and even chimeric T cells.

Expert commentary

A better understanding of the interactions between tumor and nontumor host cells, extracellular matrix and secreted molecules is revolutionizing our general views on tumor initiation and malignant expansion. Tumors, including ovarian cancer, are now seen as organ-like structures where a dynamic crosstalk

between tumor and the predominant nontumor compartments is required for progression. However, preclinical optimization of targeting elements of the TME is very challenging in terms of high-throughput screening. Unlike classical screening approaches for drugs targeting tumor cells, testing the effect of interventions on the TME requires *in vivo* models that recapitulate the human disease, because the interactions between these complex networks affect multiple compartments that cannot be frequently mimicked in a Petri dish. The recent availability of oncogene-driven genetic models of cancer is opening the field for the design of alternative interventions against multiple tumors, including ovarian cancer.

Five-year view

After more than 40 years of therapeutic approaches restricted to eliminate tumor cells, the need for new complementary therapeutic targets has become urgent. The ovarian cancer microenvironment offers many cell types and molecular elements for mutually exclusive interventions (Figure 1). Although the field is only in its infancy, emerging results from targeting vascular compartments illustrate the potential of targeting the TME. Myeloid leukocytes, lymphocytes, fibroblasts and endothelial cells, as well as their secreted products, surface molecules and paracrine survival factors, provide a fertile ground for novel therapies. The compartmentalized nature of ovarian cancer and the accessibility of its microenvironment offer extra openings for future clinical testing. We envision that the next 5 years will see a consolidation of several antiangiogenic drugs as first-line therapies. Available immunostimulatory drugs (such as combined CD40 and TLR agonists), alone or in combination with antibodies blocking crucial tolerogenic pathways (e.g., PD1), should be incorporated into the therapeutic arsenal in the near future. In addition, the adoptive transfer of tumor-reactive T cells is expected to produce the most impressive clinical results in selected patients. However, our view is that it is unlikely that engineered T cells will be routinely applied outside specialized institutions. We finally anticipate that an area of accelerated achievements in the next years will be the identification of molecular and immune signatures that predict big cohorts of patients that can benefit from specific treatments (e.g., immunotherapy). Strides towards personalized medicine and microenvironmental targeting should thus progressively reverse the dismaying prognosis of this terrible disease.

Financial & competing interests disclosure

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Key issues

- Last December, results from two positive Phase III trials illustrated for the first time the potential of targeting the ovarian cancer microenvironment with bevacizumab.
- The compartmentalized nature of ovarian cancer, its immunogenicity, abundance of inflammatory cells, aggressiveness and the accessibility of its microenvironment make it an ideal disease for new microenvironmental interventions and offers extra opportunities for future clinical testing.
- T cells engineered to express chimeric receptors targeting specific tumor markers should produce impressive clinical results in a particular group of patients treated at selected institutions in the near future. However, routine implementation of this approach may be limited to a few hospitals with the required technical expertise and sophisticated facilities.
- By contrast, immunostimulatory adjuvants such as CD40 and Toll-like receptor agonists are already approved for clinical testing and could synergize *in vivo* at activating ovarian cancer microenvironmental leukocytes.
- Similarly, antibody-based neutralization of common immunosuppressive pathways in the tumor microenvironment (TME; primarily, PD1) may prove successful in current clinical testing.
- Combinatorial targeting of different compartments of the TME, including endothelial cells, myeloid leukocytes and fibroblasts, is expected to result in synergistic effects by breaking their crosstalk.
- Molecular signatures that predict sets of patients to benefit from specific treatments targeting the TME (thus advancing towards the goal of personalized medicine) are expected to revolutionize the future management of ovarian cancer.

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Review

Anti-tumor immunity: Myeloid leukocytes control the immune landscape

Melanie R. Rutkowski, Tom L. Stephen, Jose R. Conejo-Garcia *

Tumor Microenvironment and Metastasis Program, The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104, USA

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ABSTRACT

The immune surveillance hypothesis proposed over 50 years ago that many precancerous lesions are eliminated without a histological trace due to immunological pressure. Since then, it has become apparent that both the tumor and the anti-cancer immune response evolve over a long period to allow the eventual escape of nascent precancerous lesions into full-blown tumors. Although primarily focusing on loss of antigenicity, the immunoediting hypothesis has gradually evolved to appreciate the role of active immunosuppression in tumor progression, where myeloid leukocytes are increasingly recognized as the major driving force. This review highlights recent studies implicating how myeloid cells with antigen-presenting capabilities are co-opted by tumors to promote malignant progression. Because at least some advanced tumors remain significantly immunogenic, these new studies add a tweak to the immunoediting hypothesis as well as a rationale to block immunosuppressive mechanisms as a first-line intervention in cancer patients.

1. Introduction

Burnet [1] and Thomas [2] originally posited that nascent tumor lesions were eliminated by the immune system without a pathological trace. Since then, overwhelming experimental evidence demonstrates that both the innate and adaptive immune systems play a non-redundant role in the prevention or promotion of tumorigenesis. Immune recognition of tumor antigens lead to the formulation of the cancer immunoediting hypothesis, which supports that immune pressure - primarily mediated by T cells - results in progressive loss of antigens (editing) by tumor cells, eventually allowing them to escape from accumulating immune pressure [3]. Loss of natural, spontaneous (relevant) antigens has been conclusively demonstrated in carcinogen-induced tumor models [4]. However, T cell infiltration is clearly associated with superior outcomes in patients with many different tumors [5–8], while clinically relevant responses have been achieved against many tumors using T cell based immunotherapies [9–11]. Most importantly, emerging clinical evidence indicates that blockade of immunosuppressive signals such as CTLA4 and, especially, PD-1/PD-L1, allows the immune system to regain control of the progression of a variety of tumors [12,13]. These clinical data and recent experimental evidence produced by our group [14] support that advanced tumors remain sufficiently immunogenic for effective control by the immune system, adding weight to the role of immunosuppression as a major driver of malignant progression. Pathological expansion of a heterogeneous population of immature myeloid cells with immunosuppressive activity is a hallmark of virtually all solid tumor-bearing hosts, and these cells are emerging as key players of immune regulation in the tumor microenvironment (TME) [11]. Paradoxically, myeloid leukocytes with antigen-presenting capabilities are required for the orchestration of tumor-specific T cell responses. Correspondingly, we recently identified a progressive phenotypic and numerical switch in dendritic cell (DC) populations in tumor-draining lymph nodes, parallel to both malignant progression and the abrogation of T cell-mediated protection [14]. The pivotal interplay between lymphoid and myeloid cells in the TME for preventing tumorigenesis *vs.* dampening the anti-tumor immune responses, and how to modulate it in vivo to control established tumors, will be the focus of this review.

2. Innate and adaptive immunity during tumor initiation and malignant progression

Studies using mice deficient in immune effector molecules have emphasized the critical role of innate and adaptive immunity in tumor initiation and malignant progression. Challenge of these immune-deficient mice with chemical carcinogens such as methylcholanthrene (MCA) or 7,12-dimenthylbenz[a]-anthracen (DMBA)/12-*O*-tetradecanoyl phorbol-13 acetate (TPA), resulted in accelerated generation of sarcomas or skin tumors compared to control WT mice with fully functional immune effector molecules (reviewed in [15]). Innate cells such as NK, NKT cells, γδ T cells, eosinophils [15,16] and neutrophils [17–19] mediate immune

* Corresponding author. Fax: +1 215 495 6817.
E-mail addresses: mrutkowski@wistar.org (M.R. Rutkowski), tstephen@wistar.org (T.L. Stephen), jrconejo@Wistar.org (J.R. Conejo-Garcia).

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protective or tumor promoting functions in experimental models for cancers, similar to what was observed in cancer patients. NK cells in particular appear to be critical for the rejection of nascent tumors [20]. However, so far only T cells in the TME have been associated with clinically relevant immune pressure against the progression of the established tumors that are detectable in the clinic. Thus, although it is theoretically possible that NK cells "hit and run", still being important although absent from tumor locations, current clinical evidence supports that the adaptive immune system, and in particular T cells, are the crucial effector immune cells that remain able to exert some significant (although obviously suboptimal) anti-tumor activity in advanced malignancies.

The specific T cell subsets empowered with anti-tumor activity are the subject of intense debate, because both γδ T cells and CD4 αβ lymphocytes include cells with enough spontaneous regulatory activity to dampen protective immunity [21,22]. Consequently, some studies have restricted the protective role of tumor-infiltrating lymphocytes to CD8 T cells [23], although both CD4 and γδ T cells are known to contribute to the orchestration and maintenance of adaptive effective immune responses through a variety of cytotoxic [24–26] and non-cytotoxic mechanisms [27–29]. Nevertheless, the prognostic value of T cell responses implies that antigen-presenting cells are able to effectively prime T lymphocytes at some point during tumor progression. Consequently, it has been shown that CD8α+ DCs are important in cross-presenting the tumor antigens to CD8+ T cells, so that in Batf3-deficient CD8α+ DCs, T cell mediated tumor rejection is impaired [30]. Furthermore, we have shown that the elimination of DCs in nascent tumor-bearing hosts dramatically accelerates malignant progression in an ovarian cancer model, which is restrained by CD8 T cells [14]. Because T cell infiltration is clinically relevant, and also because antibodies against tumor antigens are detected in a variety of cancer patients (which requires the activation of at least CD4 T cells), effective cooperation between T cells and DCs presenting tumor antigens appears to be taken place at initial stages of tumor progression. However, despite the fact that immune system can mount strong anti-tumor responses, tumors still evade the immune pressure [31]. Although the "self" nature of non-viral tumor antigens partially explains suboptimal T cell responses, the activity of tumor-specific T cells is further paralyzed in the TME through multiple complementary mechanisms.

3. Tumor immunoediting

Seminal studies by Schreiber and colleagues using chemical carcinogens such as MCA in immunocompromised mice conclusively demonstrated that tumors developed in the absence of adaptive immune system are more immunogenic in subsequent transplantation into WT mice [32]. IFNγ was found to be the principle molecule involved in tumor cell editing and both CD4 and CD8 T cells are the mediators of this strong anti-tumor response [32]. These studies are the cornerstone of the immunoediting hypothesis, which is the current framework accepted by most tumor immunologists [3,33,34]. The immunoediting hypothesis proposes that adaptive immune response not only regulates the quantity but also the quality of anti-tumor immunity, and has three important windows in which anti-tumor immune responses occurs. It starts with an elimination phase, during which cells of the innate and adaptive immune system eliminate cells undergoing transformation. If this elimination is complete, tumors disappear at this stage. Though experimental data supports this elimination phase, it cannot be characterized in humans because these events take place before tumors become detectable, if they ever become established. If tumor cells escape immune rejection, tumor progression goes through an equilibrium phase whereby tumors are kept under the control of effective immune responses, primarily mediated by components of the adaptive immune system. This phase culminates with three possible outcomes: First, the immune system can override the tumor cells and eliminate them. Second, this phase is continual and individuals remain free of clinically relevant tumors for their life-time. The third possibility is that adaptive immunity edits the tumors in such a way that new tumor cell variants develop, for which no T cell clones exist in the immune system. In that the case, edited tumor cells were proposed to evade the immune pressure, leading to accelerated expansion and, subsequently, development of clinical symptoms [3,33–35].

While progressive loss of antigenicity has been experimentally supported and has provided a valuable framework for years, most data supporting the editing hypothesis derive from chemically (MCA or DMBA/TPA) induced tumors, or cell lines derived from them (reviewed in [15]). The value of artificial antigens that do not reflect the mild responses induced by tumor antigens (such as ova [36]) should be interpreted with more caution. The issue associated with chemically induced tumor models is that high degree of variability in mutated antigens between each mouse in the study groups as chemicals induce random mutations. Therefore, immune responses as well as editing will be variable in these models. Furthermore, experiments in which secondary transfer of transplanted tumor cells result in tumor escape may be due to deregulation and enhancement of the proliferative capacities of tumor cells [37], and not necessarily alterations in the antigenic repertoire. A seminal step to define these mechanisms was provided by a recent cancer exome analysis of MCA induced sarcomas, which identified spectrin-b2 as a potential tumor rejection antigen in MCA and T cells selectively exclude the cells expressing this mutations during the course of tumor evasion [4]. However, the fact that multiple established tumors become at least partially immunologically controlled simply by blocking T cell checkpoints [12], indicates that tumors cells can be still recognized by the immune system and therefore retain the expression of relevant antigens, which is further supported by our recent experimental observations [14]. The cancer immunoediting hypothesis has correspondingly evolved to integrate immunosuppression (in addition to loss of immunogenicity) as a relevant mechanism behind the escape phase of tumor progression [35]. The question is which is the predominant mechanism in the oncogene-driven tumors that take place in humans?

4. T cell unresponsiveness in the tumor microenvironment

Recently the hallmarks of cancer have been modified to incorporate additional characteristics of cancer in the context of how cancer subverts the immune system. These include tumor-promoting inflammation, reprogramming energy metabolism, and evasion of the immune system [38]. Thus, solid tumors maintain an immunosuppressive, hypoxic and hostile environment that directly affects the effector function of T cells. Sustained exposure to suboptimal antigen levels and multiple suppressive factors can result in unresponsiveness through T cell exhaustion, anergy or senescence, three mechanisms that use different molecular pathways [39]. Studies in chronic viral infections have unveiled that T cell exhaustion is characterized by a progressive weakening of effector activity, expression of inhibitory receptors (e.g., PD-1 TIM3, LAG-3 and CTLA-4 (reviewed in [40]) and a transcriptional state that includes the overexpression of Blimp-1 and T-bet, along with up-regulation of NFAT2 in the presence of suboptimal levels of AP1 [39]. An identical phenotype is identified in the microenvironment of many tumors, particularly in CD8 T cells, where the expression of inhibitory receptors is required for induction and maintenance of T cells in exhausted state. Ligands for these recep-

tors are generally expressed by regulatory DCs and myeloid derived suppressor cells (MDSCs), in addition to tumor cells.

In contrast to the progressive nature of T cell exhaustion, anergy is rapidly initiated at the time of priming, and is characterized by the up-regulation of Rnf128, Egr2 and Egr3, and diminished Ras activation, along with excessive NFAT [41]. Maintenance of anergy is antigen independent while maintenance of exhaustion dependents on persistent antigen availability/TCR signaling [42]. Importantly, both exhaustion and anergy can be reversed through, respectively, the blockade of inhibitory pathways and cytokines [41,43,44]. As commented above, emerging clinical evidence supports the promise of blocking some of these inhibitory receptors [12].

In addition to anergic and exhausted T cells, senescent lymphocytes with shortened telomeres that have reached their terminal replicative potential are also found in the TME, particularly in elderly patients. These cells are characterized by the expression of CD57 and the absence of CD28 [45], and unresponsiveness is considered to be permanent. Irreversible cell cycle arrest can also be caused by a signal transduction program induced by cellular stress [46], although these molecular pathways remain largely uninvestigated in T cells.

Besides intrinsic transcriptional programs leading to T cell unresponsiveness, many factors in the TME abrogate the activity of effector T cells. Interestingly, some of these mediators are not only produced by tumor cells, but also by DCs that, rather than promoting anti-tumor immunity, are transformed into immunosuppressive players (see Fig. 1). Those factors include indoleamine 2,3-dioxygenase (IDO) and L-arginase, enzymes secreted by tumor cells, CD8α$^+$ DCs with tolerogenic phenotypes and MDSCs [47,48]. These enzymes deplete amino acids that are required for T cell functions from the TME [48,49]. IDO catalyzes the tryptophan degradation in the kynurenine pathway [50]. Both the reduction in tryptophan concentration as well as accumulation of tryptophan metabolites is immunosuppressive. In addition, tumor-infiltrating DCs and MDSCs actively contribute to the suppression of anti-tumor CD8 T cells through the production of L-arginase [48,51,52]. Other potent immunosuppressive factors are secreted by both myeloid leukocytes and tumor cells, including TGFβb [40]. Therefore, tumor immune evasion is the outcome of complex immunosuppressive mechanisms paradoxically driven by myeloid leukocytes, which eventually paralyze protective T cell responses.

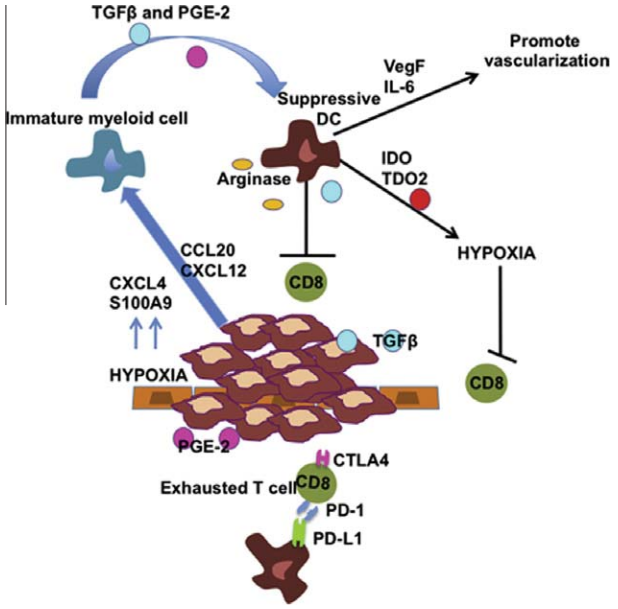

Fig. 1. Dendritic cell plasticity influences tumor progression. During aggressive malignant expansion of tumor cells, immature myeloid cells are recruited into the tumor microenvironment (TME) by CCL20 and CXCL12 produced by tumor cells or CXCL4 and S100A9 (upregulated in hypoxic environments). In the TME immature myeloid cells are converted into suppressive regulatory DCs by TGFβ and PGE-2 produced by the tumor cells. Suppressive DCs cooperate with the developing tumor mass to promote escape by secreting VegF and Il-6 (supporting angiogenesis), producing IDO and TDO2 (establishing a more hypoxic and immunosuppressive microenvironment) and secretion of immunosuppressive factors such as TGFβ and arginase (directly impeding T cell function). T cells become exhausted characterized by upregulation of inhibitory receptors such as PD-1 and CTLA4. This immunosuppressive tumor microenvironment impairs CD8 T cell anti-tumor responses, resulting in tumor escape.

5. Myeloid leukocytes and tumor-induced immunosuppression

The presence of exhausted tumor-specific T cells and (CD4-dependent) tumor antigen-specific antibodies in most cancer patients indicates that at least a fraction of tumor-reactive lymphocytes are effectively primed at early stages of tumor progression. So, how are myeloid leukocytes responsible for the orchestration of adaptive immune responses turned into immunosuppressive cells in tumor-bearing hosts? The answer is that a hallmark of virtually all advanced solid tumors is excessive mobilization of bone marrow precursors of myeloid leukocytes (including macrophages, dendritic cells and granulocytes), in response to multiple inflammatory cytokines [53–56]. This heterogeneous population, globally termed MDSCs, home to tumor locations in response to multiple chemokines, but they also exert immunosuppressive activity beyond the TME (reviewed in [57]). Among the multiple tolerogenic mechanisms that they promote, nitration of tyrosines in TCR–CD8 complex appears to be particularly relevant [55,58]. Once inside the TME, maturation of these myeloid cells into immunocompetent antigen-presenting cells is derailed, resulting in diminished adaptive immunity and eventual tumor escape. Thus, under hypoxic conditions, Ly6C$^+$ MDSCs differentiate into immunosuppressive macrophages and DCs in solid tumors [59]. Correspondingly, the categorization of the highly heterogeneous myeloid populations that massively accumulate in solid tumors is complicated by a high degree of phenotypic overlap, different stages of differentiation, predominant inflammatory signals produced by every specific tumor, and the location and histological type of the tumor itself, among other factors. Applying the markers and functional attributes of leukocytes categorically defined under steady-state conditions to immune cells in the TME is therefore very challenging. Nevertheless, immunosuppressive, pro-angiogenic CD11b$^+$CD68$^+$MHC-II$^+$ macrophages are represented in virtually all solid tumors. In addition, we have repeatedly demonstrated that the predominant leukocyte subset found in *solid* ovarian tumors (but not in human tumor ascites) co-expresses determinants of *bona fide* DCs, including CD11c, DEC205, CD86 and MHC-II, and in at least a third of clinical specimens lacks the macrophage markers CD11b and CD14 [60–66]. From their perivascular location, these myeloid cells abrogate the activity of anti-tumor T cells extravassating from blood vessels into the tumor microenvironment [65]. The expression of PD-L1 by ovarian cancer-associated DCs appears to be particularly relevant immunosuppressive mechanism, based on multiple converging lines of evidence [23,62,67]. Additionally, pDCs isolated from tumors in the prostate expressed high amounts of IDO and TGFβ to promote immune suppression and VEGF-A, and IL-6 to promote angiogenesis and metastasis [68]. Therefore, although the role of other immunosuppressive leukocyte subsets such as Treg is also relevant for tumor progression [21], the abundance and per cell immunosuppressive activity of myeloid cells in the TME indicates that this heterogeneous population is the major driving force for the abrogation of anti-tumor immunity in the TME. In addition, how and

to what extent myeloid leukocytes control the conversion of inducible Treg remains largely uninvestigated.

6. Tumor mediated escape: Dendritic cell conversion

DCs and macrophages are sentinels in immunity, and are required to respond rapidly to infection or to be able to quickly modulate robust inflammatory responses. Because of this plasticity in function and phenotype, myeloid-derived cells are vulnerable to the polarizing signals elicited by the tumor and tumor microenvironment. For instance, mobilization of monocytes from the periphery due to recognition of bacterial ligands [69] or during inflammation in the intestine [70], can give rise to inflammatory dendritic cells and possibly conventional $CD103^+$ DCs capable of inducing potent T cell responses. Conversely, tumor associated fibroblasts, through depletion of GMCSF in the tumor microenvironment, are capable of converting $CD11c^+$ dendritic cells into macrophages with potent immunosuppressive capabilities [71]. To investigate the dynamics of plastic antigen-presenting cells from tumor initiation to terminal malignant progression, we recently generated an inducible model of ovarian carcinoma driven by mutations in oncogenes and suppressor genes, as it happens in humans [14]. As expected, we found that measurable tumor-specific T cell responses are orchestrated shortly after tumor initiation by immunocompetent DCs. These responses were enough to keep tumors as microscopic lesions for relatively long periods. Correspondingly, depletion of DCs 7 days after tumor challenge resulted in a dramatic acceleration of tumor growth.

Paradoxically, the initiation of malignant macroscopic expansion was dependent upon the accumulation of $CD11c^+DEC205^+MHC\text{-}II^+$ DCs within the TME. However, these cells were not only unable to effectively present tumor antigens, but also abrogated the robust priming of T cells elicited by different immunocompetent DCs. Consistently, depletion of DCs at advanced stages of tumor progression significantly delayed tumor growth, allowing the immune system to regain control of tumors, again in the absence of any direct intervention on tumor cells [14]. These results demonstrate that myeloid leukocytes, and in particular DCs in ovarian tumors, govern malignant progression, as tumor growth can be modulated in opposite directions simply by eliminating this microenvironmental cell type at different stages. Our data also support that advanced tumors remain immunogenic, because cells from advanced tumors were able to induce significant T cell responses, particularly in lymphocytes derived from early tumors. Most importantly, our results provide a framework to understand the progression of aggressive epithelial tumors, whereby transition from microscopic lesions to exponentially growing masses could be occurring without a pre-malignant or dormant detectable lesion.

So, what factors induce the conversion of immunosuppressive DCs? Our data indicate that tumor cell derived PGE2 and TGFβ are sufficient to initiate the switch in DC phenotype from an immunostimulatory to immunosuppressive phenotype [14]. Ongoing studies should clarify which one is the predominant mechanism of tolerization in vivo. Other pathways potentially involved in the immunosuppressive activity of advanced tumor DCs include hypoxia within the TME, which induces DCs that are capable of presenting peptides but have impaired antigen processing capabilities and express significantly higher levels of VEGF-A, CXCL1, and CXCL8; chemokines all implicated in promoting angiogenesis in multiple forms of cancers [72]. Although we do not find high levels of IL10 in human or mouse ovarian cancers, secretion of IL10 can also lead to DC-inhibition of maturation in different tumors [73], in addition to inducing the expression of immunosuppressive OX40 ligand via production of thymic stromal lymphopoietin (TSLP) [74]. Finally, increased lipid accumulation in tumor associated DCs in both humans and mice resulted in a diminished ability to process and load antigen onto MHC, resulting in ineffective antigen presentation to T cells within the TME [75].

Other tumor-derived factors that could be significant contributors to DC conversion include IL6, which induce Socs3 upregulation in tumor-associated DCs leading to inhibition of pyruvate kinase M2 (M2-PK) [76], an enzyme involved in aerobic glycolysis [77]. S100A8/A9 [78], which induce the massive recruitment of immune cells and prevent their differentiation within the TME [79], could also participate in this process.

7. Back to normal: In vivo re-programming of tumor DCs

Due to their massive accumulation and suppressive power, macrophages and DCs in the TME emerge as major therapeutic targets. Importantly, we have demonstrated that when these leukocytes receive certain activating signals, at least in mouse models, they can process full-length OVA in vitro [60] and in vivo [62,66], and effectively present processed SIINFEKL to T cells. Therefore, interventions that achieve effective re-programing of immunosuppressive myeloid leukocytes in vivo into immunocompetent antigen-presenting cells, could be much more effective than their mere depletion, by simultaneously eliminating a major immunosuppressive driving force and boosting anti-tumor immunity in situ at tumor locations (see Fig. 2).

Ovarian cancer represents an ideal disease for these interventions because the TME is both compartmentalized and accessible. Supporting the feasibility of this approach in preclinical models, we have demonstrated that agonistic (and clinically available) CD40 and TLR agonists synergize to transform ovarian cancer-associated myeloid cells from an immunosuppressive to an immunostimulatory cell type [66]. Building on the insight of these studies, and by taking advantage of the enhanced endocytic pathways of tumor-associated DCs [62], we have more recently combined the synergy between the intrinsic TLR agonistic activity of double-stranded RNA and CD40 activation with the immunostimulatory activity of miR-155. Thus, we demonstrated that Dicer substrates mimicking the sequence and structure of endogenous miR-155 are selectively taken-up by tumor-associated $CD11c^+MHC\text{-}II^+$ DCs in mice growing aggressive orthotopic ovarian

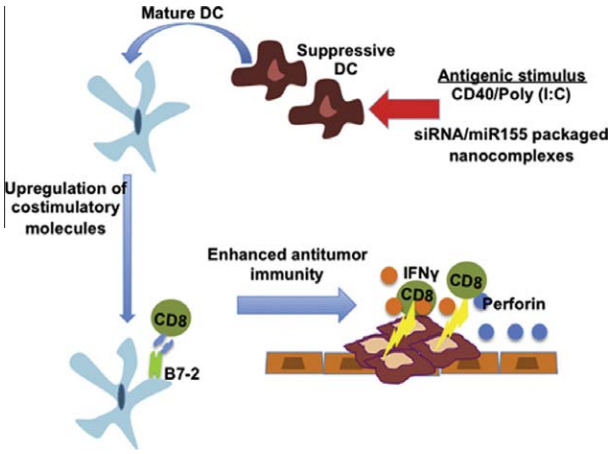

Fig. 2. In situ reversal of the immature phenotype of suppressive DCs with potent antigenic stimulus. Reversal of DC phenotype can be achieved by delivery of potent antigenic stimulation using agonistic CD40 and poly(I:C) or by delivery of immunostimulatory nanoparticles complexed with a mimetic for miR155, resulting in the conversion of immunosuppressive DCs into immunostimulatory DCs. Following stimulation, costimulatory molecules such as B7–2 are upregulated and resulting in enhanced effector T cell function and inhibition of tumor progression.

tumors when combined with biocompatible polymers, which synergizes with CD40 agonists [80]. Two important observations can be drawn from these studies; one, that DCs are major orchestrators of the immunosuppressive microenvironment, and two, in situ delivery of a potent antigenic stimulus is sufficient to reverse the tolerogenic phenotype, which provides a rationale for subsequent clinical testing.

8. Conclusions and future perspectives

Accelerated malignant growth coincides with the massive accumulation of immature myeloid leukocytes into the TME, which eventually breaks the dynamic equilibrium between protective T cell responses and proliferating tumor cells. Although tumors also lose recognizable antigens during their progression, they appear to remain significantly immunogenic to be controlled by existing anti-tumor T cells when inhibitory checkpoints are neutralized, as supported by experimental and clinical evidence. Because of their plasticity, myeloid leukocytes are highly susceptible to endogenous and exogenous signals within the tumor milieu. The presence of these cells within the tumor microenvironment is sufficient to tip the balance in favor of exponential tumor progression and escape from the immune pressure. However, myeloid cells (DCs) initially orchestrate measurable adaptive immune responses that can keep tumors in check for relatively long periods. Consequently, emerging evidence indicates that myeloid leukocytes govern cancer progression. Most importantly, partial reversal of the immunosuppressive genetic program of tumor DCs can be achieved in vivo and in situ by combining immunostimulatory agonists and delivering immune-activating miRNA mimetics. Understanding the genetic pathways and secretory factors that influence the mobilization of myeloid precursors and how to transform them from an immature to immunosuppressive phenotype should open new avenues for effective control of established tumors, besides iterations of chemotherapeutic drugs directly targeting tumor cells.

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