Synthesis and delivery of short, noncoding RNA by B lymphocytes

Gonzalo Almanza[a,1], Veronika Anufreichik[a,1], Jeffrey J. Rodvold[a,1], Kevin T. Chiu[a], Alexandra DeLaney[a], Johnny C. Akers[b], Clark C. Chen[b], and Maurizio Zanetti[a,2]

[a]Laboratory of Immunology, Department of Medicine and Moores Cancer Center, and [b]Center for Theoretical and Applied Neuro-Oncology, Division of Neurosurgery and Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093-0815

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Evolutionarily conserved short (20–30 nucleotides) noncoding RNAs (microRNAs) are powerful regulators of gene expression in a variety of physiological and pathological processes. As such, means to efficiently modulate microRNA function constitute an important therapeutic opportunity. Here we demonstrate that primary B lymphocytes can be genetically programmed with nonviral plasmid DNA for the biogenesis and delivery of antisense sequences (anti-microRNA) against microRNA-150 (miR-150). Within 18 h of transfection with an anti-miR-150 construct, primary B lymphocytes secrete ∼3,000 copies of anti-miR-150 molecules per cell. Anti-miR-150 molecules released by B lymphocytes were internalized by CD8 T lymphocytes during cross-priming in vitro and in vivo, resulting in marked down-regulation of endogenous miR-150. However, such internalization was not observed in the absence of cross-priming. These results suggest that shuttling anti-miR-150 molecules from B lymphocytes to T cells requires the activation of receiver T cells via the antigen receptor. Finally, anti-miR-150 synthesized in B cells were secreted both as free and extracellular vesicle-associated fractions, but only extracellular vesicle-associated anti-miR-150 were apparently taken up by CD8 T cells. Collectively, these data indicate that primary B lymphocytes represent an efficient platform for the synthesis and delivery of short, noncoding RNA, paving the way for an approach to immunogenomic therapies.

microvesicles | immunotherapy

In eukaryotes, evolutionarily conserved short (20–30 nucleotides) noncoding RNAs (microRNAs) regulate gene expression by binding to sequences with partial complementarity on target RNA transcripts, causing translational repression and/or messenger RNA degradation (1, 2). Each microRNA may repress up to hundreds of transcripts, and each microRNA can potentially regulate a large portion of the transcriptome (3). MicroRNAs have been implicated in the regulation of a variety of processes, including cell growth, differentiation and metabolism (4), and immunity and inflammation (5, 6). In addition to regulating processes that play an essential physiological role in mammalian organisms, microRNAs have also been associated with disease such as cancer (7), viral infection (8), and cardiac hypertrophy and fibrosis (9). Thus, in instances in which regulation of a specific process (e.g., the immune response) is desirable, or a disease appears to result from altered microRNA expression (e.g., cancer), targeting specific microRNAs with anti-microRNA holds promises for therapeutic intervention.

The in vivo therapeutic delivery of short, noncoding RNAs (microRNAs and anti-microRNAs) has been attempted using oligonucleotides that silence microRNAs (antagomirs) (10), but their usefulness in a clinical setting remains to be tested. Delivery methods include cholesterol conjugates, neutral lipid emulsions, functionalized gold nanoparticles, adeno-associated and retrolentiviral vectors encoding for antisense oligonucleotides (11), and nano-sized membrane vesicles (30–100 nm), termed exosomes (12).

Transduced or transfected primary B lymphocytes have been previously proposed as vehicles for the synthesis and delivery of proteins of immunological relevance (13, 14). B lymphocytes are an attractive cell type in which to carry gene manipulations for therapeutic purposes because B lymphocytes (*i*) are abundant in peripheral blood (∼15% of all leukocytes), (*ii*) develop a formidable translational capacity once activated through the antigen receptor, and (*iii*) do not need culture, maturation, or differentiation to be used as vehicles of DNA-based regulatory functions (15). For instance, we demonstrated that primary B lymphocytes transfected ex vivo with plasmid DNA and injected i.v. into naïve immune-competent mice synthesize and process transgenic molecules, thus initiating a systemic T-cell response in vivo (16) while persisting in secondary lymphoid organs for ∼15 d (15). Because the RNAseIII enzymes (Drosha and Dicer) that are required to process plasmid-borne RNA into small RNA, and ultimately single-stranded mature microRNA, are functional in primary B lymphocytes (17, 18), we decided to experimentally verify whether the biogenesis and secretion of short, noncoding anti-microRNA molecules could be activated in primary B lymphocytes transfected with suitable plasmid DNA. To this end, experiments were performed targeting microRNA-150 (miR-150), a microRNA involved in shaping the characteristics of memory CD8 T cells (19), control of B-cell lymphopoiesis (20), and in liquid and solid tumors (21, 22).

Here we report that primary murine B lymphocytes transfected with plasmid DNA (pCMV-MIR) comprising the coding sequence for anti-miR-150 efficiently synthesize and secrete functional anti-microRNA molecules, which are taken up by CD8 T lymphocytes during antigen presentation/T-cell activation in vitro and in vivo, apparently through small vesicles referred herein as extracellular vesicles (EVs), a collective term inclusive of exosomes and microvescicles (23). These findings are discussed with respect to the use of suitably programmed primary B lymphocytes for new forms of microRNA-based therapies.

Significance

Short, noncoding RNAs are a powerful way to regulate gene expression. As such, they are potential candidates for new forms of therapy. However, clinical translation is still hampered by lack of suitable and cost-effective delivery methods. Here we demonstrate that primary B lymphocytes can be programmed with plasmid DNA, effectively and easily, for the synthesis and delivery of antisense sequences (anti-microRNA) in vitro and in vivo. We also demonstrate that anti-microRNAs secreted by primary B lymphocytes in vivo are taken up by T lymphocytes during antigen activation. These initial observations predict that microRNA-based intervention to modulate immune responses or to target cancer cell growth may be possible through the type of cell-based therapy disclosed here.

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[1]G.A., V.A., and J.J.R. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: mzanetti@ucsd.edu.

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Results

Synthesis and Secretion of Anti-miR-150 in B Lymphocytes. To test the possibility that primary B lymphocytes could efficiently sustain the synthesis of anti-microRNAs, primary B lymphocytes purified from the spleen of naïve adult mice were transfected with plasmid DNA encoding anti-miR-150 (pCMV-MIR^{a150}). This plasmid codes for the 22 bp corresponding to anti-miR-150 (antisense) under the control of the CMV promoter (Fig. 1*A*). Assuming equal cell distribution, the input during transfection was $\sim 3 \times 10^5$ molecules of plasmid per cell. Copies of anti-miR-150 were produced in every instance as determined by copy number in transfected B lymphocytes. On the basis of the intracellular synthesis values, we estimated that, on average, primary B lymphocytes synthesize ~140 copies per cell in the first 18 h (Fig. 1*B*), with a fourfold variation in synthetic rate from experiment to experiment. The possibility that the quantitative RT-PCR (qRT-PCR) would amplify the input plasmid was ruled out because the anti-miR-150–specific primers did not amplify the plasmid (Fig. S1), suggesting that amplification was specific for the short anti-miR-150 after intracellular biogenesis.

Secretion of anti-miR-150 was assessed in the culture supernatant harvested 18 h after transfection. Anti-miR-150 molecules were abundantly secreted in the culture medium. When the copy number was adjusted for the number of transfected primary B lymphocytes, we found that over the 18-h period each cell secretes on average 3,000 copies, many more copies than those estimated inside the cell (Fig. 1*C*). This finding suggests a rapid cellular export of mature anti-miR-150 with accumulation in the extracellular compartment. Collectively, these data show that primary B lymphocytes transfected with pCMV-MIR^{a150} initiate a reproducible, high-level synthesis and secretion of specific anti-microRNA molecules.

Uptake of Anti-miR-150 by CD8 T Lymphocytes During Cross-Priming in Vitro. Cross-priming is the property of CD8 T cells to be activated by phagocytic antigen presenting cells after uptake of soluble antigen and processing/presentation in the MHC class I pathway (24). As such, cross-priming is regarded as the preferential mode of activation of CD8 T cells by host antigen presenting cells after uptake of self-tumor antigens (25). Here, we used in vitro cross-priming to test the possibility that anti-miR-150 secreted by primary B lymphocytes could be internalized by CD8 T cells specifically during antigen activation by dendritic cells (DCs) during cross-priming. Briefly, bone marrow-derived CD11b$^+$/CD11c$^+$ DC (BMDC) were cultured in vitro with the model antigen ovalbumin (OVA) for 16 h before adding (*i*) naïve CD8 T cells from transgenic OT-I mice that express a T-cell receptor (TCR) specific for the SIINFEKL OVA peptide presented in MHC class I molecules (26), and (*ii*) the anti-miR-150 containing supernatant from 18-h culture of transfected primary B lymphocytes (Fig. 2*A*). These cocultures were subsequently incubated for 96 h. To ensure that CD8 T cells were activated during cross-priming, cells were stained for CD44 and CD69, two canonical surface markers of activation, and were found to be positive in a large proportion of cross-primed CD8 T cells (Fig. 2*B*). A copy number analysis of CD8 T cells from these cocultures revealed that in every instance in which we added the anti-miR-150 enriched supernatant from transfected primary B lymphocytes, anti-miR-150 was markedly amplified in cross-primed T cells (Fig. 2*C*). In contrast, when the same B lymphocyte supernatant was added to CD8 T cells cocultured with BMDC without OVA (i.e., no cross-priming), no detectable anti-miR-150 amplification was found. This result suggests that antigen-mediated activation of CD8 T cells via cross-priming is necessary for the internalization of anti-microRNAs.

Transfer Anti-miR-150 from Transfected B Lymphocytes to CD8 T Cells During Cross-Priming in Vivo. Next, we tested the possibility that anti-miR-150 secreted by transfected primary B lymphocytes could undergo internalization by CD8 T cells during cross-priming in vivo. To this end, we used and compared two TCR transgenic strains of mice: OT-I mice specific for OVA and F5 mice whose CD8 T cells bear a TCR specific for the ASNENMDAM peptide of the nucleoprotein (NP) antigen of the influenza A virus (27), as a control. To induce cross-priming, mice were preinjected i.p. with OVA (5 mg) to cause antigen-specific activation of CD8 T cells in secondary lymphoid organs (28). Twenty-four hours after OVA administration, mice were injected i.v. with 1×10^6 primary B lymphocytes freshly (<1 h) transfected with pCMV-MIR^{a150} (Fig. 3*A*). We reasoned that because F5 mice are specific for influenza virus NP antigen, they would not respond to OVA immunization, hence representing an ideal indicator of any effect due to the transfected B lymphocytes outside the context of cross-priming (e.g., passive uptake), and also account for any effect due to OVA. As expected, OVA activated CD8 T cells in OT-I but not in F5 mice, as determined by CD44 and CD69 surface staining on spleen and lymph node-derived CD8 T cells on day 3 (Fig. 3*B*). In 7 of 7 instances, we amplified anti-miR-150 in OT-I CD8 T cells, whereas amplification in CD8 T cells from F5 mice was minimal. The average copy number per cell in OT-I T cells was 4×10^4 vs. 40 in F5 T cells (Fig. 3*C*). Thus, anti-miR-150 molecules secreted by transfected primary B lymphocytes are taken

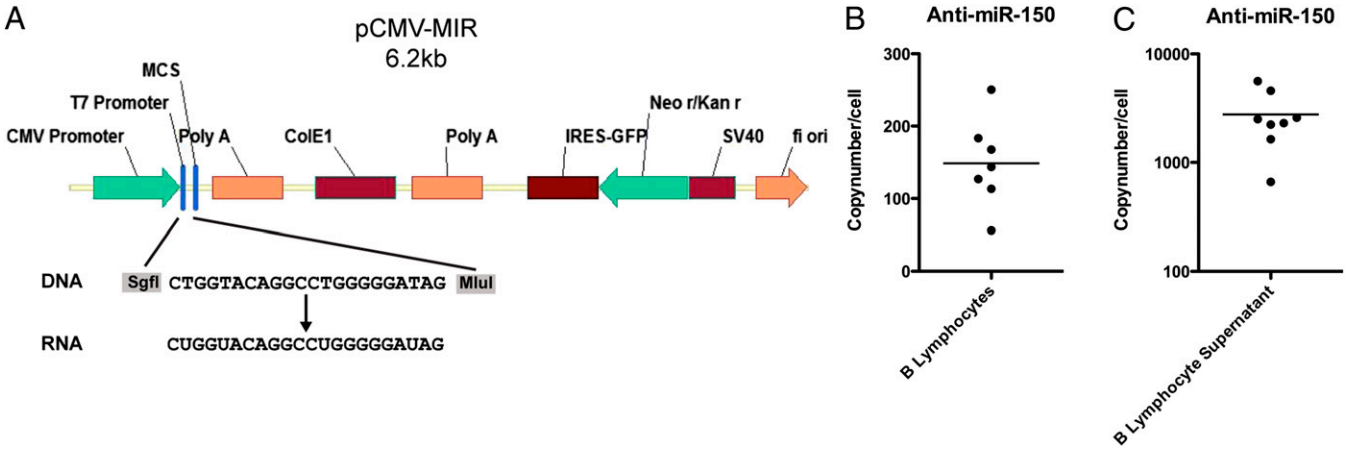

Fig. 1. Structure of pCMV-MIR^{a150} and secretion of anti-miR-150 by primary B lymphocytes transfected with pCMV-MIR^{a150}. (*A*) Schematic map of pCMV-MIR^{a150} and anti-miR-150 coding sequence and transcript. (*B*) Intracellular anti-miR-150 detection (copy number per cell) in primary B lymphocytes transfected with pCMV-MIR^{a150} harvested 18 h after transfection. (*C*) Secreted anti-miR-150 (copy number per cell) based on detection in culture supernatants harvested 18 h after transfection. Data points refer to single independent experiments.

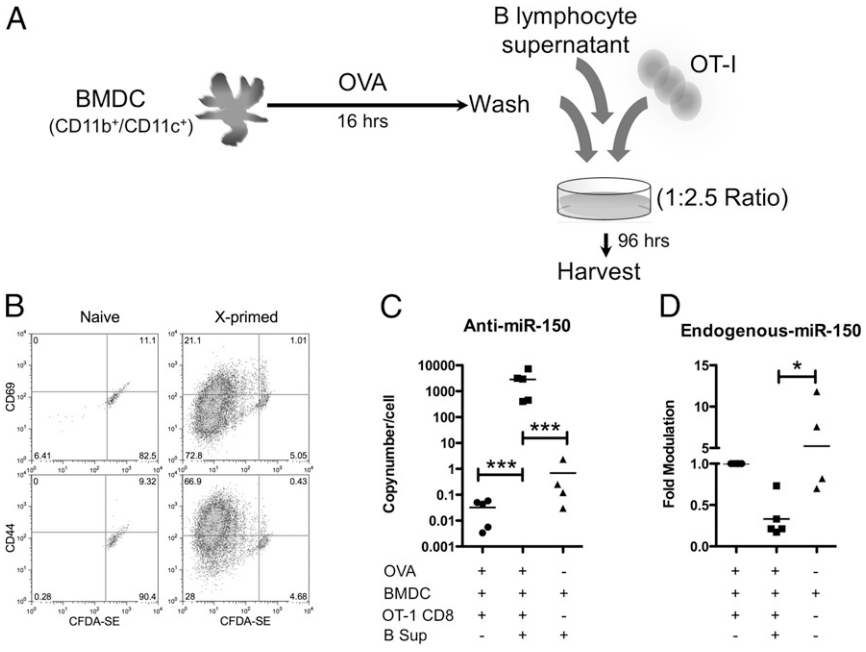

Fig. 2. Anti-miR-150 secreted by transfected primary B lymphocytes are internalized by CD8 T cells during cross-priming in vitro. (*A*) Scheme of experimental design of in vitro T-cell cross-priming. (*B*) Flow cytometry analysis showing that in vitro cross-primed CD8 T cells express CD69 and CD44 activation markers. (*C*) Detection of anti-miR-150 content (copy number per cell) in purified CD8 T lymphocytes cross-primed in the presence of supernatant from primary B lymphocytes transfected with pCMV-MIR^{a150}. CD8 T cells cross-primed without addition of transfected primary B lymphocyte supernatant or CD8 T cells from BMDC-CD8 T-cell cocultures without OVA (BMDC + OT-I CD8 + B Sup) to which the supernatant of primary B lymphocytes transfected with pCMV-MIR^{a150} was added, served as controls. Dots refer to single independent experiments. (*D*) Fold modulation (RQ) of endogenous miR-150 in CD8 T cells of corresponding cultures.

up by CD8 T cells during antigen-specific activation, suggesting local shuttling of anti-miR-150 molecules from B lymphocytes to CD8 T lymphocytes.

In Vitro and in Vivo Down-Modulation of miR-150 in T Cells During Cross-Priming. The expression of miR-150 in mature T lymphocytes is not static and is down-regulated by TCR engagement (29), making it an ideal target to assess regulation by exogenous anti-miR-150. To determine whether anti-miR-150 secreted by B lymphocytes affects miR-150 expression in target T lymphocytes, we measured miR-150 levels in CD8 T cells cross-primed in vitro and in vivo, respectively. We found that the miR-150 expression in CD8 T cells cross-primed in vitro in the presence of B lymphocyte-derived anti-miR-150 supernatant was considerably reduced (~70%) relative to CD8 T cells cross-primed only (Fig. 2*D*). miR-150 levels in CD8 T cells from cocultures in which OVA had been omitted were comparable or even increased relative to baseline controls. In these cells, the endogenous level of an unrelated microRNA (let-7a) was unaffected by anti-miR-150 treatment (Fig. S2). Thus, the uptake of anti-miR-150 by CD8 T cells during in vitro antigen cross-priming resulted in marked and specific decrease of endogenous miR-150 expression. Furthermore, we found that the level of miR-150 in CD8 T cells after cross-priming in vivo was also considerably reduced (~60%) relative to levels in naïve OT-I T cells (Fig. 3*D*) but not in control F5 CD8 T cells, in which we observed an increase. These findings suggest that anti-miR-150 molecules secreted by transfected primary B lymphocytes, once internalized by CD8 T lymphocytes cross-primed in vivo, specifically and reproducibly down-regulate endogenous miR-150.

Anti-miR-150 Is Highly Enriched in B Cell-Derived EVs, Which Are Internalized by T Cells During Cross-Priming in Vitro. EVs have been shown to mediate the intercellular transfer of short, non-coding RNA (12). To verify whether such a mechanism was operative in our model system, we isolated EVs from murine J558L

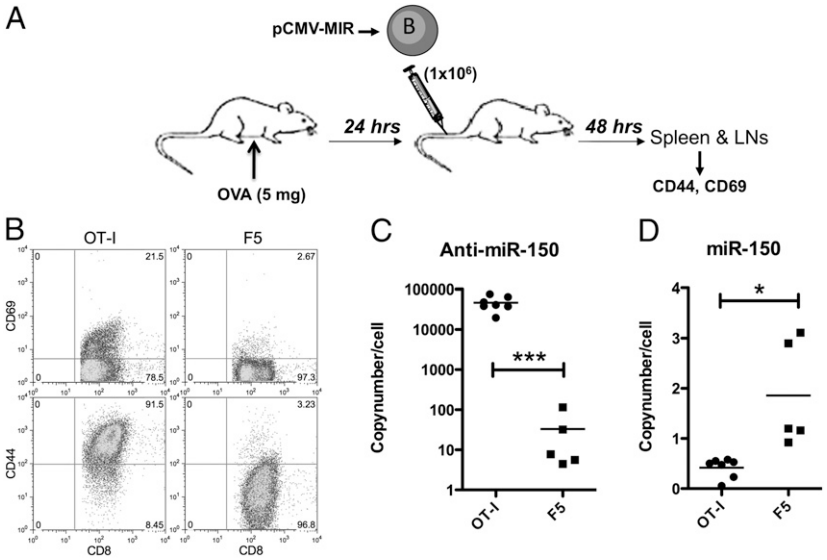

Fig. 3. Transfected B lymphocytes transfer anti-miR-150 to CD8 T cells during cross-priming in vivo. (*A*) Schematic of in vivo cross-priming. OVA (5 mg) was injected i.p. into OT-I and F5 mice. After 24 h mice were injected i.v. with 10^6 syngeneic primary B lymphocytes transfected (<24 h) with pCMV-MIR^{a150}. OT-I and F5 mice were killed 48 h later, and spleen and lymph node CD8$^+$ T cells were isolated and analyzed for expression of cell-surface activation markers by flow cytometry (*B*) as in Fig. 2. (*C*) Content (copy number per cell) of anti-miR-150 molecules, and (*D*) fold modulation of endogenous miR-150 in CD8 T cells purified from OT-I and F5 spleens after cross-priming in vivo. mir150 expression of naïve T lymphocytes from OT-I mice was set to 1.

plasmacytoma cells transfected with pCMV-MIR^{a150}. After 96 h culture in EV-free supernatant 2×10^7 transfected cells were subjected to standard centrifugation and the resulting supernatant ultracentrifuged at 120,000 × *g* (120K) to generate two fractions: an EV-free supernatant and an EV-rich pellet, respectively (Fig. 4*A*). These two fractions were then interrogated by qRT-PCR for anti-miR-150 content. Anti-miR-150 could be amplified from both the 120K EV-free supernatant and the EV-rich pellet (Fig. 4*B*). Thus, anti-miRs produced in B cells are released both as free and EV-associated RNA molecules. Next we sought to determine whether both short RNA species were involved in uptake/internalization by CD8 T cells during cross-priming in vitro. By qRT-PCR we determined that anti-miR-150 could be amplified predominantly in CD8 T cells cross-primed in the presence of EVs (Fig. 4*C*) but not in cells cross-primed without the addition of EVs or in CD8 T cells cocultured with BMDC without OVA (i.e., no cross-priming), as expected from previous experiments. Surprisingly, amplification in CD8 T cells cross-primed in the presence of the 120K EV-free supernatant was very low (Fig. 4*C*). To directly visualize the internalization of EVs in cross-primed CD8 T cells, EVs produced by transfected J558L cells were isolated and labeled with the green fluorescent lipid dye PKH67 and added to OT-I CD8 T cells cocultured with BMDC with or without OVA as illustrated in Fig. 2*A*. By fluorescence microscopy, PKH67-labeled EVs could be visualized inside CD8 T cells during cross-priming but not inside CD8 T cells cocultured with DCs without OVA (i.e., no cross-priming) in the presence of PKH67-labeled EVs (Fig. 4*D*), indicating that PKH67-labeled EVs translocate to CD8 T cells specifically during antigen activation. We estimated that 40% of cross-primed CD8 T cells contain labeled EVs, with ~50 EVs per cell (Table S1). Collectively, these experiments demonstrate that upon transfection, primary B lymphocytes secrete EVs with an anti-miR-150 cargo that are taken up by activated CD8 T cells upon antigen activation.

Discussion

We report that primary B lymphocytes transfected with suitably engineered plasmid DNA efficiently synthesize and secrete anti-miR-150 molecules that can be internalized by CD8 T cells during antigen-mediated activation, in vitro and in vivo. Anti-miR-150 molecules produced in primary B lymphocytes also effectively down-regulate endogenous miR-150 levels in cross-primed CD8 T lymphocytes. Finally, we show that EVs serve as the likely vehicle through which anti-miR-150 molecules are shuttled into receiver CD8 T cells. Collectively, our results disclose a unique application for B lymphocytes: the synthesis and secretion of functional short, noncoding RNAs, including their release in the form of EVs.

MicroRNAs are powerful regulators of biological processes through translational repression and/or mRNA degradation, mechanisms different from the canonical role of mRNA. Through these mechanisms of action, microRNAs regulate immunity, inflammation, and cancer. For instance, the fate of memory CD8 T cells is likely regulated by a discrete number of selected microRNAs, including miR-150, whose manipulation may enable one to direct CD8 T-cell fate predictably (19). The data presented here suggest that B lymphocyte-derived anti-miR-150 molecules are internalized in CD8 T cells when these cells are activated by antigen-presenting cells. This implies that the use of B lymphocytes as synthesis and delivery vehicles of short, noncoding RNAs to regulate adaptive T-cell immunity is not only possible but also endowed with an intrinsic fail-safe mechanism that limits the effect to antigen-activated CD8 T cells. Our data point to the fact that T cells are permissive to internalization of regulatory RNA only during an antigen-driven immune response [i.e., antigen

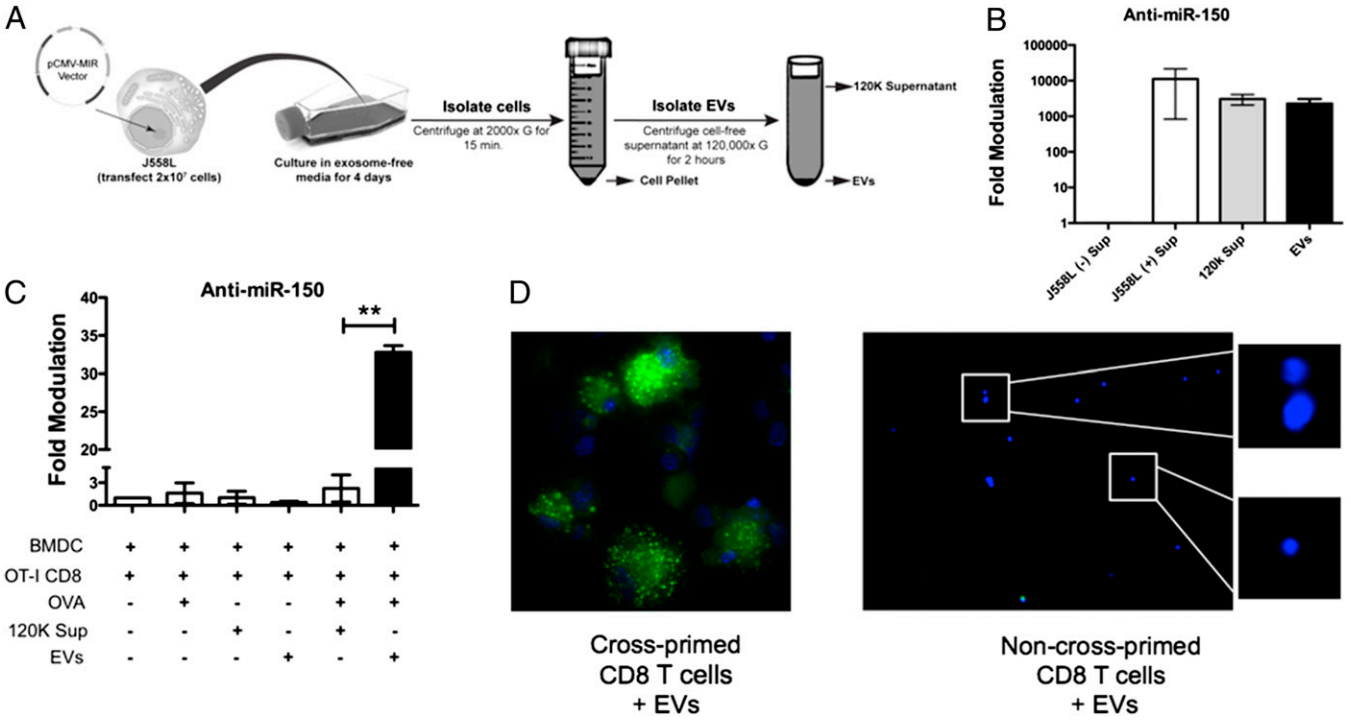

Fig. 4. Isolation of anti-miR-150 activity in EVs and EV visualization inside CD8 T cells after cross-priming in vitro. (*A*) Schematic of in vitro production and isolation of EVs from J558L cells transfected with pCMV-MIR^{a150}. (*B*) Detection of anti-miR-150 in the 120K EV-free supernatant and in the EV-rich pellet of J558L cells after short-term (96 h) transfection with pCMV-MIR^{a150}. Results are expressed as fold modulation and refer to the results of two independent experiments (mean ± SEM). (*C*) Fold modulation of anti-miR-150 inside in vitro cross-primed CD8 T cells, and appropriate controls, with or without addition of the 120K EV-free supernatant or EV-rich ultracentrifugation pellet. Results refer to two independent experiments (mean ± SEM). (*D*) Fluorescence microscopy analysis of CD8 T cells cross-primed in vitro in the presence of PKH67-labeled EVs (*Left*) or cocultured with DCs without OVA (no cross-priming) but in the presence of PKH67-labeled EVs (*Right*). (Magnification: *Left*, 20×; *Right*, 10×.)

presentation and activation by antigen-presenting cells (DCs)]. As such, the system described herein may be used as an adjuvant strategy to determine the fate of T cells during vaccination (19), restrict the development of FoxP3$^+$ T cells (30), and modulate inflammation (31). In general terms, a similar approach could be used in inflammatory conditions and autoimmune diseases (6).

Over the past decade, microRNA "signatures" have been increasingly associated with various types of cancer, different stages of tumorigenesis, and cancer prognosis (32–34). Overall, the role of microRNAs in cancer stem from either (*i*) the overexpression of oncogenic microRNAs ("oncomirs") caused by genomic deletion, mutation, epigenetic silencing, and/or microRNA processing alterations, or (*ii*) the loss of suppressor microRNAs (reviewed in ref. 35). As an initial proof of principle, we found that murine cancer cells treated in vitro with a primary B lymphocyte supernatant containing anti-miR-150 molecules markedly down-regulate the levels of endogenous miR-150 (Fig. S3). Additionally, although the mechanism of microRNA transmission to cancer cells needs to be further investigated, our findings suggest that primary B lymphocytes programmed for the synthesis and secretion of short, noncoding RNAs may be used to target cancer cells to either (*i*) suppress oncomirs (36), or (*ii*) restore microRNAs that suppress oncogenes or metastases (37, 38). Similarly, the approach may be used to modulate the tumor microenvironment by targeting microRNAs that drive mutator activity (39) or promote the metastatic potential of cancer cells (40).

EVs have been implicated in the transfer of microRNAs and mRNAs as a novel mechanism of genetic exchange between cells (41). Many cell types can form and secrete EVs: B lymphocytes in particular have been the object of two reports (42, 43). However, in one case the microRNA content of the EVs was not interrogated, and in the other, B cells were infected with Epstein Barr virus. At variance, here we show that primary B lymphocytes can be programmed with plasmid DNA to form and secrete EVs containing a cargo of anti-miR-150 molecules, and that these EVs apparently enable and mediate internalization by CD8 T cells (Fig. 4 *C* and *D*). Transmission by EVs likely protects the cargo (e.g., microRNA) from RNase degradation, ensuring stability and providing for a mechanism to cross the hydrophobic cell membrane. Whereas the exact mechanism of EV internalization by activated CD8 T cells remains to be clarified, EV-encapsulation of microRNA by B lymphocytes may offer advantages over soluble microRNA molecules that are also released by B lymphocytes.

In conclusion, we show that primary B lymphocytes can be programmed for the synthesis and secretion of short, noncoding RNA molecules, opening unique possibilities for microRNA-based therapy. Because autologous B lymphocytes transfected with plasmid DNA have already been used in humans in the context of therapeutic vaccination (44), the type of "immunogenomic therapy" exemplified here could undergo rapid clinical translation.

Materials and Methods

Mice. C57BL/6 mice were originally purchased from the Jackson Laboratories. TCR transgenic OT-I mice (C57BL/6; Thy1.2$^+$) that are specific for the SIINFEKL OVA peptide (26) were obtained from Stephen Hedrick [University of California, San Diego (UCSD), La Jolla, CA]. TCR transgenic RAG$^{-/-}$ F5 mice are specific for the ASNENMDAM peptide of the NP antigen of the influenza A virus (27) and were obtained from the National Institutes of Health (Bethesda) courtesy of Jonathan Yewdell. All mice were maintained in the animal facility of the UCSD Moores Cancer Center. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was performed according to a protocol approved by the Institutional Animal Subject Committee (UCSD no. S00023).

Plasmid DNA, MicroRNA, Oligonucleotides, and Antigens. Plasmid DNA expressing anti-miR-150 (pCMV-MIR^{a150}) is a 6.2-kb vector (Origene) into which an 82-bp insert, containing the coding sequence for the 22 bp corresponding to anti-miR-150 (Fig.1*A*), was cloned using Sgf I and Mlu I restriction sites. The expression of the anti-microRNA precursor is driven by a CMV promoter (Fig. 1*A*). Synthetic anti-miR-150 and snoRNA202 were purchased from Integrated DNA Technology. Gene-specific primers for mmu-miR-150, and anti-miR150, let-7a, and snoRNA202 (endogenous control) were purchased from Applied Biosystems. Ovalbumin (OVA) was purchased from Sigma (grade II; lot no. 20H0763).

Transfection Procedures. Primary B lymphocytes were isolated by negative selection (StemCell Tech) from the spleen of C57BL/6 or F5 mice and were transfected with plasmid pCMV-MIR^{a150} using the Amaxa Cell Line Nucleofector Kit (Lonza). Briefly, 5×10^6 cells were transfected with 2 μg of plasmid DNA in the buffer solution provided by the manufacturer. After transfection, the cells were resuspended in 2 mL of complete RPMI medium, plated on a six-well tissue culture plate, and incubated a 37 °C in a 5% CO_2 atmosphere. Untransfected B lymphocytes were plated and used as a negative control. Transfected and negative control cells and their supernatants were harvested at the end of 18-h culture, unless otherwise specified.

qRT-PCR. MicroRNA was extracted from the cells using either the RNAGEM Tissue PLUS (Zygem) or the mirVana PARIS Kit (Life Technologies). MicroRNAs in supernatant samples were isolated using either the mirVANA PARIS Kit (Life Technologies) or the miRNeasy Serum/Plasma Kit (Qiagen). cDNA was synthesized from the purified microRNA using the High Capacity cDNA Synthesis kit (Life Technologies) with snoRNA202, miR-150, let-7a, or anti-miR150 primers (ABI). qRT-PCR was performed on an ABI StepOne system using TaqMan reagents for 50 cycles using validated FAM-labeled mouse snoRNA202, miR-150, anti-miR150, and let-7a TaqMan primer/probe sets (Life Technologies) under universal cycling conditions. Target gene expression was normalized to snoRNA202 and analyzed using the -ΔΔCt relative quantification method.

Relative Quantification and Copy Number Determination. To determine the copy number of anti-miR-150, samples normalized at 100 ng cDNA per reaction were run concomitantly with a standard curve constructed with known amounts (100–0.01 ng) of anti-miR-150 cDNA. The endogenous control standard curve was constructed using known amounts (100–0.01 ng) of snoRNA202 cDNA. Let-7a total cDNA was similarly extracted, quantified, and adjusted to 100 ng/μL. cDNA was generated with Applied Biosystems let-7a (002478) and snoRNA202 (001232) specific RT primers. Samples were run in duplicate with anti-miR150 and snoRNA202 FAM-labeled probe/primer sets. Relative expression was determined by comparing untreated to experimental samples. In all instances, the Ct value of the endogenous control was subtracted from the Ct value of target. Once the amount (ng) of specific target was determined, the copy number present in each reaction was calculated using the following formula: (ng × 6.0223 × 10^{23})/(number of nucleotides × 1.0 × 10^9 × 650) as indicated at http://www.uic.edu/depts/rrc/cgf/realtime/stdcurve.html.

BMDC Generation and CD8 T-Cell Cross-Priming in Vitro. The preparation of BMDC and the isolation of CD8 T cells by negative selection (StemCell Tech) are described in ref. 19. As indicated, BMDC were supplemented or not with heat-treated (63 °C × 25 min) OVA (1 mg/mL) for 16 h before adding naïve CD8$^+$ T cells isolated from spleen and lymph node cells of OT-I mice. The yield and purity of transgenic OT-I CD8 T cells was determined by Vα2/CD8 positivity by flow cytometry and was >90%. Vα2$^+$/CD8$^+$ T cells (2.5×10^5) were then cocultured with 10^5 BMDC in complete RPMI medium or in complete RPMI medium containing 50% vol/vol supernatant from pCMV-MIR^{a150} transfected primary B lymphocytes for 96 h. T cells were recovered from 96-h cocultures using Lympholyte M (Cedar Lane) and analyzed by flow cytometry and qPCR.

In Vivo Studies. Six- to fourteen-week-old OT-I or F5 mice were injected i.p. with 5 mg heat-treated (63 °C × 25 min) OVA according to ref. 28. Twenty-four hours later mice were injected i.v. with 10^6 primary C57BL/6 B lymphocytes that had been negatively selected from a spleen cell suspension and transfected with plasmid pCMV-MIR^{a150}. B lymphocytes were used within 1 h from transfection. Mice were killed after 48 h (i.e., 3 d after OVA injection), and CD8$^+$ T cells were negatively selected from spleen and lymph nodes and analyzed as indicated in the text.

Flow Cytometry. Single-cell suspensions of CD8+ T cells were stained with fluorophore-conjugated anti-CD8α (eBioscience, clone Ly-2), anti-CD69 (BD Biosciences, clone H12F3), anti-CD44 (BD Biosciences, clone IM7), and anti-Vα2 (BD Biosciences, clone B20.1) antibodies, or appropriate isotype controls. Viability was determined by 7-AAD exclusion. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using CellQuest Pro (BD Biosciences) and FlowJo software (Tree Star).

EVs Isolation. For the purpose of isolating EVs, transfection experiments were performed in J558L mouse plasmacytoma cells (45). Briefly, 5×10^6 J558L cells were transfected using the Lonza Amaxa Cell Line Nucleofector Kit V. Cells were transfected with plasmid pCMV-MIR^{a150} (2 μg). After transfection, cells were placed in fresh EV-depleted medium prepared by ultracentrifugation of RPMI supplemented with 20% FBS at 120,000 × *g* for 18 h at 4 °C. The medium was then diluted to a final concentration of 10% FBS before use. Transfected J558L cells were cultured in EV-free RPMI at 37 °C for 96 h, after which the EV fraction was isolated by differential centrifugation. Briefly, conditioned media were first centrifuged at 2000 × g for 20 min to remove cellular debris. The supernatant was collected and further centrifuged at 10,000 × *g* for 30 min. The resultant supernatant was then transferred to ultracentrifuge tubes for ultracentrifugation at 120,000 × *g* for 2 h. The supernatant was discarded, and the EV pellets were resuspended in PBS for storage at −80 °C before RNA isolation. All centrifugation steps were performed at 4 °C.

Fluorescence Microscopy Study. To visualize the uptake of vesicles by cross-primed OT-I CD8 T cells (Fig. 4), EVs were labeled with the fluorescent dye PKH67 (Sigma) according to the manufacturer's protocol. Briefly, 4 μL of PKH67 was added to 1 mL of Diluent C and mixed thoroughly before the dye solution was combined with EVs that had been resuspended in 1 mL of Diluent C. After gently mixing for 5 min, 2 mL of 1% BSA was added to bind the excess dye. Labeled EVs were pelleted and washed with PBS by ultracentrifugation at 120,000 × *g* for 2 h at 4 °C. Freshly prepared PKH67-labeled EVs were added to cocultures of BMDC and OT-I CD8 T cells on day 1 using 50 μL of EVs in 1 mL of standard culture medium. Cocultures grown in 1 mL of the 120,000 × *g* EV-free spin supernatant served as controls. In both instances, CD8 T cells were harvested on day 4 as detailed above and centrifuged onto a glass slide using a CytoSpin 2 centrifuge (Shandon) and mounted using ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were analyzed on a BZ-9000 Bioevo fluorescence microscope (Keyence Corporation of America).

Statistical Methods. Unpaired, two-tailed *t* test was used to analyze results in Fig. 3*D*, Fig. 4*C*, and Fig. S2. Fig. 2*D* was analyzed using nonparametric, Mann-Whitney test. Data in Figs. 2*C* and 3*C* were log-transformed before unpaired, two-tailed *t* test. Significance is reported as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

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