Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity

Krzysztof Bryniarski, PhD,[a] Wlodzimierz Ptak, MD,[a] Asha Jayakumar, PhD,[b] Kerstin Püllmann, MD,[c] Michael J. Caplan, MD, PhD,[d] Arthit Chairoungdua, PhD,[d] Jun Lu, MD, PhD,[e] Brian D. Adams, PhD,[e] Emilia Sikora, PhD,[a] Katarzyna Nazimek, MS,[a] Susanna Marquez, MS,[f] Steven H. Kleinstein, PhD,[f,g] Panjamaporn Sangwung, BS,[h] Yasuko Iwakiri, PhD,[h] Eric Delgato, BS,[b] Frank Redegeld, PhD,[i] Bart R. Blokhuis, MS,[i] Jacek Wojcikowski, PhD,[j] Anna Wladyslawa Daniel, PhD,[j] Tom Groot Kormelink, PhD,[b] and Philip W. Askenase, MD[b] *Krakow, Poland, New Haven, Conn, Hannover, Germany, and Utrecht, The Netherlands*

Background: T-cell tolerance of allergic cutaneous contact sensitivity (CS) induced in mice by high doses of reactive hapten is mediated by suppressor cells that release antigen-specific suppressive nanovesicles.

Objective: We sought to determine the mechanism or mechanisms of immune suppression mediated by the nanovesicles.

Methods: T-cell tolerance was induced by means of intravenous injection of hapten conjugated to self-antigens of syngeneic erythrocytes and subsequent contact immunization with the same hapten. Lymph node and spleen cells from tolerized or control donors were harvested and cultured to produce a supernatant containing suppressive nanovesicles that were isolated from the tolerized mice for testing in active and adoptive cell-transfer models of CS.

Results: Tolerance was shown due to exosome-like nanovesicles in the supernatants of $CD8^+$ suppressor T cells that were not regulatory T cells. Antigen specificity of the suppressive nanovesicles was conferred by a surface coat of antibody light chains or possibly whole antibody, allowing targeted delivery of selected inhibitory microRNA (miRNA)–150 to CS effector T cells. Nanovesicles also inhibited CS in actively sensitized mice after systemic injection at the peak of the responses. The role of antibody and miRNA-150 was established by tolerizing either panimmunoglobulin-deficient $JH^{-/-}$ or miRNA-$150^{-/-}$ mice that produced nonsuppressive nanovesicles. These nanovesicles could be made suppressive by adding antigen-specific antibody light chains or miRNA-150, respectively.

Conclusions: This is the first example of T-cell regulation through systemic transit of exosome-like nanovesicles delivering a chosen inhibitory miRNA to target effector T cells in an antigen-specific manner by a surface coating of antibody light chains. (J Allergy Clin Immunol 2013;132:170-81.)

Key words: *Exosomes, exosome-like nanovesicles, nanovesicles, T-cell suppression, miRNA, miRNA-150, antibody light chains, allergic cutaneous contact dermatitis, contact sensitivity*

Exosomes are nanovesicles generated intracellularly by budding from the multivesicular bodies of the terminal endosomal pathway, where they accumulate and are released from the cell during exocytosis of the multivesicular bodies.[1,2] Exosomes or related vesicles are produced by all cell types in virtually all species and have been found in all fluids studied. Their outstanding property is that they contain a cargo of donor cell proteins, mRNAs, and microRNAs (miRNAs) that are delivered extracellularly to acceptor cells, where they can function.[3-6] Thus the vesicular transport of proteins can drive or inhibit signaling pathways.[5-7] mRNA can translate donor cell proteins,[3,4] and delivered miRNA can bind acceptor cell mRNA to regulate protein translation.[3,4,8,9]

Contact sensitivity (CS) in mice is a major model of the clinical allergic skin diseases contact dermatitis and atopic dermatitis. Additionally, CS is a model of delayed-type hypersensitivity mechanisms that participate in other T cell–mediated processes, such as in T-cell aspects of autoimmunity, transplantation, infection resistance, and cancer. Furthermore, the effector phase of CS has been shown recently to have unanticipated complexity. The new findings established that sensitization involves Toll-like receptors[10]; initiation of elicitation involves B-1 B cells, invariant natural killer (NK) T cells, IL-4, mast cells, platelets, endothelial cells, and complement[11]; and responses can be mediated by CD4, CD8,[12] or T_H17[13] T cells and even NK cells.[14] Finally, there is now recognition of regulation of CS by either regulatory T

From [a]the Department of Immunology, Jagiellonian University College of Medicine, Krakow; [b]the Section of Allergy and Clinical Immunology, Department of Medicine, [c]the Department of Genetics, and [f]the Department of Pathology, Yale University School of Medicine, New Haven; [c]the Department of Hematology, University of Hannover; [d]the Department of Cellular and Molecular Physiology, [g]the Interdepartmental Program in Computational Biology and Bioinformatics, and [h]the Section of Gastroenterology, Department of Medicine, Yale University, New Haven; [i]the Division of Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University; and [j]the Institute of Pharmacology, Polish Academy of Sciences, Krakow.

K.B. was supported by grants from the Polish Ministry of Science and Higher Education: N401 092 31/2176 and K/ZDS/003718. T.G.K. was supported by a grant from Lung Foundation Netherlands; 3.2.11.09FE. P.W.A. was supported by grants AI-076366, AI-07174, and AI-1053786 from the National Institutes of Health.

Disclosure of potential conflict of interest: K. Bryniarski has received research support from the Polish Ministry of Science and Higher Education. A. Jayakumar, M. J. Caplan, A. Chairoungdua, S. Marquez, S. H. Kleinstein, and P. W. Askenase have received research support from the National Institutes of Health (NIH). K. Püllmann has received research support from the Hannover Medical School. Y. Iwakiri has received research support from the NIH/National Institute of Diabetes and Digestive and Kidney Diseases. T. Groot Kormelink has received research support from the Dutch Asthma Foundation. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication December 27, 2012; revised April 9, 2013; accepted for publication April 22, 2013.

Available online May 31, 2013.

Corresponding author: Philip W. Askenase, MD, Section of Allergy and Clinical Immunology, Department of Medicine, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06520. E-mail: philip.askenase@yale.edu.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2013.04.048

Abbreviations used

Ab LC: Free antibody light chain
CS: Contact sensitivity
DPBS: Dulbecco PBS
Foxp3: Forkhead box protein 3
FT: Flow through
hrIL-2: Human recombinant IL-2
miRNA: MicroRNA
NK: Natural killer
Nl Cell Sup: Supernatant from culture of lymph and spleen cells from normal (nonimmunized) mice
NTA: Nanoparticle tracking analysis
OX: Oxazolone
TNP: Trinitrophenyl
Treg: Regulatory T
Ts: Suppressor T cells from antigen-tolerized mice
Ts Sup: Supernatant from culture of lymph and spleen suppressor T cells from tolerized mice

(Treg) cells[15] or myeloid suppressor cells.[16] The present study presents evidence of yet another regulatory pathway involving suppressor T cells producing antigen-specific exosome-like nanovesicles that deliver inhibitory miRNA.

Such exosomal transport of functional miRNAs passing genetic information between donor and acceptor cells has been confirmed in diverse instances[3,4,8,9] and has provided insight into new levels of regulation between cells in the immune system.[10-12] Exosome targeting usually is paracrine,[5,6,17-23] but there also is endocrine transport of exosome-like nanovesicles through the bloodstream, enabling regulation of distant acceptor cell function,[24-26] as seen here. The current study presents new evidence of a cell-to-cell suppressive pathway involving $CD8^+$ suppressor T cell–derived exosome-like nanovesicles that antigen-specifically target the effector T-cell mixture of CS by delivering inhibitory miRNAs. Selection of the particular antigen specificity and the inhibitory miRNA shown here opens up significant translational possibilities for the treatment of a variety of human diseases.

METHODS

Description of the materials and methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

High-dose antigen tolerance induces suppressor T cells that produce inhibitory supernatant

We found that high trinitrophenyl (TNP) antigen dose tolerance induced suppressor T cells from antigen-tolerized mice (Ts) and that supernatant from culture of lymph and spleen suppressor T cells from tolerized mice (Ts Sup) contained all their suppressive activity for CS effector cells (Fig 1, *A*, group C vs groups B and D). We suspected that vesicles in the Ts Sup might be responsible for the suppression. Therefore putative vesicles were enriched by means of progressive ultrafiltration and differential centrifugation, culminating in pelleting by means of two 100,000*g* ultracentrifugations.[1,2] The final pellet contained 130-nm nanovesicles resembling exosomes, as determined by means of electron microscopy (Fig 1, *B*, right) and nanoparticle tracking analysis (NTA; Fig 1, *C*).[27] Like exosomes, these nanovesicles expressed tetraspanins, such as CD9, by means of immunoblotting (Fig 1, *D*) and CD3 and T-cell receptor β by means of flow cytometry (data not shown), confirming their T-cell origin.

The final 100,000*g* pellet from oxazolone (OX)–Ts-Sup from mice tolerized with OX-labeled mouse red blood cells compared with the supernatant above the pellet contained the Ts Sup ability to suppress adoptive transfer of OX CS effector T cells (Fig 2, *A*, group D vs group C). Identical results were obtained in the TNP CS system (data not shown). Furthermore, a dose-response experiment in the TNP CS system was done to test the potency and validity of the suppressive nanovesicles and showed a decreasing suppression of adoptive CS by the resuspended serially diluted Ts Sup pellet nanovesicles (see Fig E1, groups D, E, and F, in this article's Online Repository at www.jacionline.org), whereas those from the supernatant from culture of lymph and spleen cells from normal (nonimmunized) mice (Nl Cell Sup) pellet at the high dose were not suppressive (group C). Finally, resuspension of the 100,000*g* pellet and repeated ultracentrifugation on a sucrose gradient resulted in buoyant fractions. Only the fraction that showed buoyancy identical to that of exosomes[1,2] suppressed adoptive transfer of CS (Fig 2, *B*, group E), like the starting TNP–Ts Sup nanovesicles (Fig 2, *B*, group D). Considering all the above characteristics, we henceforth called these suppressive $CD8^+$ T cell–derived vesicles exosome-like nanovesicles.

An *in vitro* non–antigen-specific assay confirms the *in vivo* suppressive function of the Ts Sup–derived nanovesicles

To further confirm the above, a non–antigen-specific *in vitro* assay was used to test inhibition of the HT-2 T-cell line responsiveness to IL-2 by the exosome-like nanovesicles. The end point was the lowest number of serially diluted nanovesicles that resulted in at least 50% HT-2 cell viability. This assay confirmed the suppressive activity of the Ts Sup exosome-like nanovesicles (Fig 3, *C*, group B).

Another *in vitro* but antigen-specific assay confirmed suppressive activity of Ts Sup exosome-like nanovesicles

Here immunobead-isolated $CD4^+$ CS effector T cells responded *in vitro* to TNP-linked dendritic cells by producing IFN-γ. Shown are 4 separate experiments confirming that the 100,000*g* pellet–derived exosome-like nanovesicles from Ts Sup suppressed IFN-γ production, whereas similar Nl Cell Sup nanovesicles did not (see Fig E2 in this article's Online Repository at www.jacionline.org).

Suppressive exosome-like nanovesicles are derived from $CD8^+$ T cells, are present in the plasma of Ts donors, and are not derived from Treg cells

Depletion of $CD8^+$ cells from the Ts Sup cell culture with anti-CD8 mAb plus complement (Fig 3, *A*, group C) or with anti-CD8–conjugated versus anti-CD4–conjugated beads (data not shown) removed the ability to generate suppressive supernatant. Furthermore, blood plasma from the high-dose antigen–tolerized

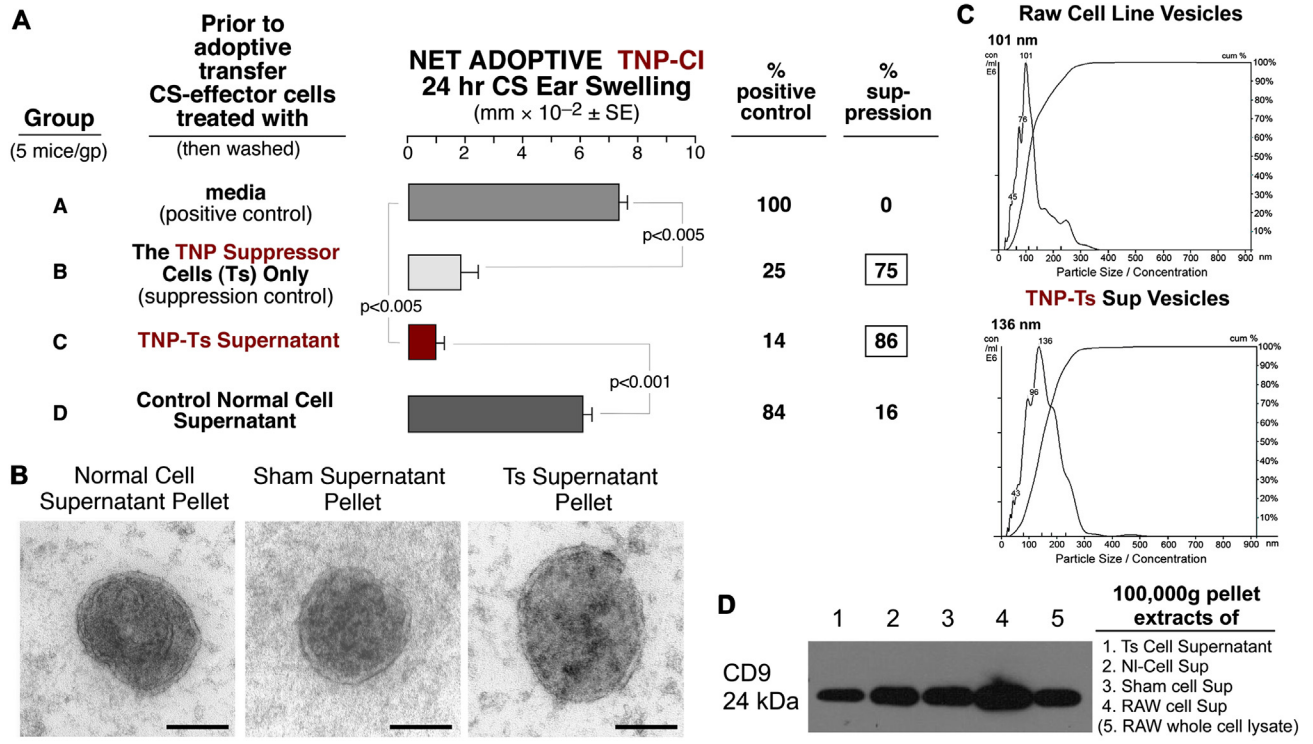


FIG 1. High-dose antigen tolerization of the CS immune response induces Ts cells producing Ts Sup containing exosome-like nanovesicles. **A,** Ts cells *(group B)* or their culture supernatant (Ts Sup, *group C*) suppressed adoptive transfer of CS. **B,** Electron microscopy revealed that NI Sup, sham supernatant, and TNP–Ts Sup pellets contained nanovesicles resembling exosomes at 80,000 times magnification. *Bar* = 65 nm. **C,** NTA showed homogenously sized nanovesicles from control RAW cell line supernatant and from TNP–Ts Sup pellets, indicating particle size/concentration. **D,** Western immunoblotting showed CD9 tetraspanin expression by extracts of pellets from TNP–Ts Sup, NI Cell Sup, sham supernatant *(Sham cell Sup)*, and RAW cell line Sup compared with control RAW cell lysate.

donors of Ts cells processed for exosomes to the 100,000*g* pellet also contained suppressive nanovesicles (Fig 3, *B*, group E), whereas similar NI Cell and sham plasma-derived nanovesicles had none (Fig 3, *B*, groups F and G). In support of these findings, the *in vitro*, IL-2–dependent, HT-2 cell, non–antigen-specific assay showed strong suppressive activity of plasma exosome-like nanovesicles from tolerized mice versus normal mice (Fig 3, *C*, group D vs group C). Finally, we tested whether Treg cells were involved using DEREG mice.[28] High-dose antigen tolerance resulted in exosome-like nanovesicles that had equivalent suppressive ability when derived from the Treg cell–depleted mice compared with wild-type mice (Fig 3, *D*).

Suppressor T-cell exosome-like nanovesicles inhibit active cutaneous CS responses *in vivo*

We tested whether the nanovesicles could act *in vivo* when directly injected into actively sensitized mice that were already expressing a CS response. Nanovesicles were administered intraperitoneally at the 24-hour peak response (Fig 4, open circles). Then the subsequent time course of ear swelling was compared with that of actively sensitized untreated and ear-challenged mice (Fig 4, squares) and with that of recipients of control vesicles from sham-tolerized mice (Fig 4, triangles). Ts Sup exosome-like nanovesicles strongly suppressed subsequent ear swelling at 48 and 72 hours by 53% and 60%, respectively (Fig 4), whereas sham supernatant nanovesicles did not. Furthermore, similar *in vivo* treatment with the nanovesicles showed that suppression could last up to 120 hours after a single injection (see Fig E3, *A*, triangles, in this article's Online Repository at www.jacionline.org), and significant inhibition even occurred when nanovesicles were administered orally (see Fig E3, *B*, group D).

Suppression by exosome-like nanovesicles is antigen specific through a dual reciprocal antigen specificity test

Preliminary results suggested functional antigen specificity of the suppressive nanovesicles. This was confirmed by using a dual reciprocal antigen criss-cross experiment that demonstrated that nanovesicles from TNP-tolerized mice only suppressed TNP CS effector cells (Fig 5, *A*, group B) and not CS responses to OX, another hapten antigen (Fig 5, *A*, group E). Similarly, exosome-like nanovesicles from OX hapten–tolerized mice suppressed OX CS effector cells (Fig 5, *A*, group F) but not TNP CS effector T cells (Fig 5, *A*, group C). To possibly account for antigen specificity, flow cytometry showed antibody kappa light chains (Ab kappa LC) on the surfaces of nanovesicles from tolerized mice (Fig 5, *B*, right, red peak vs gray isotype control) compared with the control macrophage cell line (Fig 5, *B*, left, blue peak vs gray isotype

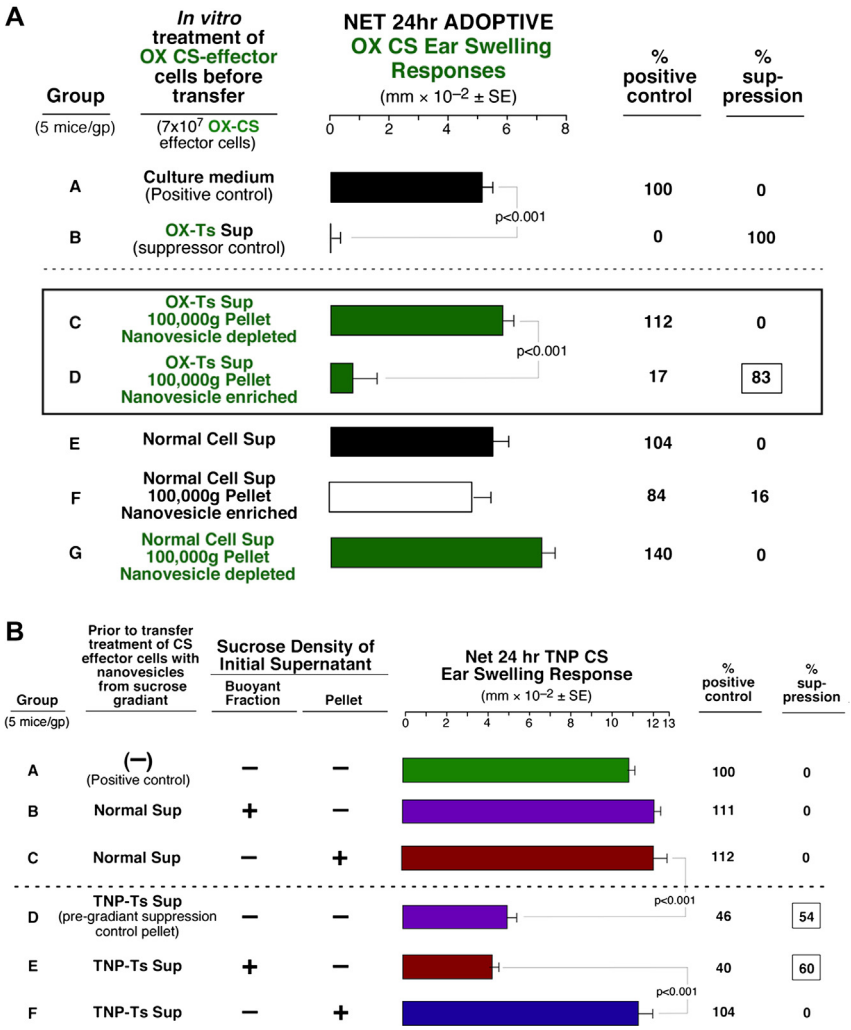


FIG 2. Ts Sup function is entirely in the 100,000*g* pellet and in the buoyant fraction of a discontinuous sucrose gradient. **A,** The OX–Ts Sup pellet was suppressive *(group D)*, whereas OX–Ts Sup depleted of nanovesicles *(group C)*, starting NI Sup *(group E)*, NI Sup pellet *(group F)*, and NI Sup depleted of nanovesicles *(group G)* were nonsuppressive. **B,** Vesicles from the TNP–Ts Sup 1.86/1.08 buoyant fraction *(group E)* and vesicles from the original Ts Sup pellet *(group D)* were suppressive, whereas the pellet depleted of buoyant material *(group F)* and NI Cell Sup fractions (*groups B* and *C*) were not inhibitory.

control). This suggested that antigen-specific antibody or free antibody light chain (Ab LC) on the nanovesicle surface could provide a mechanism for antigen specificity.

Tolerization of panimmunoglobulin-deficient JH$^{-/-}$ mice confirms that antigen specificity was due to antibody on the surface of the exosome-like nanovesicles

We found that nanovesicles from tolerized panimmunoglobulin-deficient JH$^{-/-}$ mice[29] were nonsuppressive (Fig 5, *C*, group C). Furthermore, after the first 100,000*g* pelleting, we incubated the exosome-like nanovesicles with monoclonal anti-TNP Ab LC[30] *in vitro* for 30 minutes at 37°C and then washed away free Ab LC by means of a second ultracentrifugation step. Very importantly, these likely Ab LC–sensitized nanovesicles were suppressive (Fig 5, *C*, group D, and see Fig E4, group C, in this article's Online Repository at www.jacionline.org), whereas comparable antibody heavy chain–exposed vesicles were not (see Fig E4, groups D and E).

Lack of inhibition of cell transfer by nanovesicles from the tolerized JH$^{-/-}$ donors[29] might have been due to a lack of surface antibody on intrinsically suppressive exosome-like nanovesicles. Thus we tested the nanovesicles in the non–antigen-specific assay for inhibition of HT-2 T-cell line responsiveness to IL-2. Interestingly, despite their inability to suppress CS effector cell adoptive transfer *in vivo*, JH$^{-/-}$ Ts Sup exosome-like nanovesicles were suppressive in this non–antigen-specific assay, as were the wild-type TNP Ts Sup nanovesicles (Fig 5, *D*, groups B and C vs group A).

Antigen affinity chromatography isolates a minor subpopulation that has all the suppressive activity

We considered that if the exosomes had antigen-specific Ab LC or antibody on their surface, then they might be antigen

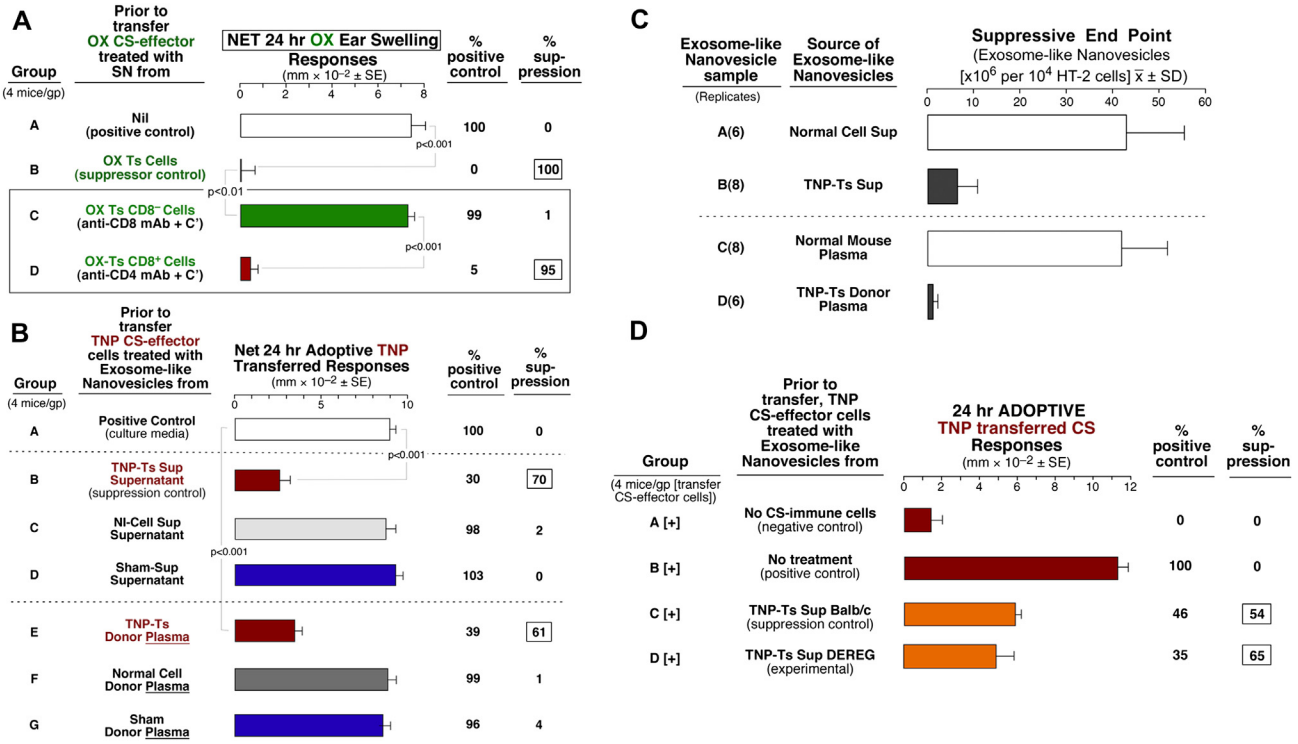


FIG 3. Determination that the Ts Sup suppressive exosome-like nanovesicles are derived from $CD8^+$ cells, are present in plasma of Ts cell donors, and are not produced by Treg cells. **A,** Treatment of Ts cells from tolerized mice with anti-CD8 mAb plus complement *(C')* before culture to derive Ts Sup eliminated suppression of adoptively transferred CS *(group C)*. Similar anti-CD4 mAb treatment of the OX-Ts had no effect *(group D)*. **B,** Tolerized Ts donor plasma nanovesicles were suppressive *(group E)*, whereas nanovesicles from other sources (*groups C, D, F,* and *G*) were noninhibitory. **C,** Only TNP–Ts Sup and Ts cell donor plasma exosome-like nanovesicles inhibited the *in vitro* HT-2 cell response to IL-2 (*groups B* and *D*). **D,** DEREG mice depleted of Treg cells and wild-type mice were tolerized with a high antigen dose and showed similar suppressive ability (*groups C* and *D*).

binding. Thus we attempted antigen affinity chromatography of the suppressive nanovesicles (Fig 6, *A*) and recovered an antigen-binding subfraction (12% of the total) with all the suppressive activity in the TNP CS model (Fig 6, *B*, group D). Furthermore, Fig 6, *C*, shows that there was suppression of the HT-2 T-cell response to IL-2 by using the antigen-binding nanovesicles eluted from the column (Fig 6, *C*, group C), whereas the flow through (FT) and column wash fractions were nonsuppressive (Fig 6, *C*, groups A and B), confirming the findings from the CS model.

Cloning, sequencing, and bioinformatic comparison of reads from the exosome-like nanovesicle populations separated by the TNP antigen affinity column

Comparison and ranking of the frequency of sequences between the 2 nanovesicle fractions from the TNP column (ie, the antigen-binding and antigen-suppressive vs the nonbinding and nonsuppressive) were performed (see Table E1 in this article's Online Repository at www.jacionline.org). This suggested that miRNA-150 (line 7), which was previously associated with T-cell regulation,[31-37] might be a candidate for mediating suppression by the T cell–derived exosome-like nanovesicles. In contrast, among the sequences more frequent in the opposite FT versus the eluate sequences was miRNA-155 (see Table E2, line 12, in this article's Online Repository at www.jacionline.org), which therefore was depleted from the Ts cell nanovesicles. This miRNA is strongly associated with Treg cells and guides expression of forkhead box protein 3 (Foxp3).[38] These data support our findings that Treg cells were not involved in the $CD8^+$ T-cell suppression described here.

Anti-miRs confirm the potential involvement of miRNA-150 in the suppression by high-dose antigen tolerization

Fig 7, *A*, group C, shows that anti-miRs antagonistic to miR-150 reversed suppression mediated by the exosome-like nanovesicles in adoptive transfer of CS. In contrast, a set of 5 anti-miR controls aimed at the other prominent miRNAs more frequent in the column eluate compared with FT vesicles (see Table E1) or mimic controls did not reverse suppression. Similarly, in addition to these *in vivo* data, the miR-150 antagonist reversed Ts Sup nanovesicle suppression of the HT-2 cell response to IL-2, again compared with these controls (Fig 7, *B*, group B), suggesting that miR-150 also was involved in the *in vitro* inhibition of HT-2 cell responses to IL-2.

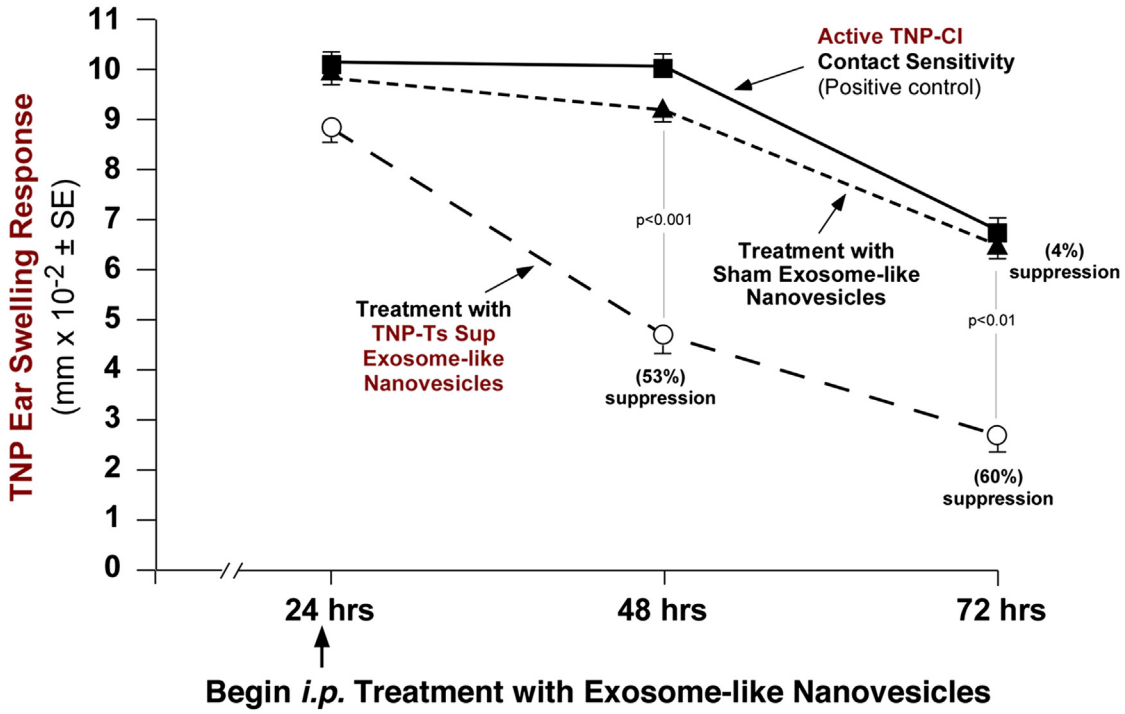


FIG 4. *In vivo* treatment with suppressive exosome-like nanovesicles inhibits established CS responses in actively sensitized mice. Ts Sup versus control sham supernatant nanovesicles were injected intraperitoneally *(i.p.)* at 24 hours of an ongoing CS response in actively sensitized and ear-challenged mice. The Ts Sup exosomes suppressed CS at 48 and 72 hours, whereas the sham supernatant vesicles were noninhibitory.

Experiments with mice deficient in miR-150 definitively identified miR-150 as crucial to *in vivo* suppression by the exosome-like nanovesicles

The miR-150$^{-/-}$ mice[31] could not be tolerized (Fig 8, *A*, group D) compared with the wild-type control mice (Fig 8, *A*, group B), and after attempted tolerization, their exosome-like nanovesicles were not suppressive compared with those of the wild-type mice (Fig 8, *B*, group C vs group B). Very importantly, these nonsuppressive exosome-like nanovesicles could be transfected for reconstitution of suppression by mere *in vitro* incubation with miR-150 alone (Fig 8, *C*, group D). Again, this procedure was performed between the two 100,000*g* pelleting steps.

Performance of the HT-2 assay showed that miR-150 was also crucial to this *in vitro* assay

The tolerized miR-150$^{-/-}$ exosome-like nanovesicles were nonsuppressive, whereas miR-150 transfection reconstituted their suppression *in vitro* (Fig 8, *D*, group C vs group D). This finding confirmed that nanovesicle inhibition of the *in vitro* correlative HT-2 T-cell assay also was dependent on delivery of miR-150. Overall, we have shown that activated exosome-like nanovesicles from tolerized suppressor T cells were responsible for the *in vivo* and *in vitro* suppression and differed greatly from normal nanovesicles. The results suggest that the acquired ability of activated nanovesicles to permit chosen antibody coating and loading with selected miRNA might be used to generate very specific therapeutic exosome-like nanovesicles.

DISCUSSION

Synopsis of new findings

This study of T cell–derived immunosuppressive exosome-like nanovesicles in allergic cutaneous CS generated 2 important discoveries. First, the nanovesicles were antigen specific, which enabled them to suppress antigen-specific CS effector T cells and bind to specific antigen-linked affinity columns. This antigen specificity resulted from a coating of Ab LC or antibody that we believe was produced by B cells activated during the tolerogenic procedure. The second discovery was that these nanovesicles could easily be transfected with selected miRNAs to therefore deliver a chosen regulatory double-stranded RNA cargo to genetically affect particular functions of antigen-specifically targeted cells. Thus suppressive function depended on the exosome-like nanovesicles from the Ts cells and B cell–produced antigen-specific Ab LC or antibody. Use of unprecedented techniques, such as antigen-specific affinity chromatography of the nanovesicles, led to isolation of a suppressive subset we subjected to molecular cloning. Deep sequencing and comparative bioinformatics of suppression- versus non–suppression-associated miRNAs led to preliminary identification of the inhibitory miRNA as the previously T cell–associated miRNA-150.[31-37]

Postulated pathway of effector T-cell suppression by exosome-like nanovesicles

We hypothesize that the described procedure of tolerance induction results in activation of 2 essential cell types. One is the CD8^{+} suppressor T-cell population, which produces the exosome-like nanovesicles containing inhibitory miRNA-150.

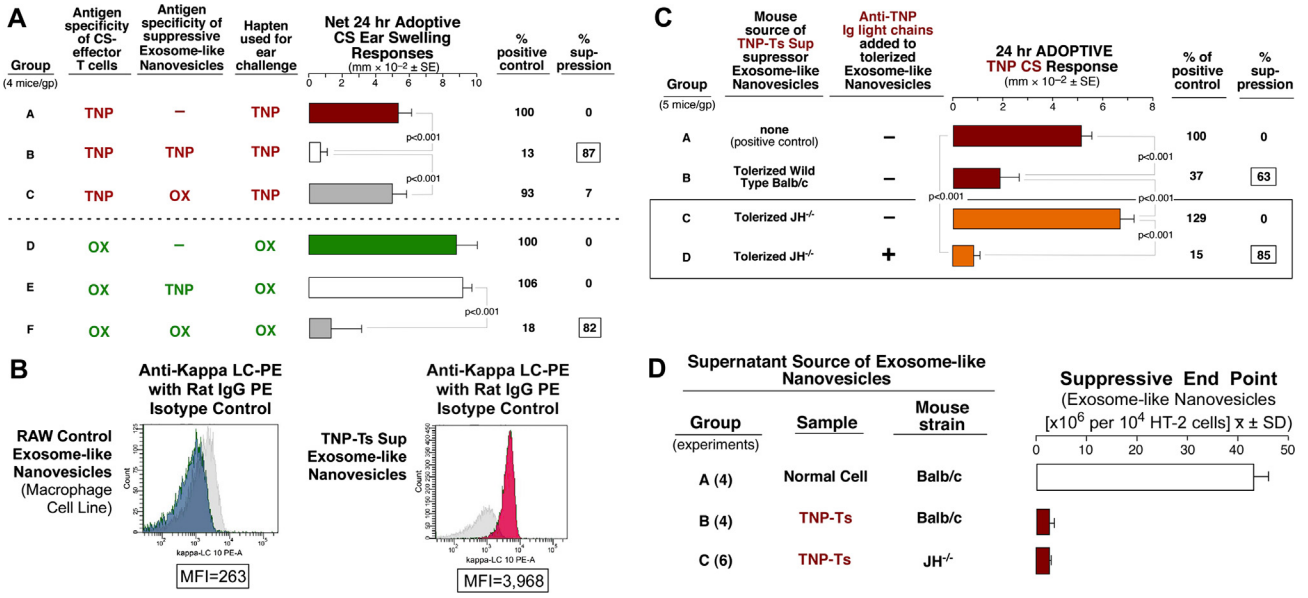


FIG 5. The suppressive exosome-like nanovesicles are antigen specific. **A,** Dual reciprocal antigen specificity of Ts Sup exosome-like nanovesicles. TNP–CS effector cells only, positive control *(group A)*, suppression in the TNP homologous system *(group B)*, no suppression in the TNP heterologous system *(group C)*, OX–CS effector cells only, positive control *(group D)*, no suppression in the OX heterologous system *(group E)*, and suppression in the OX homologous system *(group F)* are shown. **B,** Flow cytometric analysis of kappa light chain expression on TNP–Ts Sup (*right, red*) and RAW cell–derived (*left, blue*) nanovesicles. Isotype controls are shown in gray. *MFI*, Mean fluorescence intensity. **C,** Nanovesicles from TNP-tolerized $JH^{-/-}$ mice did not mediate suppression *(group C)*. *In vitro* addition of monoclonal anti-TNP Ab LC to nanovesicles from tolerized $JH^{-/-}$ mice reconstituted suppression *(group D)*. **D,** The non–antigen-specific assay of HT-2 T-cell responsiveness to IL-2 showed that nanovesicles from tolerized $JH^{-/-}$ mice had strong non–antigen-specific suppressive activity (*group C* vs *group A*), which is equivalent to that of wild-type Ts Sup nanovesicles (*group B* vs *group A*).

The other collaborating cells likely are B1 B cells, probably antigen-specifically activated in the peritoneal cavity through contact skin immunization during tolerogenesis.[11] After migration to the spleen, B1 B cells were shown to release antigen-specific IgM and Ab LC into the circulation.[11] Therefore the suppressive nanovesicles produced during tolerogenesis could be coated with Ab LC or antibody *in vivo*, as shown by means of flow cytometry (Fig 5, *B*). Moreover, coating of exosome-like nanovesicles with Ab LC could be performed *in vitro* (Fig 5, *C*).

Treatment of the CS effector cell mixture with nanovesicles was effective *in vitro*, as shown in adoptive transfer experiments. Moreover, the exosome-like nanovesicles injected systemically at the peak response into actively sensitized mice were able to suppress CS responses, likely by targeting the activated CS effector T cells *in vivo* at the CS elicitation site.

The exact mechanism of tolerance and the targeted cell type are subject to ongoing research. The CS effector T cells themselves might be the direct target of the suppressive nanovesicle action. However, preliminary results suggest that the regulatory signal could also be transmitted to CS effector cells by targeted antigen-presenting cells, such as dendritic cells or macrophages, the functions of which are altered by the suppressive exosome-like nanovesicles (unpublished data).

Antigen specificity of suppressive exosome-like nanovesicles

The antigen specificity we identified is an important new property of exosome-like nanovesicles. Not only is this the first demonstration of antigen-specific nanovesicles, but also it is the first demonstration that such vesicles with biological activity can be separated into at least 2 functional subpopulations: a minor antigen-binding fraction having all the activity and a major non–antigen-binding fraction that was nonsuppressive. The potential surface antigen specificity of the suppressive exosome-like nanovesicles was based on 4 findings: (1) flow cytometry showing Ab kappa LC on their surfaces, (2) dual reciprocal antigen-specific suppressive function, (3) specific antigen binding for antigen affinity chromatography, and (4) reconstitution of suppression in nonsuppressive nanovesicles from $JH^{-/-}$ panimmunoglobulin-deficient tolerized mice by coating with antigen-specific monoclonal Ab LC.

Our data show that nanovesicle antigen specificity and suppressive activity can be conferred by a coating with free Ab LC and not antibody heavy chain that therefore likely binds an unknown site on the activated exosome-like nanovesicles. An alternative possibility is that intact, antigen-specific IgM or IgG is responsible. Despite the usual low-affinity antigen binding of isolated Ab LC, they can mediate antigen specificity.[11,39] Furthermore, when Ab LC are multiply displayed on the nanovesicle surface, the overall avidity for antigen likely increases, particularly in this hapten system, where there is only one antigen determinant. Free immunoglobulin light chain previously has been implicated in a variety of immune and allergic inflammatory diseases[40-43] and might be one mechanism for the beneficial effect of anti-CD20 B-cell therapy with rituximab.[44,45] The effects of Ab LC in diverse responses first were ascribed to binding and activating mast cells,[11,30,40-43,46] but binding of

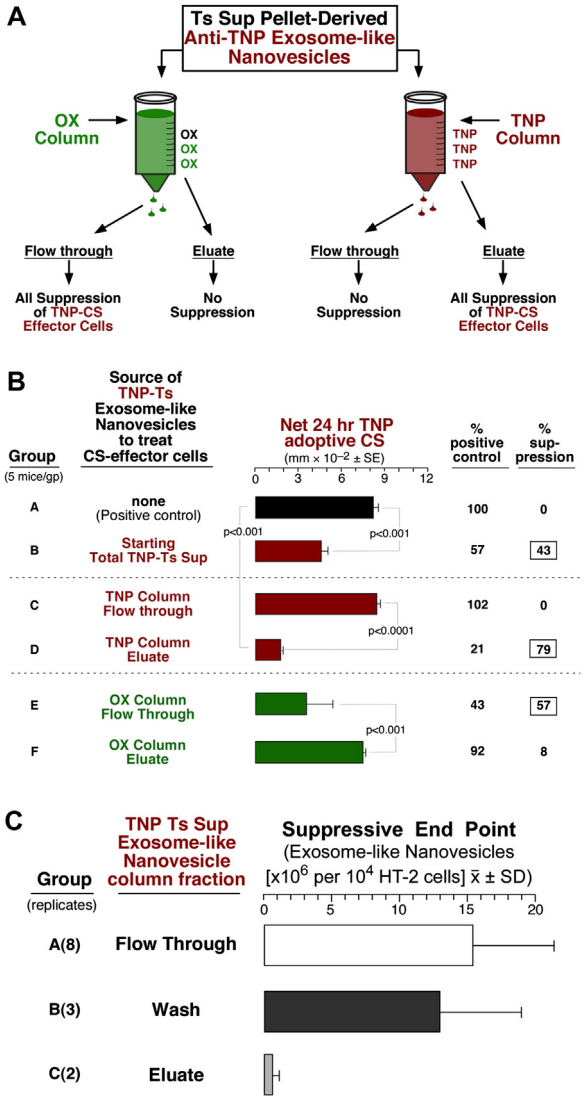


FIG 6. Isolation of a small suppressive nanovesicle subpopulation by means of antigen affinity chromatography. **A,** Suppressive TNP–Ts Sup nanovesicles were applied to a column conjugated with either TNP or OX. Only 12% of applied nanovesicles adhered to the TNP column and were eluted with dilute guanidine. **B,** TNP–Ts Sup vesicles from the TNP column FT mediated no suppression *(group C)*, whereas the TNP nanovesicles from the eluate had all the activity *(group D)*. The OX column FT, but not eluate, had all the suppressive activity (*group E* vs *group F*). **C,** The eluate fraction from the TNP column strongly inhibited HT-2 cell viability *(group C)*, whereas the column wash *(group B)* and the FT *(group A)* were not suppressive.

Ab LC to human T cells, B cells, and monocytes has been recently demonstrated.[47]

Suppression by antigen-specific exosome-like nanovesicles

Suppressive nanovesicles can be compared with the immunosuppressive extracellular vesicles described previously in allergy and immunity[48-51] and other conditions, such as pregnancy,[52-54] breast-feeding,[55] and especially cancers, in which tumor-derived exosomes subvert a variety of host responses.[56-62] However, in none of these other systems have exosomes been shown to exhibit antigen specificity. This was suggested previously but without antigen binding or dual reciprocal testing.[59] Our system of high-dose antigen-induced suppressive Ts cells and Ts Sup previously was elegantly characterized biologically and noted to be antigen specific.[63-65] However, neither the mechanisms for antigen specificity (here shown to be antibody) nor elucidation of how suppression was mediated (here shown because of miRNA-150 contained in exosome-like nanovesicles) was determined.

Suppression is mediated by miRNA-150 in exosome-like nanovesicles from tolerized CD8$^+$ suppressor T cells

A crucial step leading to the identification of miR-150 as a mediator of suppression was isolation by means of antigen affinity column chromatography of a suppressive antigen-binding subpopulation of nanovesicles representing only 12% of the total. Comparing the miRNA sequences of this subpopulation with those of the nonsuppressive non–antigen-binding nanovesicles led to miR-150 as a candidate, and inhibition of suppressive activity by an miR-150 antagonist confirmed this idea. Finally, experiments with miR-150$^{-/-}$ mice definitively established miR-150 as the major suppressive small RNA carried by the exosome-like nanovesicles to antigen-specifically inhibit targeted cells in the CS effector cell mixture. Of further importance, the miRNA-150 in the exosome-like nanovesicles also could act non–antigen-specifically to inhibit HT-2 cell responsiveness to IL-2, an *in vitro* assay[66] that turned out to be truly correlative with the *in vivo* nanovesicle suppression of CS. This should prove to be an excellent system to determine the molecular mechanisms of the effects of miR-150 on targeted cells, here possibly taken up by nonspecific mechanisms, such as pinocytosis, instead of by an antibody-dependent antigen-specific mechanism. miR-150 was described originally in positive T-cell mediation of B-cell, T-cell, and myeloid/erythroid development[31,33] and, more recently, development of T, NK, and NKT cells.[35,37] As would be expected for regulation by an miRNA, activating versus suppressive effects might depend on particular targeted transcription factors other than the strongly miR-150–associated c-Myb.[8,31,34,35] Accordingly, miR-150 also can inhibit B-cell development depending on timing[32] and is considered a tumor suppressor.[67] The intracellular target of miR-150 in nanovesicle-mediated suppression of CS will be the subject of future investigations.

Relation of these studies of T-cell suppression to human patients with contact dermatitis and other inflammatory diseases

The present study demonstrates a mechanism of T-cell tolerance in mice mediated by exosome-like nanovesicles carrying miRNA-150 produced by suppressor CD8$^+$ T cells and possibly delivered at the cutaneous site of CS.

Human studies have noted Treg cells defined by Foxp3, cytokine, or both associations at skin lesions of patients with contact dermatitis.[68] In addition to CD4$^+$ Treg cells, there are many clinical instances in which a role for CD8$^+$ Treg cells has been described. These include regulation of IgE-mediated allergy,[69] autoimmunity,[70,71] viral diseases,[72-74] cancer,[75,76] and transplantation alloimmunity.[77] CD8$^+$ Treg cells are being appreciated to play a role in a variety of regulatory processes.[78-80]

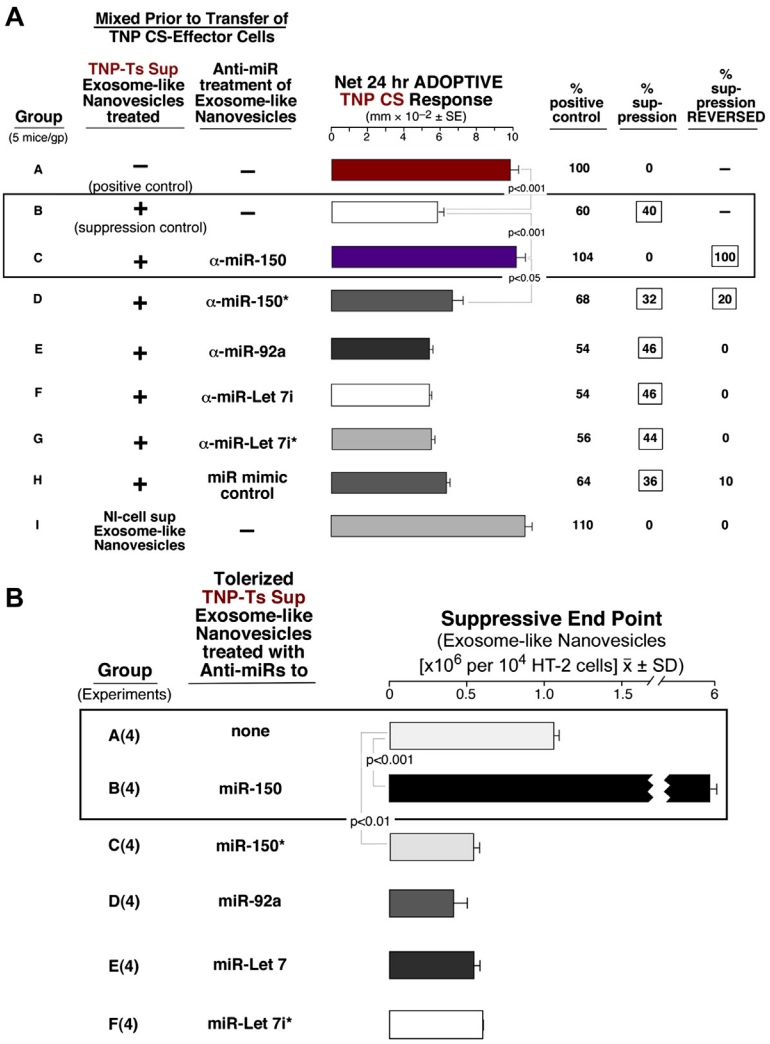


FIG 7. Use of specific anti-miRs to test for candidate suppressive cargo of the Ts Sup exosome-like nanovesicles. **A,** TNP–Ts Sup nanovesicles preincubated with anti–miR-150 completely inhibited CS suppression *(group C)*. Similar treatment with anti–miR-150* (the antisense passenger strand) only resulted in 20% reversed suppression *(group D)*. Treatment with anti-miR to miR-92a *(group E)*, Let 7i *(group F)*, Let 7i* *(group G)*, or the mimic control *(group H)* did not reverse suppression. **B,** The same anti-miRs were tested in the in HT-2 assay. Only the anti-miR to miR-150 reversed suppression *(group B)*.

However, in the current study we ruled out $Foxp3^+$ Treg cell participation in this tolerogenesis.

Therefore, it is important to consider whether similar antigen-specific $CD8^+$ suppressor T cells or analogous tolerance mechanisms also exist in human subjects and possibly modulate clinical diseases through the release of comparable exosome-like nanovesicles. On the other hand, cell populations mediating regulatory mechanisms observed in murine models might not always be directly translatable to human subjects. However, even when different regulatory cell types are involved in mediating tolerance, the same clinical effect can be observed. This was demonstrated in a clinical trial studying induction of suppression of the autoimmune response in patients with multiple sclerosis, in which the same tolerogenic procedure was performed in both species but the resulting suppressive T cells had distinctly different phenotypes.[81,82] Because the activity of the suppressive nanovesicles could also be modulated by easy transfection with miRNA and surface coating with Ab LC, this newly described mechanism of suppression might have important potential in regulating immune responses.

Translation potentials

The discovery of antigen-specific exosome-like nanovesicles suggests that they could be targeted to specific cells by sensitizing their surfaces with a coat of a chosen Ab LC against a marker of the desired target cells and loaded with selected miRNA cargo for specific intracellular genetic therapy. This might enable suppression of specific effector cells in patients with allergic, autoimmune, and inflammatory diseases. Alternatively, the suppressive function of Treg cells in patients with cancer or small RNA derived from oncogenes in patients with leukemia can be antagonized by nanovesicle-derived cargo. Although our findings pertain to hapten-induced skin allergy, we are extending them

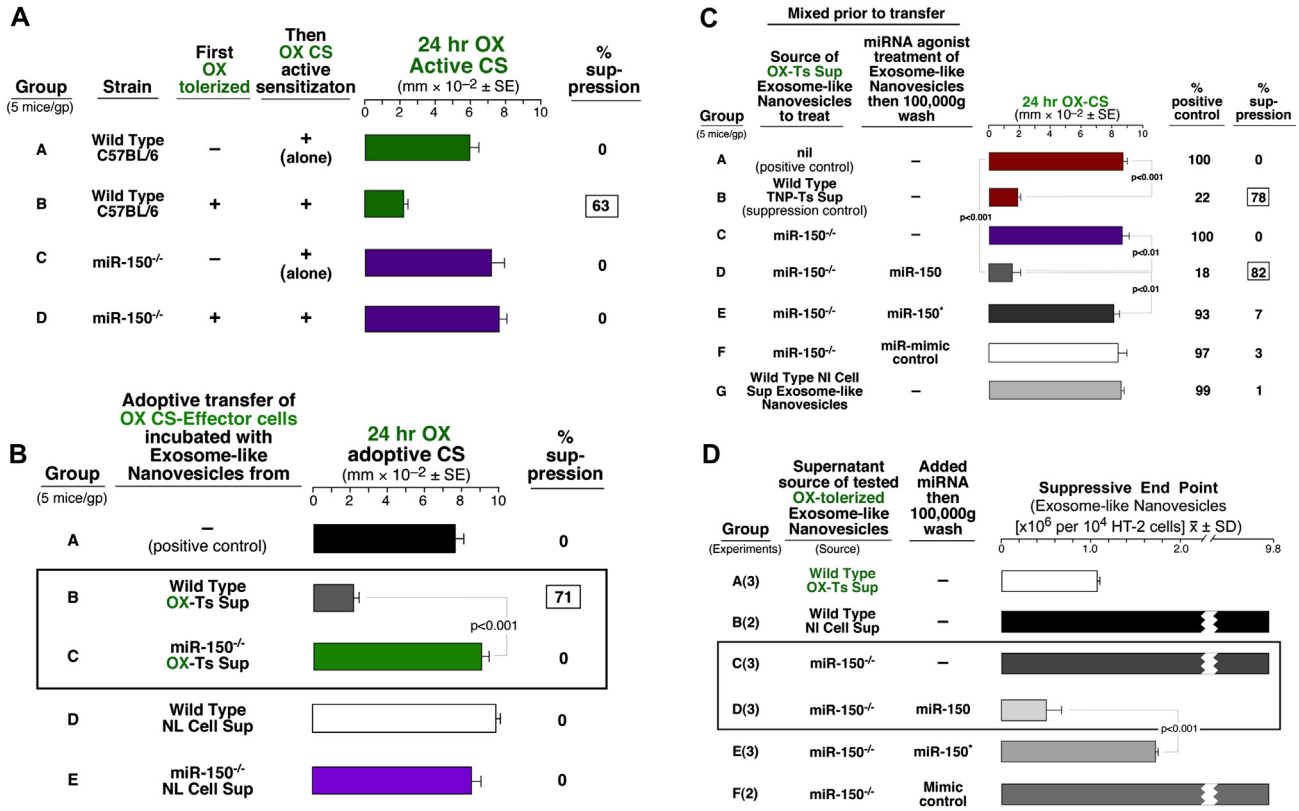


FIG 8. miRNA-150$^{-/-}$ mice show definitively that miR-150 is the suppressive entity in the exosome-like nanovesicles from tolerized mice. **A,** Wild-type C57BL/6 and miR-150$^{-/-}$ mice were actively contact sensitized and had normal responses at 24 hours (*groups A* and *C*). Wild-type mice first tolerized and then actively sensitized showed strong suppression *(group B)* in contrast to miR-150$^{-/-}$ mice, which showed no suppression after tolerance induction *(group D)*. **B,** Treatment of OX CS effector cells with miR-150$^{-/-}$ OX Ts Sup nanovesicles *(group C)*, as well as with WT NI Cell Sup *(group D)* and miR-150$^{-/-}$ NI Cell Sup *(group E)* nanovesicles did not cause suppression of CS. **C,** Nanovesicles from OX-tolerized miR-150$^{-/-}$ mice transfected with miR-150 mediated suppression, whereas transfection with miR-150* *(group E)* or miR-mimic control *(group F)* did not result in suppression. **D,** Non–antigen-specific assay of HT-2 T-cell responsiveness to IL-2 showed that nanovesicles from miR-150$^{-/-}$ OX-tolerized mice *(group C)* were noninhibitory, but transfection with miR-150 rendered them suppressive *(group D)*.

to protein antigens in patients with delayed-type hypersensitivity and allergic asthma (Groot Kormelink et al, unpublished).

The ability of activated exosome-like nanovesicles to be transfected with selected small RNA cargo to bind surface antigens preferentially expressed on targeted cells and alter specific target cell functions could achieve a high therapeutic index as a new physiologic and specific delivery vehicle. Finally, our data suggest that therapeutic exosome-like vesicles are able to act at great distances through the blood for prolonged times after a single dose and might even work when administered orally. Furthermore, as therapy, they seem to be able to suppress active disease through cooperating immunologic and genetic mechanisms. In summary, the unique and very important potential translational properties of the suppressive exosome-like nanovesicles that we have described are their easy transfection with miRNA and, above all, their manipulable antigen specificity.

We thank Madeleine Michaud for her secretarial and administrative skills; David Fedson, Ivana Kawikova, Timur Yarovinsky, and Avrion Mitchison for their advice on the manuscript; Jordan Pober for his continuing sage advice on the project; Jerry Domian for his tireless adjustments of the figures; and Esther Nolte-t Hoen and Marca Wauben of Utrecht University for helpful preliminary sucrose gradient experiments. We especially thank Markus Hafner and Thomas Tuschl of the Rockefeller University for the preparation and sequencing of small RNA cDNA libraries from provided samples. This was essential to the eventual identification of the crucial role of miR-150.

Key messages

- **Antigen-specific exosome-like nanovesicles delivering selected inhibitory miRNA represent a new form of regulation shown here to inhibit allergic contact dermatitis.**
- **The described mechanism of tolerance enables antigen-specific targeting of particular cell function through miRNA interference.**
- **This process might lead to establishment of a new form of natural immunologic and genetic therapy of many human diseases.**

REFERENCES

1. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 2009;9:581-93.

2. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3.22.
3. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-9.
4. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008;10:1470-6.
5. Korkut C, Ataman B, Ramachandran P, Korkut C, Ashley J, Barria R, et al. Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. Cell 2009; 139:393-404.
6. Koles K, Nunnari J, Korkut C, Barria R, Brewer C, Li Y, et al. Mechanism of evenness interrupted (evi)-exosome release at synaptic moutons. J Biol Chem 2012;287:16820-34.
7. Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. Exosome release of betacatenin: a novel mechanism that antagonizes Wnt signaling. J Cell Biol 2010; 190:1079-91.
8. Zhang J, Luo N, Luo Y, Peng Z, Zhang T, Li S. microRNA-150 inhibits human CD133- positive liver cancer stem cells through negative regulation of the transcription factor c-Myb. Int J Oncol 2012;40:747-56.
9. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res 2012;22:107-26.
10. Martin SF, Dudda JC, Bachtanian E, Lembo A, Liller S, Durr C, et al. Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. J Exp Med 2008;205:2151-62.
11. Askenase PW, Szczepanik M, Itakura A, Kiener C, Campos RA. Extravascular T cell recruitment requires initiation begun by V14+ NKT cells and B-1 B cells. Trends Immunol 2004;25:441-9.
12. Vocanson M, Hennino A, Cluzel-Tailhardat M, Saint-Mezard P, Benetiere J, Chavagnac C, et al. CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. J Invest Dermatol 2006;126:815-20.
13. Lee DS, Gulati N, Martiniuk F, Levis WR. CD70 and Th17 are involved in human contact sensitivity. J Drugs Dermatol 2011;10:1192-4.
14. Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol 2010;11:1127-35.
15. Simonetta F, Chiali A, Cordier C, Urrutia A, Girault I, Bloquet S, et al. Increased CD127 expression on activated FOXP3+CD4+ regulatory cells. Eur J Immunol 2010;40:2528-38.
16. Ilkovitch D. Role of immune-regulatory cells in skin pathology. J Leukoc Biol 2011;89:41-9.
17. Choudhuri K, Llodra J, Kam L, Stokes D, Dustin M. Antigen-induced release and retroviral subversion of TCR-enriched microvesicles at the CD4+ T cell immunological synapse. J Immunol 2012;188:58-65.
18. Mittelbrunn M, Gutierrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun 2011;2:282-91.
19. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012;119:756-66.
20. Sahoo S, Klychko E, Thorne T, Misener S, Schultz KM, Millay M, et al. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. Circ Res 2011;109:724-8.
21. Hergenreider E, Heydt S, Tréguer K, Boettger T, Horrevoets AJ, Zeiher AM, et al. Atheroprotective communication between endothelia cells and smooth muscle cells through miRNAs. Nat Cell Biol 2012;14:249-56.
22. Lachenal G, Pernet-Gallay K, Chivet M, Hemming FJ, Belly A, Bodon G, et al. Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. Mol Cell Neurosci 2011;46:409-18.
23. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci 2011;124:447-58.
24. Eldh M, Ekström K, Valadi H, Sjöstrand M, Olsson B, Jernas M, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS One 2010;5:e15353.
25. Meckes DG Jr, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. Proc Natl Acad Sci U S A 2010;107:20370-5.
26. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, et al. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 2010;107:6328-33.
27. Soo CY, Song Y, Zheng Y, Campbell EC, Riches AC, Gun-Moore E, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology 2012;136:192-7.
28. KaLahl K, Sparwasser T. In vivo depletion of FoxP3+Tregs using the DEREG mouse model. Methods Mol Biol 2011;707:157-72.
29. Chen J, Trounstine M, Alt FW, Young F, Kurahara C, Loring JF, et al. Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. Int Immunol 1993;5:647-56.
30. Redegeld FA, van der Heijden MW, Kool M, Heijdra BM, Garssen J, Kraneveld AD, et al. Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. Nat Med 2002;8:694-701.
31. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 2007;131:146-59.
32. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci U S A 2007;104:7080-5.
33. Bruchova-Votavova H, Yoon D, Prchal JT. MiR-451 enhances erythroid differentiation in K562 cells. Leuk Lymph 2010;51:686-93.
34. Hussein K, Theophile K, Büsche G, Schlegelberger B, Göhring G, Kreipe H, et al. Significant inverse correlation of microRNA-150/MYB and microRNA-222/p27 in myelodysplastic syndrome. Leuk Res 2010;34:328-34.
35. Bezman NA, Chakraborty T, Bender T, Lanier LL. MiR-150 regulates the development of NK and iNKT cells. J Exp Med 2011;208:2717-31.
36. Ghisi M, Corradin A, Basso K, Frasson C, Serafin V, Mukherjee S, et al. Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150. Blood 2011;117:7053-62.
37. Zheng Q, Zhou L, Mi QS. MicroRNA miR-150 is involved in Valpha14 invariant NKT cell development and function. J Immunol 2012;188:2118-26.
38. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3- Dependent MicroRNA155 confers competitive fitness to regulatory t cells by targeting SOCS1 protein. Immunity 2009;30:80-91.
39. Housden NG, Harrison S, Housden HR, Thomas KA, Beckingham JA, Roberts SE, et al. Observation and characterization of the interaction between a single immunoglobulin binding domain of protein L and two equivalents of human kappa light chains. J Biol Chem 2004;279:9370-8.
40. Powe DG, Groot Kormelink T, Sisson M, Blokhuis BJ, Kramer MF, Jones NS, et al. Evidence for the involvement of free light chain immunoglobulins in allergic and nonallergic rhinitis. J Allergy Clin Immunol 2010;125:139-45.
41. Groot Kormelink T, Calus L, De Ruyck N, Holtappels G, Bachert C, Redegeld FA, et al. Local free light chain expression is increased in chronic rhinosinusitis with nasal polyps. Allergy 2012;67:1165-72.
42. Thio M, Groot Kormelink T, Fischer MJ, Blokhuis BR, Nijkamp F, Redegeld FA. Characteristics of immunoglobulin free light chains: crosslinking by antigen is essential to induce allergic inflammation. PLoS One 2012;7:e40986.
43. Groot Kormelink T, Askenase PW, Redegeld FA. Immunobiology of antigen-specific immunoglobulin free light chains in chronic inflammatory diseases. Curr Pharm Des 2012;18:2278-89.
44. Groot Kormelink T, Tekstra J, Thurlings RM, Boumans MH, Vos K, Tak PP, et al. Decrease in immunoglobulin free light chains in patients with rheumatoid arthritis upon rituximab (anti-CD20) treatment correlates with decrease in disease activity. Ann Rheum Dis 2010;69:2137-44.
45. Tekstra J, van Roon J, Groot Kormelink T, Redegeld F. Immunoglobulin free lightchain levels and rituximab response in rheumatoid arthritis: comment on the article by Sellam et al. Arthritis Rheum 2011;63:4034-5.
46. Paliwal V, Tsuji RF, Szczepanik M, Kawikova I, Campos RA, Kneilling M, et al. Subunits of IgM reconstitute defective contact sensitivity in B-1 cell-deficient xid mice: kappa light chains recruit T cells independent of complement. J Immunol 2002;169:4113-23.
47. Hutchinson AT, Jones DR, Raison RL. The ability to interact with cell membranes suggests possible biological roles for free light chain. Immunol Lett 2012;142: 75-7.
48. Almqvist N, Lonnqvist A, Hultkrantz S, Rask C, Telemo E. Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. Immunology 2008;125:21-7.
49. Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, et al. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. Mol Ther 2006;13:289-300.
50. Kim SH, Bianco NR, Shufesky WJ, Morelli AE, Robbins PD. MHC class II+ exosomes in plasma suppress inflammation in an antigen-specific and Fas ligand/Fas-dependent manner. J Immunol 2007;179:2235-41.
51. Prado N, Marazuela EG, Segura E, Fernández-García H, Villalba M, Théry C, et al. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. J Immunol 2008;181:1519-25.

52. Hedlund M, Stenqvist AC, Nagaeva O, Kjellberg L, Wulff M, Baranov V, et al. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. J Immunol 2009;183:340-51.
53. Sabapatha A, Gercel-Taylor C, Taylor DD. Specific isolation of placenta-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences. Am J Reprod Immunol 2006;56:345-55.
54. Taylor DD, Akyol S, Gercel-Taylor C. Pregnancy-associated exosomes and their modulation of T cell signaling. J Immunol 2006;176:1534-42.
55. Admyre C, Johansson SM, Qazi KR, Filén JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol 2007;179:1969-78.
56. Hao S, Ye Z, Li F, Meng Q, Qureshi M, Yang J, et al. Epigenetic transfer of metastatic activity by uptake of highly metastatic B16 melanoma cell-released exosomes. Exp Oncol 2006;28:126-31.
57. Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z. Human tumor-derived exosomes down-modulate NKG2D expression. J Immunol 2008;180:7249-58.
58. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent Immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest 2010;120:457-71.
59. Yang C, Kim SH, Bianco NR, Robbins PD. Tumor-derived exosomes confer antigen-specific immunosuppression in a murine delayed-type hypersensitivity model. PLoS One 2011;6:e22517.
60. Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. J Immunol 2011;187:676-83.
61. Cai Z, Yang F, Yu L, Yu Z, Jiang L, Wang Q, et al. Activated T cell exosomes promote tumor invasion via Fas signaling pathway. J Immunol 2012;188:5954-61.
62. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 2012;18:883-91.
63. Ptak W, Rozycka D, Rewicka M. Induction of suppressor cells and cells producing antigen-specific suppressor factors by haptens bound to self-carriers. Immunobiology 1980;156:400-9.
64. Ptak W, Rosenstein RW, Gershon RK. Interactions between molecules (subfactors) released by different T cell sets that yield a complete factor with biological (suppressive) activity. Proc Natl Acad Sci U S A 1982;79:2375-8.
65. Ptak W, Gershon RK, Rosenstein RW, Murray JH, Cone RE. Purification and characterization of TNP- specific immunoregulatory molecules produced by T cells sensitized by picrylchloride (PC1F). J Immunol 1983;131:2859-63.
66. Ferreri NR, Herzog WR, Askenase PW. Inhibition of IL-2-dependent proliferation by a prostaglandin- dependent suppressor factor. J Immunol 1993;150:2102-11.
67. Watanabe A, Tagawa H, Yamashita J, Teshima K, Nara M, Iwamoto K, et al. The role of microRNA-150 as a tumor suppressor in malignant lymphoma. Leukemia 2011;25:1324-34.
68. Matsushima H, Takashima A. Bidirectional homing of Tregs between the skin and lymph nodes. J Clin Invest 2010;120:653-6.
69. Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D, Lee TH. The role of CD8+ T cells in immunoglobulin E regulation. Allergy 1995;50:9-14.
70. Suzuki M, Konya C, Goronzy JJ, Weyand CM. Inhibitory CD8+ T cells in autoimmune disease. Hum Immunol 2008;69:781-9.
71. Jiang H, Canfield SM, Gallagher MP, Jiang HH, Jiang Y, Zheng Z, et al. HLA-E-restricted regulatory CD8(+) T cells are involved in development and control of human autoimmune type 1 diabetes. J Clin Invest 2010;120:3641-50.
72. Accapezzato D, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 2004;113:963-72.
73. Buckheit RW 3rd, Salgado M, Silciano RF, Blankson JN. Inhibitory potential of subpopulations of CD8+ T cells in HIV-1-infected elite suppressors. J Virol 2012;86:13679-88.
74. Tumne A, Prasad VS, Chen Y, Stolz DB, Saha K, Ratner DM, et al. Noncytotoxic suppression of human immunodeficiency virus type 1 transcription by exosomes secreted from CD8+ T cells. J Virol 2009;83:4354-64.
75. Chiba T, Ohtani H, Mizoi T, Naito Y, Sato E, Nagura H, et al. Intraepithelial CD8+ T-cell-count becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: possible association with suppression of micrometastasis. Br J Cancer 2004;91:1711-7.
76. Olson BM, Jankowska-Gan E, Becker JT, Vignali DA, Burlingham WJ, McNeel DG. Human prostate tumor antigen-specific CD8+ regulatory T cells are inhibited by CTLA-4 or IL-35 blockade. J Immunol 2012;189:5590-601.
77. Ciubotariu R, Colovai AI, Pennesi G, Liu Z, Smith D, Berlocco P, et al. Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+CD28- regulatory T cells. J Immunol 1998;161:5193-202.
78. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med 2002;195:695-704.
79. Chang CC, Zhang QY, Liu Z, Clynes RA, Suciu-Foca N, Vlad G. Downregulation of inflammatory microRNAs by Ig-like transcript 3 is essential for the differentiation of human CD8(+) T suppressor cells. J Immunol 2012;188:3042-52.
80. Van Kaer L. Comeback kids: CD8(+) suppressor T cells are back in the game. J Clin Invest 2010;120:3432-4.
81. Tutaj M, Szczepanik M. Epicutaneous (EC) immunization with myelin basic protein (MBP) induces TCRalphabeta+ CD4+ CD8+ double positive suppressor cells that protect from experimental autoimmune encephalomyelitis (EAE). J Autoimmun 2007;28:208-15.
82. Jurynczyk M, Walczak A, Jurewicz A, Jesionek-Kupnicka D, Szczepanik M, Selmaj K. Immune regulation of multiple sclerosis by transdermally applied myelin peptides. Ann Neurol 2010;68:593-601.

METHODS

Mice

CBA/J, BALB/c, $JH^{-/-}$,[E1] and C57BL/6 mice were from the National Cancer Institute, Jackson Laboratories (Bar Harbor, Me), or Jagiellonian University (Krakow, Poland); miR-$150^{-/-}$ mice[E2] were from Klaus Rajewsky (Jackson Laboratories no. 007750); and DEREG mice depleted of Treg cells by means of intravenous *Diphtheria* toxin injection (verified by means of flow cytometry) were from Tim Sparwasser.[E3] Mice were fed autoclaved food and water. All experiments were performed according to the guidelines of both Yale and Jagiellonian University.

Reagents

The contact-sensitizing agents TNP-Cl (picryl chloride; Chemica Alta, Edmonton, Alberta, Canada), trinitrobenzenesulfonic acid, OX, and human recombinant IL-2 (hrIL-2) were from Sigma (St Louis, Mo), and guanidine HCl (pH 4.7) was from American Bioanalytical (Natick, Mass).

Media

Mishell-Dutton medium, RPMI 1640, and minimal essential medium with amino acids were from Sigma. HEPES solution (American Bioanalytical), FCS, Dulbecco PBS (DPBS), Dulbecco modified Eagle medium, Pen-Strep, sodium pyruvate, and L-glutamine were all obtained from Gibco Invitrogen (Auckland, New Zealand).

Antibodies and proteins

The anti-TNP antibody light and heavy immunoglobulin chains were produced as previously described.[E4] Anti-CD9 antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif). Hapten-protein conjugates linked to Sepharose affinity columns (TNP_{22}-BSA and OX_{18}-BSA) were produced at Jagiellonian University.

Active sensitization and adoptive transfer of CS responses

After achievement of light ether or metaphane anesthesia, mice were contact sensitized by means of application of 150 μL of 5% TNP-Cl or 3% OX in ethanol/acetone (3:1) to the skin of the shaved abdomen, chest, and feet. On day 4, they were ear challenged with 10 μL of 0.4% TNP-Cl or OX in olive oil/acetone (1:1) on both sides of both ears. The increase in ear thickness after indicated time points was measured with an engineer's micrometer (Mitutoyo, Kawasaki, Japan) and expressed in units of 10^{-2} mm ± SE.[E5-E7]

Background nonspecific increase in ear thickness (± 2 units at 24 hours) in nonsensitized similarly challenged littermates was subtracted from experimental groups to yield a net swelling value. Most ear readings were done by an observer completely unaware of the project or protocol (double blinded), and others were performed by an observer knowledgeable of the protocol but blind to the groups (single blinded).

For adoptive cell transfers of CS, actively sensitized mice were killed on day 4 by means of cervical dislocation, and spleens and axillary and inguinal lymph nodes were harvested for CS effector cells, which were subsequently transferred intravenously (about 7×10^7 cells per recipient) to naive lightly anesthetized recipients that were immediately ear challenged with reactive hapten. Ear thickness was measured as above.[E5-E8]

Induction of TNP– or OX–Ts cells (TNP-Ts or OX-Ts cells), TNP–Ts Sup or OX-Ts Sup, and control normal cell and sham supernatants

For tolerization, isogenic mouse red blood cells were conjugated with TNP or OX[E5] and injected intravenously as 10% suspension on days 0 and 4 into naive mice, that were skin painted with homologous hapten on day 9. Spleens and lymph nodes containing induced Ts cells were harvested on day 11, and single-cell suspensions were prepared for culture at 2×10^7 per milliliter in serum free Mishell-Dutton medium supplemented with pyruvic acid, glutamine, minimal essential medium with additional amino acids, 2-mercaptoethanol, HEPES, and Pen-Strep for 48 hours at 37°C in 5% CO_2 to produce Ts Sup. Subsequently, Ts Sup was separated from cells and debris by means of centrifugation and stored at 4°C if used within 2 to 4 days, at −20°C if used within 10 days, or at −70°C for longer times.[E1,E2,E9-E11]

In TNP– and OX–Ts Sup no uniformly increased levels of IL-10, TGF-β, or IL-6 were found by means of sensitive ELISA (BD PharMingen, San Jose, Calif). The sensitivities of the ELISAs used were 15 pg/mL (IL-10 and IL-6) and 30 pg/mL (TGF-β). For a control, normal cells from naive mice were cultured identically to generate Nl Cell Sup and eventually processed to nanovesicles. For an additional control, sham-tolerized mice received intravenous injections of isogenic mouse red blood cells treated chemically as for hapten conjugation but without hapten, and subsequently, skin painted with vehicle alone. Nl Cell Sup and sham supernatant nanovesicles were isolated as described above.

Separation of vesicles from cell supernatant or fresh plasma

Supernatants from 48-hour cell cultures in serum-free Mishell-Dutton medium or plasma diluted 1:1 in DPBS were centrifuged at 300*g* and then at 10,000*g* for 30 minutes, followed by sequential filtration of the cell-free supernatant or plasma with 0.45- and 0.22-μm filters. Nanovesicles were then separated by means of double ultracentrifugation in a Beckman L870M ultracentrifuge at 100,000*g* at 4°C for 70 minutes.[E12] The final pellet of enriched nanovesicles was resuspended in DPBS.

Quantitative description of immune cell treatment with nanovesicles in adoptive experiments

Culture of spleen and lymph node cells (2×10^7 per milliliter) from 40 tolerized mice yielded approximately 400 mL of supernatant that was applied to the nanovesicle enrichment procedure. This resulted in 4000 μL of resuspended final 100,000*g* pellets containing approximately 1.75×10^{12} vesicles (estimated by using NTA[E13]). In a typical adoptive transfer we used 30 μL of resuspended pellet (about 1.3×10^{10} nanovesicles) per 7×10^7 effector cells per recipient. After incubation for 30 minutes at 37°C, excess nanovesicles were removed from the immune cells by means of centrifugation at 300*g*, and the remaining vesicle-exposed CS effector cells were transferred intravenously to naive recipients to determine the effect of the nanovesicles on the adoptive cell transfer of CS responses.

In vitro non–antigen-specific bioassay to estimate the suppressive activity of exosome-like nanovesicles

This *in vitro* assay developed by us[E14] and confirmed by others[E15] measured the strength of non–antigen-specific suppression of the HT-2 T-cell line (American Type Culture Collection, Manassas, Va) responsiveness to IL-2 by exosome-like nanovesicles from tolerized mice. HT-2 T cells were cultured in high-glucose Dulbecco modified Eagle medium with 5% FCS (centrifuged at 100,000*g* to remove vesicles) and 100 U/mL hrIL-2. At least 2 hours before the test IL-2 was removed from cell culture by proper washing with IL-2 free medium. Then 10^4 cells (50 μL) were plated per microwell into 100 μL of medium containing 3 U/mL hrIL-2, and then nanovesicles were added to the first well in a volume of 50 μL. The starting vesicle concentration was estimated by using NTA before serial 3-fold dilutions. Plates were incubated at 37°C in 5% CO_2, and after 18 hours, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After another 3 to 3.5 hours, 120 μL of acidic isopropanol with 0.04 mol/L HCl was added, and the mixture was incubated overnight in darkness at 25°C. The absorbance of developed dark blue dye was measured at 570 nm, and results were compared with the standard curve. The end point of the assay was the last titered well containing the lowest number of exosomes that led to 50% viability of HT-2 cells. In the standard curve supplementation of HT-2 cells alone with 3 U/mL hrIL-2 represented 100% viability, whereas no hrIL-2 resulted in 0% viability. Then, after processing as above, the middle value of absorbance between 0% and 100% was taken as the 50% viability point to be used as the assay end point. This was repeated at each testing of nanovesicles. The strength of suppression was inversely proportional to the number of nanovesicles needed to reach the end point of 50% viability, which was expressed as the suppressive end point (ie, the number of exosomes × 10^6 per 10^4 HT-2 cells).

Antigen affinity chromatographic separation of TNP–Ts Sup exosome-like nanovesicles on TNP-Sepharose and OX-Sepharose affinity columns

TNP-conjugated BSA (TNP_{22}-BSA) and OX-conjugated BSA (OX_{18}-BSA) were linked to cyanogen bromide–activated Sepharose 4FF (fast flow; Pharmacia, Uppsala, Sweden), according to the manufacturer's procedure. TNP–Ts Sup exosome-like nanovesicles were separated on different affinity syringe columns to avoid cross-contamination. Each run used 15 mL of TNP–Ts Sup filtered through a 0.22-μm filter, omitting the 100,000*g* centrifugation step to avoid vesicle aggregation. The column FT was that portion of the applied nanovesicles that passed through the columns. Then the columns were washed with 6 × 5 mL of DPBS and eluted with guanidine (pH 4.7) at 4°C, which resulted in 2 fractions per column: the FT and the eluate.

Molecular cloning of the antigen affinity column eluate versus FT fractions

RNA obtained from the suppressive exosome-like nanovesicle eluate versus the nonsuppressive FT fractions from the TNP antigen affinity column was sequenced.[E16] RNAs at less than 100 bp from a polyacrylamide sizing gel were isolated to include pre-miRNA and mature miRNA species for cDNA in this size range. First, they were ligated to synthetic oligonucleotide at the 5′ ends and then ligated to another synthetic oligonucleotide at the 3′ ends. Primers complementary to the oligonucleotides were used for reverse transcriptase reactions, and then PCR was used to augment cDNA sequencing by means of Solexa (Illumina, San Diego, Calif). The cDNAs were ligated in a plasmid under the control of a T7 promoter. Then they were amplified and converted to RNA for bioassays attempting to determine the RNA sequence or sequences that in number pertain to the Ts Sup suppressive exosome-like nanovesicles in the eluate greater than those from the FT or *vice versa*.

Bioinformatic analysis of eluate versus FT sequences from TNP-antigen affinity column separation of suppressive TNP–Ts Sup nanovesicles

Solexa deep sequencing of the TNP affinity column–separated exosome-like nanovesicle miRNA of the eluate versus FT column produced 3,394,296 total reads (409,476 eluate and 2,984,820 FT), with 379,153 unique reads.[E17] Sequence lengths ranged between 16 and 25 nucleotides, with a median length of 20 nucleotides. Bowtie (version 0.12.5 of miRBase, http://microrna.sanger.ac.uk/sequences/) was used to map individual reads onto the mouse reference genome (mm9) from UCSC (mouse genome, http://hgdownload.cse.ucsc.edu/downloads.html#mouse) and mouse miRNA sequences in miRBase (release 15 of miRBase, http://microrna.sanger.ac.uk/sequences/). All unique alignments without mismatch were retained for further analysis. Bowtie mapped 22% of the unique reads onto the mouse genome and 0.6% onto 222 distinct mouse miRNAs.[E18] The miRNA reads were kept for further analysis if they met 2 criteria: (1) they could be mapped onto any of the mouse miRNAs without considering the reverse complement, and (2) they did not map onto other positions in the mouse reference genome. These miRNA sequences accounted for 25,963 total reads (1,148 unique reads) and covered 187 distinct mouse miRNAs. Differential expression analysis was carried out with edgeR (version 1.6.5)[E18] to compare TNP-eluate and FT samples. Significance required a *P* value of less than .01 and a false discovery rate of less than 0.05. Because these data did not include replicates, a common dispersion of 0.1 was assumed, as recommended in the edgeR user's guide for genetically identical model organisms. This was consistent with the value of 0.14 estimated for the 50 miRNAs with the lowest absolute fold differences in the eluate versus FT. miRNAs previously associated with exosomes were identified by using ExoCarta.[E19]

Attempted blockage of CS suppression by TNP–Ts Sup exosome-like nanovesicles by using *in vitro* incubation with anti–miR-150

Various anti-miRs (Dharmacon/Thermo Fisher, Uppsala, Sweden) were used to attempt blocking of the biological activity of specific miRNAs present in the mixed suppressor TNP–Ts Sup nanovesicle pool. Per each recipient, we incubated 3 μg (equals 0.2 nmol/L) of specific anti-miRs or control with exosome-like nanovesicles from the 0.22-μm filter. We used the 100,000*g* pellet from TNP–Ts Sup in a 37°C water bath for 1 hour, followed by a second ultracentrifugation in DPBS at 100,000*g* at 4°C for 70 minutes to remove the free anti-miRs. The resuspended pellets of anti-miR–treated nanovesicles were then incubated with 7×10^7 TNP CS effector cells for 30 minutes in a 37°C water bath. Then the CS effector cells were washed at 300*g* with DPBS to remove the non–cell-associated anti-miR–treated nanovesicles and used for intravenous adoptive transfer to naive recipients.

Transfection of miR-150$^{-/-}$ OX–Ts Sup exosome-like nanovesicles with synthetic miRNA-150

OX–Ts Sup nanovesicles from OX-tolerized miR-150$^{-/-}$ mice were transfected with synthetic miR-150, miR-150*, or miR mimic negative control. Nanovesicles derived from 15 mL of supernatants for each group of 5 mice had been processed to the first 100,000*g* ultracentrifugation pellets, which, after resuspension with 150 μL of DPBS, were incubated with 3 μg (equals 0.2 nmol/L) per recipient of miRNA (22 bp, Dharmacon/Thermo Fisher) in a 37°C water bath for 1 hour. This was followed by a second ultracentrifugation in DPBS at 100,000*g* at 4°C for 70 minutes to remove the free miRNA. Then the transfected exosome-like nanovesicles from the second pellet were used for incubation with OX CS effector cells at 37°C for 30 minutes. After washing by means of centrifugation at 300*g* with DPBS, the cells exposed to transfected nanovesicles were adoptively transferred into naive recipients.

Statistical analysis

Each mouse provided a statistical "n" of 1. Thus after ear challenge, the thickness of each ear of each mouse was measured in duplicate by a blinded observer using a micrometer (Mitutoyo). Four measurements per mouse were pooled to determine the mean ear swelling for each mouse, which was a reflection of CS inflammation. The average ear swelling expressed as the Δ ± SE (after subtraction of the negative control value) was calculated for each group. We used 4 to 6 mice for each experimental and control group. All experiments were carried out 2 to 4 times. The 2-tailed Student *t* test, ANOVA, and *post hoc* RIR Tukey tests were used to assess the significance of differences between groups, with *P* values of less than .05 taken as the minimum level of statistical significance.

REFERENCES

E1. Chen J, Trounstine M, Alt FW, Young F, Kurahara C, Loring JF, et al. Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. Int Immunol 1993;5:647-56.

E2. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 2007;131:146-59.

E3. KaLahl K, Sparwasser T. In vivo depletion of FoxP3+Tregs using the DEREG mouse model. Methods Mol Biol 2011;707:157-72.

E4. Redegeld FA, van der Heijden MW, Kool M, Heijdra BM, Garssen J, Kraneveld AD, et al. Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. Nat Med 2002;8:694-701.

E5. Ptak W, Rozycka D, Rewicka M. Induction of suppressor cells and cells producing antigen-specific suppressor factors by haptens bound to self-carriers. Immunobiology 1980;156:400-9.

E6. Ptak W, Rosenstein RW, Gershon RK. Interactions between molecules (subfactors) released by different T cell sets that yield a complete factor with biological (suppressive) activity. Proc Natl Acad Sci U S A 1982;79:2375-8.

E7. Ptak W, Gershon RK, Rosenstein RW, Murray JH, Cone RE. Purification and characterization of TNP- specific immunoregulatory molecules produced by T cells sensitized by picrylchloride (PC1F). J Immunol 1983; 131:2859-63.

E8. Paliwal V, Tsuji RF, Szczepanik M, Kawikova I, Campos RA, Kneilling M, et al. Subunits of IgM reconstitute defective contact sensitivity in B-1 cell-deficient xid mice: kappa light chains recruit T cells independent of complement. J Immunol 2002;169:4113-23.

E9. Eldh M, Ekström K, Valadi H, Sjöstrand M, Olsson B, Jernas M, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS One 2010;5:e15353.

E10. Meckes DG Jr, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. Proc Natl Acad Sci U S A 2010;107:20370-5.

E11. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, et al. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 2010;107:6328-33.

E12. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3.22.

E13. Soo CY, Song Y, Zheng Y, Campbell EC, Riches AC, Gun-Moore E, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology 2012;136:192-7.

E14. Ferreri NR, Herzog WR, Askenase PW. Inhibition of IL-2-dependent proliferation by a prostaglandin- dependent suppressor factor. J Immunol 1993;150:2102-11.

E15. Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Res 2007;67:7458-66.

E16. Hafner M, Landgraf P, Ludwig J, Rice A, Ojo T, Lin C, et al. Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. Methods 2008;44:3-12.

E17. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009; 10:R25.

E18. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139-40.

E19. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acid Res 2012;40:D1241-4.

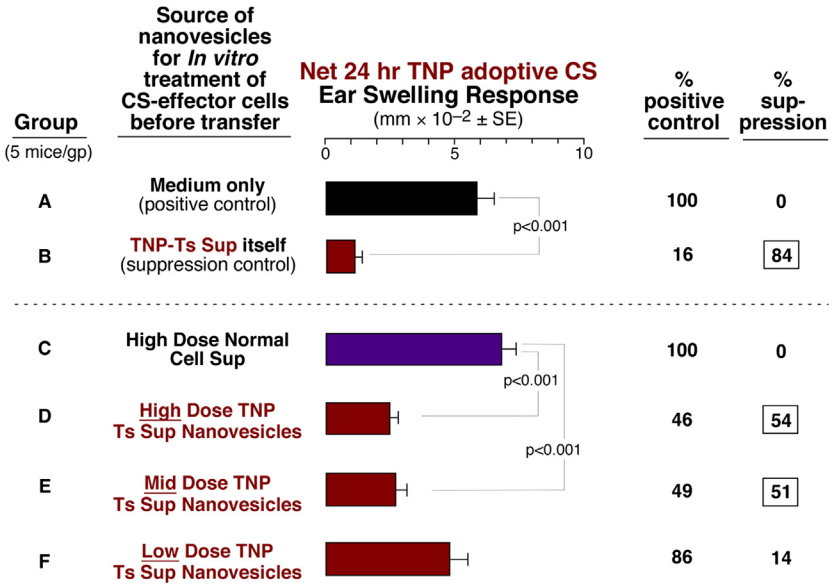


FIG E1. TNP–Ts Sup exosomes mediate a dose-dependent inhibition of TNP CS effector cells. High-dose NI Cell nanovesicles did not mediate suppression *(group C)*, whereas TNP–Ts nanovesicles administered at a dose 72×10^7 per recipient *(group D)* and serially 5-fold diluted (*groups E* and *F*) showed dose-dependent suppression.

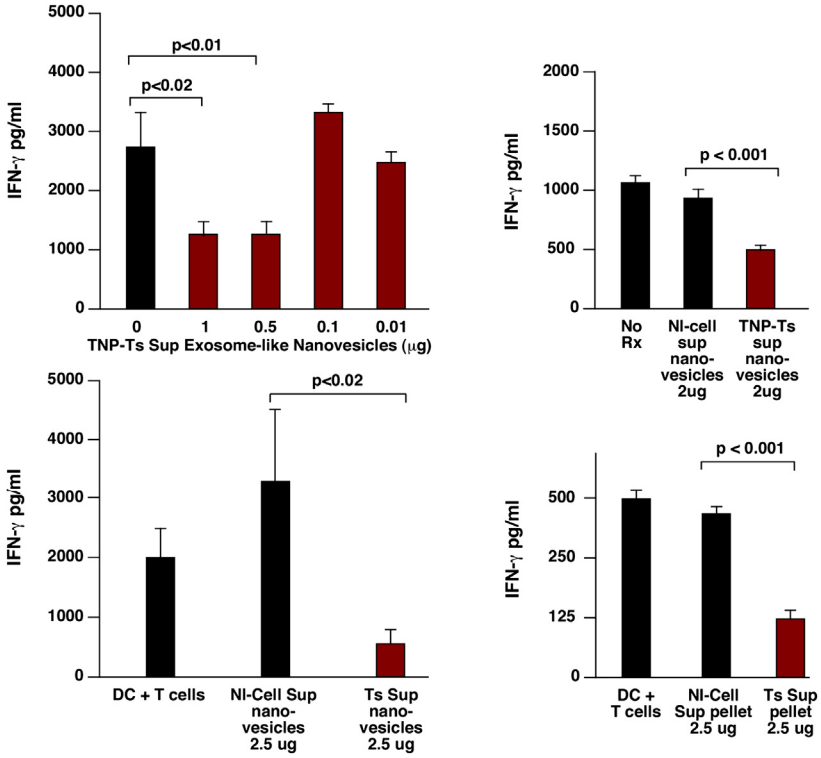
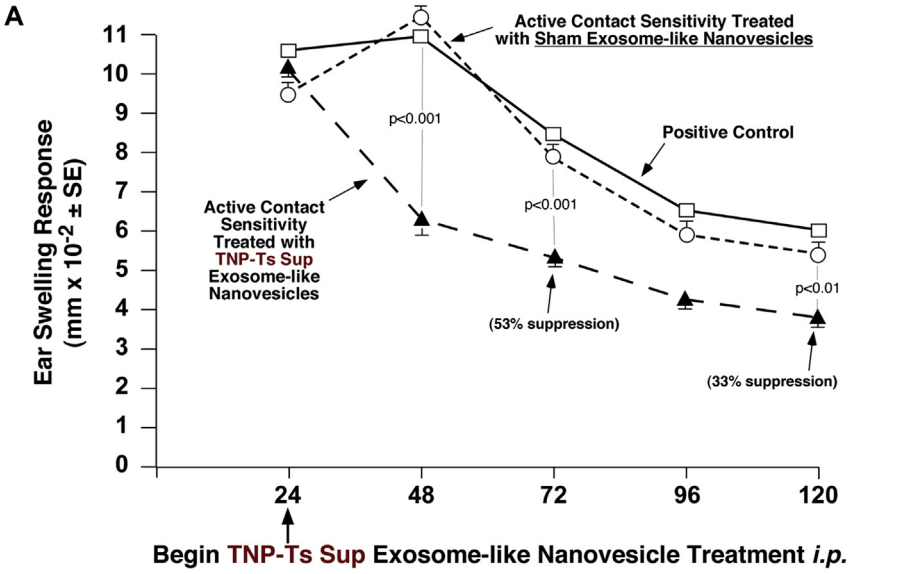


FIG E2. An antigen-specific *in vitro* assay confirms that the 100,000*g* pellet exosome-like nanovesicles have all the suppressive activity of the Ts Sup. TNP-conjugated dendritic cells *(DC)* and TNP CS effector cells were cocultured in the presence or absence of TNP-Ts nanovesicles or control NI Cell Sup nanovesicles. IFN-γ was measured as a read out for CS-effector cell activation. The 4 experiments shown confirmed *in vitro* the suppressive antigen-dependent activity of exosome-like nanovesicles derived from the Ts Sup.



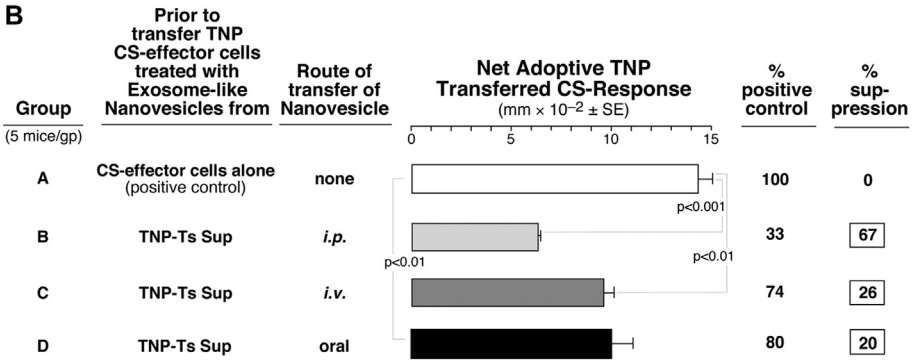


FIG E3. *In vivo* treatment with suppressive exosome-like nanovesicles shows prolonged inhibition of established CS responses in actively sensitized mice. **A,** TNP–Ts Sup versus control sham supernatant nanovesicles were injected intraperitoneally in a single dose at 24 hours of an ongoing CS response of actively sensitized and ear-challenged mice. Only TNP–Ts Sup nanovesicles significantly suppressed CS up to 120 hours. **B,** TNP–Ts Sup nanovesicles were injected intraperitoneally or intravenously or administered orally with a single dose at 24-hour responses of an ongoing CS response in actively sensitized and ear-challenged mice. Treatment through all routes was successful, with the intraperitoneal route being most effective. *i.p.*, Intraperitoneal; *i.v.*, intravenous.

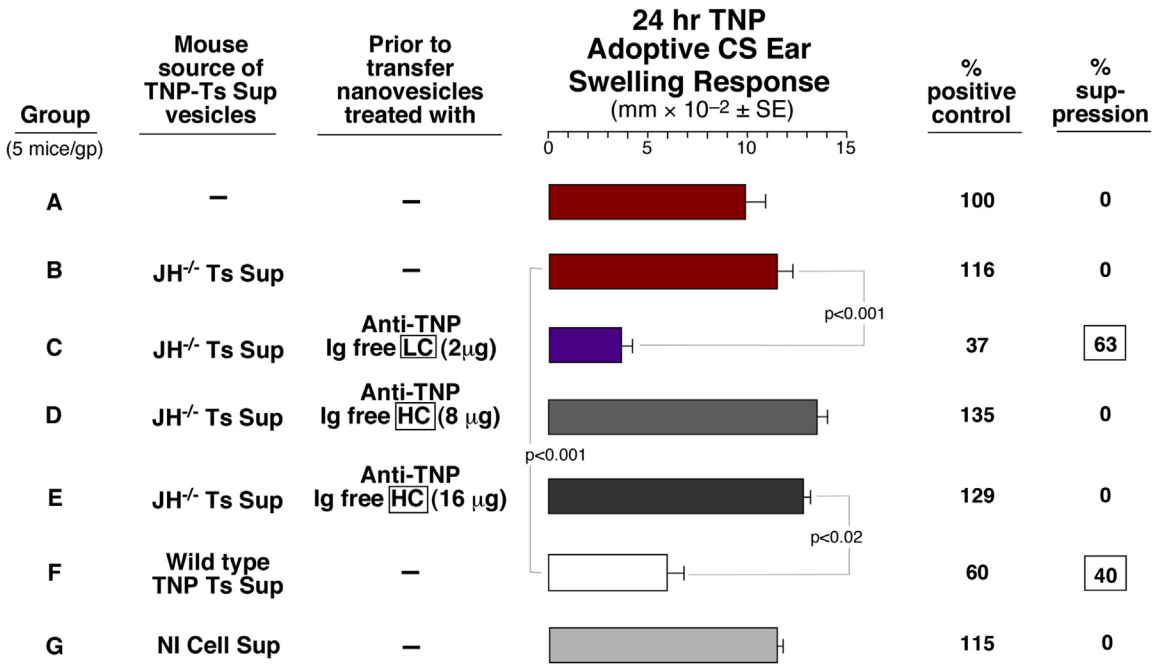


FIG E4. Anti-TNP mAb light chains *(LC)*, but not heavy chains *(HC)*, sensitize exosome-like nanovesicles for suppression of adoptive CS. Nanovesicles harvested from tolerized $JH^{-/-}$ mice were pelleted and sensitized *in vitro* with various doses of anti-TNP antibody light or heavy chains before washing of the unbound chains at 100,000*g*. Only sensitizing the nanovesicles with antibody light chains restored their suppressive capacity *(group C)*.

TABLE E1. Mouse miRNAs significantly upregulated in the TNP antigen affinity column eluate compared with the FT

	Log (concentration)	Log (fold change)	*P* value	FDR	No. of reads, FTX	No. of reads, ETX
mmu-mir-92a-1	−12.70	5.79	1.76E-12	3.19E-10	60	457
mmu-mir-33	−16.44	8.13	3.41E-12	3.19E-10	2	77
mmu-mir-27a	−15.36	5.96	1.89E-10	1.18E-08	9	77
mmu-mir-484	−16.19	6.00	7.51E-10	3.51E-08	5	44
mmu-mir-296	−31.74	36.54	5.26E-09	1.40E-07	0	36
mmu-mir-92a-2	−17.20	6.62	7.60E-08	1.78E-06	2	27
mmu-mir-150	−13.11	3.69	8.95E-07	1.67E-05	94	167
mmu-mir-210	−17.95	7.11	1.72E-06	2.52E-05	1	19
mmu-mir-486	−17.95	7.11	1.72E-06	2.52E-05	1	19
mmu-let-7b	−13.83	3.54	2.94E-06	3.67E-05	60	96
mmu-mir-184	−32.29	35.46	5.19E-06	6.06E-05	0	17
mmu-let-7i	−11.32	3.17	9.88E-06	1.09E-04	389	479
mmu-mir-423	−14.17	3.39	1.15E-05	1.19E-04	50	72
mmu-mir-500	−32.48	35.07	4.53E-05	4.46E-04	0	13
mmu-mir-153	−32.60	34.83	1.65E-04	1.47E-03	0	11
mmu-mir-328	−17.78	4.28	1.01E-03	8.55E-03	3	8
mmu-mir-222	−16.38	3.09	1.18E-03	9.56E-03	12	14
mmu-mir-1964	−32.83	34.37	1.41E-03	1.10E-02	0	8
mmu-mir-221	−15.31	2.50	2.35E-03	1.69E-02	31	24
mmu-mir-339	−17.42	3.54	4.07E-03	2.53E-02	5	8
mmu-mir-147	−33.04	33.96	6.15E-03	3.38E-02	0	6
mmu-mir-98	−18.12	3.60	9.22E-03	4.93E-02	3	5

The *P* values were adjusted for multiple testing by using the Benjamini and Hochberg approach for controlling the false discovery rate and are exact *P* values computed by using edgeR.

ETX, Eluate column; *FDR*, false discovery rate; *FTX*, FT column; *Log (concentration)*, overall concentration for an miRNA across the 2 groups being compared; *Log (fold change)*, log fold change for the counts between groups.

TABLE E2. Mouse miRNAs significantly upregulated in the TNP antigen affinity column FT (FTX) compared with the eluate (ETX)

	Log (concentration)	Log (fold change)	*P* value	FDR	No. of reads, FTX	No. of reads, ETX
mmu-mir-29a	−10.63	−4.74	9.81E-10	3.67E-08	9766	50
mmu-mir-148a	−13.75	−5.14	5.23E-09	1.40E-07	1288	5
mmu-mir-26b	−12.99	−3.90	8.93E-07	1.67E-05	1411	13
mmu-mir-148b	−15.89	−5.51	1.76E-06	2.52E-05	333	1
mmu-mir-340	−16.00	−5.29	2.88E-06	3.67E-05	285	1
mmu-mir-696	−32.48	−35.08	1.60E-04	1.47E-03	95	0
mmu-mir-2133-2	−14.35	−2.59	1.90E-03	1.42E-02	350	8
mmu-mir-20a	−15.55	−3.01	2.47E-03	1.71E-02	176	3
mmu-mir-29c	−16.74	−3.81	3.49E-03	2.33E-02	102	1
mmu-mir-191	−15.03	−2.58	3.82E-03	2.46E-02	218	5
mmu-mir-712	−14.49	−2.30	5.10E-03	3.07E-02	287	8
mmu-mir-155	−33.10	−33.83	5.48E-03	3.10E-02	40	0
mmu-mir-362	−33.10	−33.83	5.48E-03	3.10E-02	40	0

Shown here are miRNA sequences more frequent in the FT versus the eluate. For column descriptions, please see the footnote for Table E1.
ETX, Eluate column; *FDR*, false discovery rate; *FTX*, FT column.