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

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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

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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