

REVIEW

Approaches to inducing antigen-specific immune tolerance in allergy and autoimmunity: Focus on antigen-presenting cells and extracellular vesicles

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Abstract

Increasing prevalence of allergic and autoimmune diseases urges clinicians and researchers to search for new and efficient treatments. Strategies that activate antigen-specific immune tolerance and simultaneously maintain immune reactivity to all other antigens deserve special attention. Accordingly, antigen-presenting cells (APCs) seem to be the best suited for orchestrating these mechanisms by directing T cell immune responses towards a tolerant subtype. Recent advances in understanding cell-to-cell communication via extracellular vesicles (EVs) make the latter promising candidates for reprogramming APCs towards a tolerant phenotype, and for mediating tolerogenic APC function. Thus, comprehensive studies have been undertaken to describe the interactions of APCs and EVs naturally occurring during immune tolerance induction, as well as to develop EV-based manoeuvres enabling the induction of immune tolerance in an antigen-specific manner. In this review, we summarize the findings of relevant studies, with a special emphasis on future perspectives on their translation to clinical practice.

1 | INTRODUCTION

Immune suppression refers to a condition of diminished responsiveness of immune cells to various antigens, leading to significant impairment of humoral or cellular immunity. Known causes are congenital and acquired pathologies, such as primary immune deficiencies and HIV infection respectively. Besides, it is a condition often induced pharmacologically by treatment of autoimmune or allergic disorders. In contrast, immune tolerance describes an inhibition of immune responsiveness to either a group of related antigens, including self-tolerance to autoantigens and oral tolerance to food antigens, or a particular antigen, thus displaying strict antigen-specificity. From a clinical point of view, antigen-specific tolerance manifests itself as an inhibition of immune responsiveness to relevant antigenic entities that would otherwise cause unwanted immune responses, for example those

underlying autoimmune-, allergic- or hypersensitivity-related disorders. It is worth noting that in some cases, allergic or autoimmune responses are directed against multiple antigens. Thus, a concerted activation of mechanisms of specific immune tolerance to disease-related antigens, which still preserve immune reactivity to all other, non-related antigens, would offer the greatest therapeutic benefit in cases with an already identified causative antigen (antigens). Hence, current studies frequently focus on discovering novel pathways of induction, restoration or redirection of antigen-specific tolerance,¹ which are summarized in the current review.

As antigen-presenting cells (APCs) are the central players in directing T cell immune responses towards either effector or tolerant subtypes, an involvement in the induction of antigen-specific immune tolerance mechanisms seems obvious. Currently, studies on anti-cancer immunotherapies aim at breaking immune tolerance to tumour-derived antigens,

and thereby facilitate our understanding of the mechanisms behind the tolerogenic activities of APCs. However, little is known about the possibilities to redirect APCs towards this tolerant phenotype in autoimmune, allergic and hypersensitivity responses. Since currently available immune suppressive drugs impair immune responses non-selectively, elaboration of therapeutic strategies allowing for an induction of antigen (allergen)-specific immune tolerance would greatly minimize the adverse effects of clinical treatments of allergies and hypersensitivities. As similar immune mechanisms underlie the pathogenesis of various other conditions, including autoimmunity, transplant rejection and inflammatory disorders accompanying certain metabolic, cardiovascular and neuroendocrine diseases, the usage of new strategies for inhibiting unwanted (excessive) immune responses provoked by a particular antigen (like allergen, self-antigen), without any side-effects on the general immunity of the body, would greatly improve efficacy and safety of therapies against said conditions. In addition, extracellular vesicles (EVs) are considered very interesting candidates for therapeutic usage, as they can enclose regulatory molecules, and hence would allow for their delivery to desired target cells. Thus, this review summarizes the current knowledge on EV-mediated mechanisms of antigen-specific immune tolerance involving APCs, predominantly induced in the context of allergies, hypersensitivity and autoimmune responses.

1.1 | APCs as key inducers of immune tolerance

The term 'antigen-presenting cells' refers to cells that can present antigens to naive T lymphocytes to activate them for performing their specific effector functions. Apart from endogenous antigenic determinants (processed from self-proteins, and viral or tumour antigens) complexed with major histocompatibility complex (MHC) class I molecules, dendritic cells, B lymphocytes and macrophages, the professional APCs of the immune system, are able to present determinants of exogenous antigens in complex with MHC class II molecules. Thus, these cells are considered key players in T cell activation, as well as in shaping and orchestrating effector immune responses to any kind of antigen. On the other hand, APCs of the tolerant phenotype are involved in maintaining immune tolerance, but in some instances also in the deleterious suppression of specific, anti-tumour or anti-microbial responses. After breakage of immune tolerance or in the case of susceptible individuals, APCs potently present fragments of allergens or self-antigens, which, in fact, make them directly responsible for the induction of allergic or autoimmune responses. Therefore, APCs recently became an interesting

target for experimental and therapeutic strategies aiming at suppressing unwanted immune responses by phenotype switching from conventional to tolerogenic APCs. As APCs interact with antigen-specific T cells, such strategies should allow for an activation of mechanisms mediating immune tolerance solely to a particular antigen. Indeed, under physiological conditions, APCs are involved in the induction and maintenance of tolerance in immune privileged organs,² of peripheral tolerance to self-antigens,³ the natural microbiota of the body,⁴ and food-derived antigens (oral tolerance),^{5,6} and, likely, of central immune tolerance as well.^{7,8} On the other hand, APCs are responsible for immune dysregulation during chronic inflammation and deleterious immune unresponsiveness to tumour and malignant cells.^{9,10} Thus, attempts to use APCs presenting tumour-derived immunogenic determinants,¹¹⁻¹³ EVs loaded with tumour antigens that were targeted to APCs or EVs secreted by APCs, predominantly DCs (then so-called 'dexosomes'),^{14,15} may break the harmful immune unresponsiveness. However, APCs can be reprogrammed to express a tolerogenic phenotype, and in turn downregulate immune responses of antigen-specific T lymphocytes. This is of great importance for suppressing excessive inflammation and unwanted immune responses, activated, for instance, by allergens or self-antigens.

1.2 | EVs as mediators of tolerogenic APC function

Heterogeneous populations of EVs represent a recently discovered mode of intercellular communication. EVs differ in size and origin,¹⁶ and were thus classified into small EVs of bilamellar membrane that are formed inside endosomal compartments (multivesicular bodies, MVB), and EVs of various sizes that are budding off from the cell membrane.^{17,18} Small EVs, like exosomes, receive special attention from researchers due to their capability of delivering various biologically active compounds, including non-coding RNA molecules.¹⁸ Furthermore, EVs mediate intercellular signalling at the immune synapse, thus allowing for limited activity on cells that present and recognize a particular antigen.¹⁹ Firstly, B lymphocytes were observed to exocytose exosomes bearing MHC class II molecules,²⁰ which gave rise to an enormous progress in investigations of APC-derived EV biology, functions and possible applications. Other studies demonstrated that EVs derived from MHC class II-expressing J774 macrophage cells infected with intracellular pathogens possessed proinflammatory activity,²¹ in contrast to suppressive EVs secreted by dendritic cells (DCs) genetically modified to express Fas ligand.²² Finally, DCs were suggested to constitutively release exosomes that seem to bud off from the

endosomal membrane of the MHC class II compartment in a phosphatidylinositol 3-kinase-dependent manner.²³ Due to the expression of MHC class II, APC-derived EVs, especially exosomes, were suggested to be able to substitute APC function in activating T cells.²³ Accordingly, immature DCs interacting with T cells promote a tolerant rather than an effector phenotype in contrast to mature DCs, which, importantly, were shown to secrete EVs enriched in MHC class II molecules that have the potential to activate T cells.²⁴ On the other hand, one can speculate that MHC-bearing EVs may activate T cell anergy rather than proliferation in the absence of costimulatory molecules, as was proposed for synthetically generated nanoparticles.²⁵ Interestingly, DCs were suggested to amplify their function by releasing EVs that target neighbouring DCs.²⁶ Simultaneously, T cell-derived EVs that are transmitted to APCs at the immune synapse may induce their apoptosis.^{27,28} Overall, these observations provide evidence that APC-derived EVs are involved in immune regulation by modulating T cell activation processes, thereby further contributing to tolerance induction.

2 | ANTIGEN-SPECIFIC TOLERANCE IN AUTOIMMUNE RESPONSES

Autoimmune responses can be mediated by autoantibodies or by cytotoxic and helper T (Th) cells, which are involved in delayed-type hypersensitivity (DTH) reactions.²⁹ During the induction phase of autoimmunity, tissue-derived EVs may deliver self-antigens to APCs, which in turn activate self-reacting T cells.^{23,30} Furthermore, circulating EV-contained self-antigens may trigger autoimmune or autoinflammatory reactions by formation of immune complexes, as observed in systemic lupus erythematosus (SLE).^{31,32} Finally, overloading of splenic macrophages with apoptotic cell material was suggested to provoke EV-mediated transmission of autoantigens to DCs, in turn activating T cell responses,³³ which may trigger autoimmunity. On the other hand, APC-derived EVs constitute promising candidates for tolerance-inducing therapies in cases of existing autoimmunity, without undergoing a phenotype switch after systemic administration, in contrast to the parental cell.²³ Thus, EVs of various cellular sources are considered promising tools for immunotherapy.³⁴ Numerous attempts were undertaken to manipulate DCs and the resulting EVs to redirect them towards a tolerogenic phenotype and activity to eventually suppress autoimmune responses.^{23,35} Antigen-specific immune tolerance could then be induced either directly by suppressing self-antigen-specific effector T cells and/or B lymphocytes releasing autoantibodies, or indirectly by activation of antigen-specific regulatory T (Treg) cells.

2.1 | APCs in antigen-specific tolerance in autoimmunity

As APCs provide the first signal about an antigen to activate specific T lymphocytes, they are also the first cells to initiate tolerance-associated signalling, in some circumstances antigen-specifically.

2.1.1 | Tolerogenic APCs

As recently reviewed,^{36,37} several approaches have been undertaken so far to induce a tolerogenic APC phenotype in autoimmune conditions. Some enabled further induction of antigen-specific tolerance. One of them was based upon activation of human tolerogenic DCs with IL-10, which could then be transferred to patients with autoimmune diseases or allergies to induce type 1 regulatory T (Tr) cells.³⁸ Accordingly, other promising attempts to activate antigen-specific immune tolerance in autoimmunity include the delivery of self-antigens to tolerant APCs, which then inhibit effector T cells or activate Treg lymphocytes. In this regard, splenocytes were used as carriers of autoantigens to ameliorate the course of experimental autoimmune encephalomyelitis (EAE).^{39,40} In certain aspects, these effects resemble the induction of immune tolerance via intravenous injection of antigen-coupled syngeneic red blood cells described hereafter. Along these lines, senescent red blood cells are cleared by splenic and hepatic macrophages,^{41,42} which may be followed by tolerogenic antigen presentation. Thus, it was presumed that antigens bound to self-red blood cells should be similarly tolerated after presentation by tolerogenic APCs. Testing of this assumption indeed demonstrated that the development of persistent immune tolerance to red blood cell-bound antigens was mediated by Treg cells.⁴³ Furthermore, intravenously administered autoantigens covalently bound to self-red blood cells were proved to induce immune tolerance, in certain circumstances, to reverse early symptoms of EAE and to protect NOD/ShiLtJ mice against type 1 diabetes.⁴⁴ The anticipated antigen-specificity of the tolerance induced, which seems to be conferred by APCs at the time of antigen presentation, is highly possible in this scenario.

2.1.2 | Interaction of APCs with Treg cells

Differentiation of antigen-specific Treg cells for possible clinical applications usually requires APC-induced signalling, mostly by DCs,⁴⁵ which depends on the subset of DCs and the receptors involved.⁴⁶ Consequently, differentiated Treg cells are believed to suppress effector cells of

corresponding specificities, as proposed for treatment of autoimmune hepatitis.⁴⁵ Clinically, the antigen-specificities occurring in the context of tolerogenic effects could be partly determined by evaluating the percentage of antigen-specific effector and regulatory cells and their phenotypes,⁴⁷ along with the putative changes in susceptibility to infections or response to vaccination. In addition, it could be speculated that Treg cells activated by APCs may in turn inhibit this APC to prevent activation of self-reactive T lymphocytes. Along these lines, Treg cells were shown to be capable of removing costimulatory molecules, that is, CD80 and CD86, from cell membranes of neighbouring APCs via trans-endocytosis,⁴⁸ and a recent report demonstrated that DC-activated, antigen-specific Treg cells could even remove peptides complexed with MHC class II molecules from DC membranes.⁴⁹ Both processes significantly impaired antigen presentation to naive T cells, which was suggested as one of the possibilities by which Treg cells maintain immune homeostasis. Furthermore, activated Treg cells were reported to inhibit DC recruitment to lymph nodes and their mature functionalities, which led to an amelioration of EAE.⁵⁰

Transdermal application of myelin peptides serves as an example of experimentally elaborated treatment that has been successfully implemented into clinical trials for multiple sclerosis therapies.⁵¹ Under experimental conditions, treatment with antigen via the transdermal route was able to induce antigen non-specific immune suppression,⁵² which in a mouse EAE model was mediated by CD4+ CD8+ suppressor T cells.⁵³ In patients with multiple sclerosis, myelin peptides crossing the skin barrier activated skin-resident DCs, which at the periphery led to an induction of Tr1 cells that in turn alleviated the clinical symptoms of the disease.⁵⁴ Immune reactivity of skin-resident cells resembles mucosal immunity, and thus, epicutaneously induced immune suppression may reflect oral tolerance, at least to some extent.⁵² Therefore, although the specificity and involved intercellular signalling pathways have not been ascertained so far, it would be relevant to verify if this mechanism could be manipulated to become antigen-specific, with a special emphasis on skin DC-induced signalling cascades.

Along these lines, myeloid APCs were suggested to specialize in activation and maintenance of FoxP3+ sTreg cells after vaccination of mice with granulocyte-macrophage colony-stimulating factor (GM-CSF) covalently bound to MOG₃₅₋₅₅. As a consequence, induced Treg cells ameliorated EAE. GM-CSF binding to the corresponding receptors on myeloid APCs was reported to trigger selective cellular targeting and further promote tolerogenic presentation of MOG peptides to induce the generation of Treg lymphocytes instead of effector T cells.⁵⁵

Another reported possibility to induce antigen-specific immune tolerance in EAE was based on in vitro stimulation of B cells with cholera toxin B subunit-conjugated MOG₃₅₋₅₅.

After adoptive transfer, these B cells induced antigen-specific FoxP3+ Treg cells that in turn alleviated the clinical symptoms of EAE.⁵⁶ Interestingly, a similar tolerance phenotype could also be experimentally induced in DTH after oral or sublingual administration of antigen conjugated to cholera toxin.^{57,58} Moreover, oral administration of type II collagen alone was shown to induce immune tolerance resulting from Treg cell activation by DCs of gut-associated lymphoid tissue. These cells, in turn, ameliorated the course of collagen-induced arthritis (CIA) in mice fed with collagen.⁵⁹

Recently, antigen-specific Treg cells differentiated in vitro by culturing them in the presence of type II collagen, IL-2, transforming growth factor (TGF)- β 1, retinoid acid and CD11b+ or CD11c+ APCs were demonstrated to ameliorate CIA in mice after adoptive transfer, and to inhibit proliferation of CD4+ effector T cells in the presence of irradiated splenocytes.⁶⁰ These observations strongly suggested that, in particular circumstances, APCs could promote the development of antigen-specific Treg cells to alleviate the symptoms of autoimmune diseases, and are likely to induce immune tolerance. In contrast, already acknowledged means of in vitro activation of Treg cells via T cell receptor (TCR) stimulation with antigen generally lead to antigen-independent immune suppression in vivo.^{49,61} This suggests that APCs may provide crucial signals that make Treg cells capable of further antigen-specific action in vivo.

2.2 | EVs in antigen-specific tolerance in autoimmunity

At present, EVs of various sources are frequently studied for their actual involvement in the induction of antigen-specific immune tolerance in autoimmunity.

2.2.1 | EVs carrying self-antigens

Dendritic cells may be modified by EVs to generate split tolerance, as shown in the case of maternal microchimerism associated with cross-dressing of host DCs by EV-transmitted alloantigens.⁶² This research finding greatly contributes to increasing the knowledge on tolerance induction to alloantigens in transplantation or reproduction, but it might also provide the possibility to manipulate EVs carrying autoantigens for an autologous tolerogenic function. In this regard, split tolerance may be defined as the suppression of a pathogenic self-reactive T cell subset with no influence on T cells of other specificities. Accordingly, in the case of Th1-dependent autoimmune responses, delivery of self-antigen by EVs should reprogram DCs in a manner that allows suppressing only self-reactive Th1 lymphocytes, with no impact on the interaction of these DCs with other CD4+ T cell subsets.

CD22 and sialic acid-binding immunoglobulin-like lectin (Siglec)-G inhibiting B cell receptor (BCR)-dependent signalling in B lymphocytes were demonstrated to mediate peripheral B cell tolerance to self-antigens after binding of sialic acids,^{63,64} which was associated with apoptosis of B cells.⁶⁵ Thus, decorating antigens with high affinity ligands of these receptors was proposed as a tool to induce

antigen-specific B cell tolerance.^{64,66} This has proved effective in the case of mouse red blood cells originally deprived of such ligands.⁶⁵ These research findings suggest the possibility of engineering autoantigen-bearing EVs expressing ligands for Siglec receptors, such as sialic acid, which could greatly enhance their tolerogenic potential in an attempt to suppress or deplete autoreactive B cells in

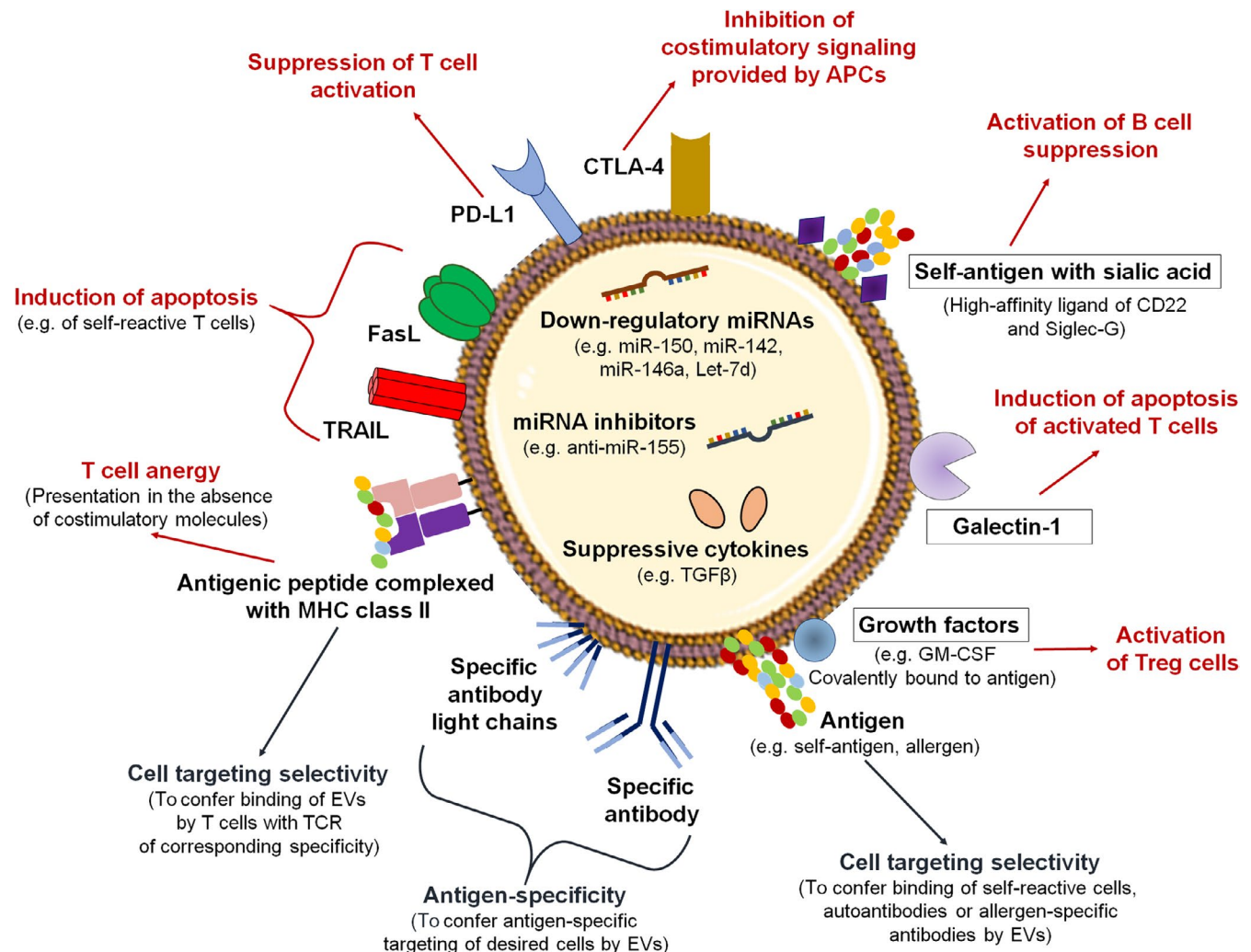


FIGURE 1 Perspectives in arming extracellular vesicles with a tolerance-inducing weapon. Extracellular vesicles (EVs) have been proposed to constitute an effective tool for induction of immune tolerance. For this purpose, EVs could be equipped with antigen-specific antibodies or light chains ensuring the strict antigen-specificity of cell targeting, or with surface-linked native antigen or antigenic peptide bound to major histocompatibility complex (MHC) groove, which would confer the targeting selectivity by favouring EV binding by, respectively, antibody/B cell receptor or T cell receptor of corresponding antigen-specificity. Interaction of T cells with EVs bearing peptide-MHC complexes may lead to T cell anergy due to the lack of costimulatory signalling. Similarly, costimulatory molecules on antigen-presenting cells (APCs) can be blocked by cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) on EV surface. By expression of Fas ligand, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) or galectin-1, EVs could induce apoptosis of targeted cells, while the expression of programmed death-1 ligand-1 (PD-L1) may prevent activation of T lymphocytes. B cells may be suppressed through the interaction of EV-transmitted sialic acid coupled to self-antigens with CD22 or Siglec-G expressed by B2 or B1a lymphocytes respectively. EVs may also be decorated with self-antigens with covalently bound growth factors, which have been suggested to activate regulatory T (Treg) cells, similarly to EV-contained suppressive cytokines, transforming growth factor beta (TGF-β) especially. Finally, EVs can deliver either regulatory microRNAs (miRNAs) interfering with messenger RNA of targeted cells or miRNA antagonists (anti-miRs) inhibiting miRNA activity in acceptor cell, both to induce long-lasting effects at genetic level. Combination of these factors, suited to the type of targeted cell presumably, would induce the expected tolerogenic effect in an antigen-specific manner. Molecules that have not been yet reported to mediate EV-induced effects are framed

the periphery (Figure 1). Similarly, one can speculate that using self-EVs as vehicles for autoantigens may produce more effective results than encapsulating autoantigens in synthetic liposomes or nanoparticles. The latter constructs were proposed for treatment of rheumatoid arthritis via targeted DC-T cell interaction, and some of these strategies have already entered phase I clinical trials, as recently reviewed.⁶⁷

It should be stressed that dysregulated responses of follicular Th cells and follicular Treg cells are linked to the pathogenesis of SLE and other antibody-mediated autoimmune diseases,⁶⁸ as well as to transplant rejection.⁶⁹ During germinal centre reactions, follicular Th cells promote B cell maturation and antibody formation, while follicular Treg cells suppress autoimmune responses by the expression of potentially self-reactive TCRs.⁷⁰ However, recent findings suggest that follicular Th cells, but not Treg cells, could benefit from interactions with antigen-pulsed DCs.⁷⁰ This, in turn, may result in an imbalanced expansion of follicular Th cells, leading to excessive, unregulated production of autoantibodies or transplant-reactive antibodies.⁶⁹ Thus, strategies skewing the ratio of regulatory and helper T cells in germinal centres would allow us to control the process of autoantibody formation. These strategies could be based on autologous EVs targeting cells in germinal centres,⁷¹ since allogeneic EVs seem to potently activate anti-tumour immunity by enhancing follicular Th cell function.⁷²

2.2.2 | APC-derived EVs

Attempts to evoke antigen-specific tolerance in inflammatory and autoimmune colitis, including Crohn's disease, are limited, as the causative antigens are poorly characterized. However, in those conditions, APCs are also considered promising mediators of immune tolerance. Accordingly, DCs tolerized with IL-10 were demonstrated to secrete EVs that attenuated experimentally induced colitis in rats, likely by downregulating proinflammatory cytokine production and activating Treg cell infiltration in colonic lamina propria tissues.⁷³ From another point of view, outer membrane vesicles secreted by the commensal microbiota could be sensed by DCs, that further activate Treg cells to ameliorate the course of experimentally induced colitis.⁷⁴ Conversely, EVs secreted by DCs treated with pathogen-derived antigens *in vitro* were shown to suppress the clinical symptoms of colitis when transferred to mice.⁷⁵ Both observations suggest an important role for microbial products in the regulation of autoimmune responses. However, one can speculate that the desired suppressive effects of pathogenic antigens depend solely on redirecting DCs

towards an anti-microbial function, but not on inducing immune tolerance.

Inducible overexpression of miRNA-146a impaired maturation of DCs after LPS stimulation. Consequently, these immature DCs released EVs with decreased expression of CD80 and CD86 costimulatory molecules, when compared to EVs secreted by DCs treated with LPS only. Furthermore, EVs released by miRNA-146a-overexpressing DCs cultured in the presence of T $\alpha_{146-162}$ peptide were demonstrated to ameliorate clinical symptoms of ongoing experimental myasthenia gravis after intravenous administration in mice. The actions of EVs could be considered at least antigen-dependent, as they inhibited the proliferation of mouse splenocytes stimulated with T $\alpha_{146-162}$ peptide or torpedo acetylcholine receptor, but not with concanavalin A.⁷⁶ However, it is worth mentioning that APC-induced effects are commonly declared antigen-specific when they lead to an inhibition of antigen-specific effector T cell function and/or proliferation, or when found to be MHC-restricted.⁷⁷

According to the above considerations and studies on Treg cell activation by APCs,⁵⁵ one can speculate that surface expression of specific cytokines or growth factors on EVs can not only drive cellular trafficking and targeting of the latter, but also greatly support their tolerogenic function (Figure 1). In addition, our research findings showed that orally administered EVs may also efficiently suppress an ongoing immune response, as discussed below. Since treatment is generally well accepted by patients when administered orally, this route should be considered an interesting modality for antigen-specific immunotherapy.

2.2.3 | Natural Tr cell-derived EVs

Reduced cytotoxic activity of CD8+ T cells was observed in mice immunized with ovalbumin-pulsed DCs co-cultured with natural CD8+ CD25+ Tr cells or EVs secreted by those cells. Furthermore, this study demonstrated a diminished cytotoxic killing capacity of CD8+ effector T cells against both ovalbumin-bearing melanoma cells and target cells loaded with ovalbumin peptide, when compared to cells loaded with control peptide, suggesting that EVs may substitute Tr cells in the maintenance of self-tolerance and prevention of autoimmunity.⁷⁸ EV-secreting CD8+ CD25+ Tr cells were presumably prestimulated by co-incubation with ovalbumin-pulsed DCs, which allowed to assume an antigen dependence of the expanded T cells. Similarly, polyclonal CD8+ CD25+ Tr cells were suggested to be converted to an antigen-specific, immune suppressive cell population after treatment with EVs-carrying peptide/MHC complexes that were secreted by ovalbumin-pulsed DCs.⁷⁹

2.2.4 | Treg cell-derived EVs and induction of tolerogenic APCs

As mentioned above, numerous experimental attempts were undertaken to verify if APCs could be redirected towards a tolerogenic phenotype in autoimmunity. Recently, Treg lymphocytes were demonstrated to release EVs carrying immune-regulatory miRNA-150 and miRNA-142, which induced a tolerogenic phenotype in the DCs targeted.⁸⁰ In addition, these cells were shown to secrete miRNA-containing EVs that suppressed effector Th1 cells.⁸¹ Taking into account the studied possibilities of directing EVs to selected cells, discovering miRNAs involved in immune regulation at the gene expression level should greatly improve the duration and selectivity of specifically induced effects. Accordingly, miRNA-carrying EVs released by apoptotic cells seem to possess weaker proinflammatory activity than those produced by healthy cells, but their miRNA content is dysregulated in patients with SLE.⁸² This implies the need to elaborate laboratory methods to selectively load EVs with the miRNAs of choice (Figure 1).

2.2.5 | EVs of other cellular sources

In various circumstances, B lymphocytes have formerly been reported to regulate immune responses, albeit regulatory B (Breg) cell phenotype and functions remain a matter of current studies.⁸³ In this regard, special emphasis is given to the investigation of a role of Breg cells in patients with autoimmune diseases.^{84–86} One can speculate that Breg cells may exert their tolerogenic effects in an EV-dependent, antigen-specific manner; however, this still requires further investigation. Accumulating evidence suggests that mesenchymal stem/stromal cells may also possess immune tolerogenic potential. However, due to an unpredictable risk of mutagenesis associated with the use of stem cells,⁸⁷ derived EVs attract researchers' bigger attention. Recently, mesenchymal stem/stromal cell-derived EVs were demonstrated to attenuate pathogenic Th1 and Th17 lymphocyte responses by inhibiting APCs in mouse models of autoimmune type 1 diabetes and uveoretinitis.⁸⁸ They were also considered promising candidates for cell-free immunotherapy in SLE and other autoimmune diseases due to their ability to suppress DC maturation and antigen-presenting function.^{87,89,90} Mesenchymal stem/stromal cell-derived EVs were also shown to tolerize DCs of diabetic patients.⁹¹ Although, in other circumstances, stem cell-derived EVs were suggested to trigger an autoimmune response in diabetes.⁹² In the cases described, stem cells were obtained from different sources, that is, bone marrow and the islet of Langerhans, respectively, opening the discussions on two issues: firstly, on the impact of tissue microenvironment on the composition and function of stem cell-secreted EVs,

and secondly, on the contribution of tissue-derived EVs to the stem cell secretome. Moreover, these interactions may possibly impact the secretome of each cell population, including APCs, which remains to be addressed. On the other hand, the placenta was recently described as a source of immunosuppressive EVs.⁹³ Nonetheless, it remains an open question whether mesenchymal stem/stromal cell- or trophoblast-derived EVs could support the induction of antigen-specific immune tolerance. Similarly, EVs originating from apoptotic cells may also contribute to the activation of immune tolerance. Recently, EVs derived from apoptotic thymocytes have been shown to ameliorate experimental colitis by promoting macrophage release of TGF- β in mice.⁹⁴ Finally, a potentially beneficial role of EVs released by activated effector T cells in immune tolerance cannot be excluded.⁹⁵

3 | ANTIGEN-SPECIFIC TOLERANCE IN ALLERGIES AND HYPERSENSITIVITY RESPONSES

In the case of allergic and hypersensitivity responses, methods for defining the actual antigen-specificity of tolerance mechanisms could be more easily accessible than in autoimmunity due to better awareness of the identity of the causative antigens. Those are usually non-self, with the exception of self-antigens inducing DTH-related autoimmune responses, as discussed above. In addition, various non-cross-reactive allergens may follow the same mechanism of allergic reaction, which greatly expands the possibilities for testing antigen-specificity of immune tolerance. In contrast, virtually any autoimmune disease is induced by related self-antigens or even determinants of the same autoantigen, which to some extent makes the experimental assessment of antigen-specificity difficult. In allergies, it was suggested that the biophysical properties of an allergen could determine the efficacy of antigen-specific immune tolerance. Accordingly, it was proposed that Treg cells could mediate tolerance against corpuscular, but not soluble antigens, which supports the concept underlying other mechanisms of antigen-specific peripheral tolerance.⁹⁶ Therefore, dissecting the mechanisms, by which antigen-specific tolerance is broken, could be crucial for developing the strategies of its preserving and restoration.^{97,98} The risk of tolerance breaking was also ascertained to be allergen dose-dependent, where even a single allergen particle may be processed by an APC to generate a great number of antigenic determinants that suffice to activate effector T cells.⁹⁶ Analogous effects may result from concentrating allergen molecules by packing into EVs. On the contrary, one can speculate that allergens accumulated in EVs may as well be used for allergen-specific immunotherapies, that is, as inducers of immune tolerance. However, while the role of EVs of various cellular sources in the pathogenesis or

exacerbation of allergic inflammation has been already acknowledged,^{99,100} much less is known about their roles in the activation processes of allergen-specific tolerance.

Allergen immunotherapy constitutes a common method used in clinical practice for desensitization of patients with IgE-mediated anaphylactic allergy, especially those associated with inhaled allergens.¹⁰¹ Exposure to high doses of a particular allergen induces allergen-specific tolerance, similar to the naturally occurring mechanisms observed in cat owners and beekeepers. Among other pathways, this mechanism involves the activation of a tolerant phenotype of DCs that in turn activate Treg cells.¹⁰² While a possible role of EVs in allergen immunotherapy has not been confirmed yet, DC-derived EVs have already been proved to carry allergens, and consequently induce allergic immune responses.¹⁰³

3.1 | APCs in antigen-specific tolerance in allergy and hypersensitivity

APCs provide crucial signals for inducing allergen tolerance at different stages of their activity and via multiple mechanisms.^{36,104} These include activation of Treg cells during immunotherapy with allergen administered by different routes, for instance, epicutaneously or subcutaneously.¹⁰⁴ Conversely, a recent report revealed a substantial role for skin-resident Treg cells in limiting the migration of antigen-primed APCs to draining lymph nodes after percutaneous sensitization.¹⁰⁵

One of the first immunotherapies proposed for autoimmune and allergic disorders, which directly involved APCs and might be considered at least antigen-dependent, was based on the use of either native antigenic peptides or altered peptide ligands (APLs) constituting native immunogenic peptides, usually with single amino acid substitutions. Recognition of APLs complexed with MHC molecules by T cells of corresponding specificities alters their activation and subsequent effector function. Consequently, the induced immune tolerance results from T cell anergy, clonal deletion or shifted polarization.¹⁰⁶ However, the clinical effects of such means of treatment greatly depend firstly on the course of peptide or APL binding that is largely unpredictable due to the physiological diversity of MHC molecules among treated individuals, and secondly, on the activation state of APCs targeted by peptides or APLs, thereby highlighting the need to improve the design of this therapeutic approach.¹⁰⁷ A similar experimental attempt to induce antigen-specific tolerance was based on the administration of allergen-adjuvant complexes. These were suggested to modify APCs for promoting the expansion of allergen-specific Treg cells.¹⁰⁴

Obviously, the complex interactions of APCs and Treg cells are also involved in the regulation of hypersensitivity reactions of types II and III, but they are less known. Type

II hypersensitivity reactions may be provoked by haptens binding to self-blood cells, alloantigens of transplanted or transfused cells or by foetal alloantigens. In some instances, this could induce autoimmune responses, like in the case of autoimmune haemolytic anaemia. Similarly, formation of immune complexes in type III hypersensitivity is also observed during autoimmune disorders, including rheumatoid arthritis. Certain studies suggested the possibility to sensitize recipients of erythrocyte transfusion against non-self alloantigens of blood groups other than ABO, thus initializing the exploration of possible mechanisms for inducing immune tolerance in post-transfusion type II hypersensitivity. One of the approaches considered was based on the blockade of CD40 ligand signalling at the time of interaction of APCs with Th cells,¹⁰⁸ while another study implied that erythrocyte transfusion in the absence of active inflammation provokes immune tolerance rather than sensitization.¹⁰⁹ Nonetheless, regarding type II and III hypersensitivities, there is still very little known on the mechanisms of induction of antigen-specific immune tolerance, especially when involving APCs and EVs, possibly due to more complex cellular circuits responsible for eventual development of the clinical symptoms defining these hypersensitivity reactions.

So far, studies on the regulation of contact dermatitis as a clinical symptom of cutaneous DTH reactions, so-called contact hypersensitivity (CHS), have been more successful in exploring the possible mechanisms of downregulating immune responses against contact allergens, including those mediated by EVs.¹¹⁰

3.2 | EVs in antigen-specific tolerance in allergy and hypersensitivity

At present, growing evidence from numerous studies supports our awareness of the complex functions of EVs in the up- and downregulation of hypersensitivity reactions. Special emphasis is now put on a better understanding of the tolerogenic role of EVs and means of induction of their antigen-specific action.

3.2.1 | Postulated role of EVs in antigen-specific regulation of type I allergy

An interesting possibility of tolerance induction in allergy, with a possible role for APCs, was also revealed by studies on intranasal tolerization. It was demonstrated that mice intranasally tolerized with olive pollen peptide produced EVs, which were found in their bronchoalveolar fluid, and reduced allergic inflammation after intranasal administration to sensitized animals.^{111,112} These findings strongly suggest the possibility to administer therapeutic EVs via an intranasal

route. It should be stressed that an intranasal route of EV administration may be more acceptable to patients, as it is non-invasive and also amenable for repetitive dispensation.

Analogously to treatment of autoimmune diseases, modified APCs, especially DCs, have been experimentally evaluated for their tolerogenic potential in hypersensitivities.¹¹³ Allergy is often associated with decreased production of IL-10 by immune cells. Thus, studies on IL-10-treated DCs showed that both manipulated cells and the resulting EVs were capable of suppressing allergen-specific Th2 cells.¹¹⁴ One can speculate that such an inhibitory effect may be induced by cytokines, TGF- β especially,¹¹⁵ that could be delivered by EVs.

Mesenchymal stem/stromal cells were suggested to regulate allergic asthma through their actions on APCs and Treg lymphocytes.¹¹⁶ Furthermore, *in vitro* studies revealed the possibility of ameliorating the course of allergic asthma by mesenchymal stem/stromal cell-secreted EVs enabling Treg cell activation in an APC-dependent manner.¹¹⁷ However, the specificity of putative clinical effects still requires further investigation. On the other hand, mast cell-derived EVs, which are known to mediate allergen presentation,¹¹⁸ and thus to exacerbate the course of allergy,¹¹⁹ may also suppress allergic inflammation by capturing IgE antibodies.¹²⁰ Other studies implied a suppressive activity of EV-contained miRNAs present in mother's milk or even commercially available bovine milk for possible therapeutic usage in allergic diseases.¹²¹⁻¹²³ However, the actual mechanism of their actions requires detailed elucidation, but implies that food may be the best carrier for delivering immune-regulatory EVs.

3.2.2 | Postulated role of EVs in antigen-specific regulation of DTH

One of the first tolerogenic activities of EVs observed in DTH reactions was based on the isolation of EVs from plasma or serum of stimulated animals. Accordingly, MHC class II-expressing EVs from plasma of mice immunized with keyhole limpet haemocyanin, but not with ovalbumin, suppressed the DTH reaction to keyhole limpet haemocyanin in a Fas-dependent manner,¹²⁴ which allows to assume an antigen-dependency of observed effects. Similarly, using the oral route for antigen administration may lead to the development of EV-mediated immune tolerance. Former studies revealed the presence of a tolerogenic factor in serum of rats fed with ovalbumin, which suppressed ovalbumin-induced DTH in tolerized rats and, when transferred, in immunized rats.^{125,126} Subsequently, the suppressive activity of this serum factor from antigen-fed mice occurred to be mediated by MHC class II-expressing EVs, called 'tolerosomes', produced by intestinal epithelial cells.¹²⁷ Furthermore, these 'tolerosomes' acted in a MHC-restricted manner, that is, only after transfer to

syngeneic recipients.¹²⁸ Finally, the 'tolerosomes' were also demonstrated as downregulators of mouse allergic asthma.¹²⁹ However, the mechanism of 'tolerosome'-mediated immune tolerance still requires further investigation.

Studies on DCs loaded *in vitro* with antigen and manipulated to express Fas ligand revealed that their suppressive activity could be efficiently substituted by the produced EVs. After local administration before antigen challenge, those EVs suppressed DTH footpad skin swelling. EVs acted in a Fas/Fas ligand interaction-dependent manner and were internalized by CD11c⁺ DCs.²² Analogously, IL-10-treated DC-secreted EVs were demonstrated to suppress the keyhole limpet haemocyanin-induced DTH reaction, and this effect depended on the expression of costimulatory CD80 and CD86 molecules.¹³⁰ Altogether, these observations suggest that EVs may regulate immune cell functions in both auto-crine and paracrine manners (Figure 1).

Another hypothesis about the induction of antigen-specific tolerance in DTH was based on the manipulation of a tumour cell line to release ovalbumin-expressing EVs, which proved capable of suppressing the ovalbumin-induced DTH reaction. Interestingly, the same ovalbumin-bearing EVs failed to affect DTH to keyhole limpet haemocyanin, which indeed suggests that the observed tolerogenic effect was antigen-specific.¹³¹ On the one hand, the generally non-selective immune suppressive potential of tumour cell-derived EVs may support the efficacy of modified tumour cell-derived EVs' actions,¹³² but on the other hand, might hamper their antigen-specific functions *in vivo*. Thus, tumour-derived EVs seem to require *ex vivo* modifications to uniquely standardize their tolerogenic potential, for example by loading them with selected, suppressive miRNAs.

3.2.3 | Possibilities to manipulate EVs for antigen-specific downregulation of hypersensitivity reactions

Antigen challenges in DTH influence the profile of miRNAs in human skin samples.¹³³ This may be associated with a dysregulation of the miRNA expression pattern reported in drug-specific T cells, which contributed to the severity of the drug-induced hypersensitivity reaction.¹³⁴ Thus, one can speculate that either direct antagonism of upregulated miRNAs by EV-mediated delivery of anti-miRNA (anti-miR) sequences or a reprogramming of APCs to repress the miRNA-induced effects in T cells would constitute promising alternatives for genetically encoded therapies of hypersensitivities (Figure 1). Considering the limitations of APL-based immunotherapy discussed above, one can assume that loading of peptides or APLs with desired MHC molecules onto APC-derived EVs, additionally armed with enclosed immune-regulatory miRNAs, could greatly improve

and prolong the beneficial effects of such immunotherapeutic strategies (Figure 1).

Apart from other important immune-regulatory functions,¹³⁵⁻¹³⁷ galectin-1 is involved in central clonal deletion of thymocytes. Thus, it was suggested that DCs expressing galectin-1 may suppress T cell responses but remarkably, the induced effect was shown to depend on the phase of the DTH reaction, that is, galectin-1-expressing DCs enhanced T cell sensitization, but impaired the effector phase of DTH by induction of effector T cell apoptosis.¹³⁸ This implies that the expression of galectin-1 by APC-derived EVs may support tolerance development by promoting the deletion of effector T cells (Figure 1).

B1 cell-secreted, hapten-specific antibody light chains (LCs) are involved in the upregulation of CHS reactions by stimulating mast cell degranulation at an early phase of the effector response. However, our recent studies demonstrated that these hapten-specific LCs, when coating immune

suppressive EVs, ensure antigen-specificity of CHS down-regulation by promoting antigen-specific EVs' binding to acceptor cells (Figure 2).^{139,140} As in autoimmune reactions, allergic responses remain under the control of Breg lymphocytes as well.¹⁴¹ Furthermore, the efficacy of allergen immunotherapy partly depends on the activation of Breg cells.¹⁴² The already acknowledged mechanism of Breg cell-induced immune suppression is mediated by IL-10.¹⁴¹ However, it is still unclear whether Breg cells act in a EV-dependent manner, and if they possess the ability to present antigen in a tolerogenic manner.¹⁴³

Finally, it is also worth noting that miRNAs, mostly those carried by EVs, were proposed to constitute potential biomarkers of a patient's susceptibility to contact allergy and of clinical severity of contact dermatitis or contact allergen-specificity of effector cells.¹⁴⁴ The latter diagnostic approach might utilize APC-derived EVs. Furthermore, EV-delivered miRNAs constitute the natural candidates for

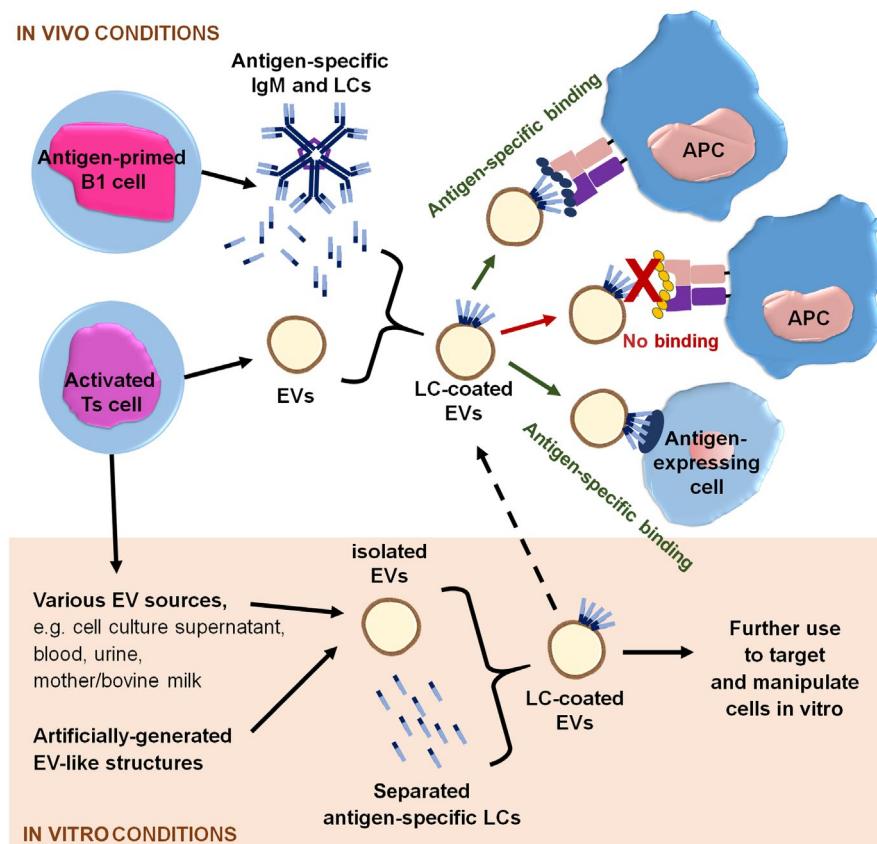


FIGURE 2 Postulated role of antigen-specific antibody light chains in directing extracellular vesicles towards target cell. Antigen-primed B1 cells synthesize IgM antibodies with a slight excess of the antibody light chains (LCs) that are secreted extracellularly. They can mediate antigen-specific immune responses due to their ability to recognize and bind the antigen. In addition, antibody kappa LCs were detected on the surface of extracellular vesicles (EVs) released by suppressor T (Ts) cells of antigen-tolerized mice. Thus, based upon existing research findings, antigen-specific LCs are considered a promising tool for directing EVs towards desired acceptor cells. It was proposed that free LCs may bind to the surface of EVs and form the multiple adjacent arrangement, which greatly increases their avidity for an antigen. As a consequence, LC-coated EVs may target particular cell by specific binding to an antigen in plasma membrane. In addition, LC-coated EVs can target antigen-presenting cells (APC) by specific binding to an antigenic determinant in the groove of the major histocompatibility complex. Besides, one can speculate that EVs isolated from various biological sources or artificially engineered EV-like nanovesicles may also be coated with LCs of chosen antigen-specificity in vitro, and then used both in vivo and in vitro to selectively target the desired cells as therapeutic agents

induction of the expected, long-lasting effects, including immune tolerance, by interfering with genome activity in target cells. Physiologically, miRNAs seem to be selectively sorted into EVs, as recently proposed for house dust mite allergen-provoked airway inflammation.¹⁴⁵ Based on these considerations, our research findings allowed us to describe a novel mechanism of antigen-specific immune tolerance induction, along with the selective means of cell targeting by miRNA-carrying EVs due to their surface coating with antigen-specific antibody LCs.

4 | THE APC-EV-MIRNA-150 CIRCUIT IN THE REGULATION OF DTH TO SELF-ANTIGENS AND ALLERGENS

Former studies reported that mice intravenously administered with a high dose of haptenated syngeneic red blood cells are much less prone to the development of CHS to this hapten, but not to another, non-cross-reacting hapten.¹⁴⁶ This immune suppression, assumed antigen-specific, was then found to be mediated by CD8⁺ suppressor T (Ts) cells, releasing a regulatory entity, so-called T suppressor factor.¹⁴⁷

4.1 | Role of EV-contained miRNA-150 in antigen-specific suppression of DTH responses

Recently, we have discovered that the enigmatic T suppressor factor is actually composed of two subfactors produced by two different cellular populations, that is, CD8⁺ FoxP3^{neg} Ts cell-derived EVs carrying regulatory miRNA-150, and B1 cell-secreted, antigen-specific antibody LCs that coat the surface of these EVs.^{118,139,140,146-148} After sequencing of miRNAs in Ts cell-derived EVs, the crucial role of miRNA-150 was proved by experiments making use of the miRNA-150 antisense strand, which blocks EV-mediated suppression, and with tolerized miRNA-150 knock-out mice, whose EVs were non-inhibitory, unless when pretreated with synthetic miRNA-150.^{139,148} Interestingly, we observed that mere mixing of EVs with miRNA-150 molecules provided miRNA-mediated EV functions, which suggest that, in particular circumstances, miRNAs may directly bind to or drop into a subpopulation of EVs by an as yet unknown mechanism.^{139,148,149} Then, Ts cell-derived EVs carrying miRNA-150, additionally coated with hapten-specific LCs, were shown to suppress the hapten-induced CHS reaction antigen-specifically in vivo (Figure 2).^{139,148} Recently, we have found that an analogous tolerance mechanism could be induced in DTH to ovalbumin and bovine casein administered without an adjuvant.¹⁵⁰ The antigen-specificity of the observed mechanism of DTH suppression by miRNA-150-carrying EVs

was proved by testing of EVs mediating tolerance to keyhole limpet haemocyanin in active, ovalbumin-induced DTH,¹⁵¹ and of ovalbumin-specific EVs in casein-induced DTH.¹⁵⁰ Interestingly, suppressed DTH reaction was observed in mice treated with Ts cell-derived EVs administered via intraperitoneal, intravenous, intradermal or even oral routes.¹⁵⁰ The highest therapeutic effect was achieved after oral administration of EVs, which greatly supports the eventual application of oral route of treatment with EVs to clinical practice.

4.2 | Role of APCs in antigen-specific suppression mediated by miRNA-150-carrying EVs

Stimulation of mouse macrophages with EV-derived miRNA-150 affected their ability to induce B cells for production of antibodies, and to activate the CHS reaction in recipients after hapten conjugation and intravenous transfer.¹⁵² Afterwards, we have shown that macrophages are required to activate the generation of tolerogenic EVs transmitting miRNA-150 by Ts cells, since the CHS reaction elicited in tolerized mice depleted of clodronate-sensitive cells was only weakly suppressed.¹⁵² Furthermore, macrophages stimulated with EV-delivered miRNA-150 gained the ability to inhibit the proliferation of CHS effector cells.¹⁵² Similarly, APCs pretreated with miRNA-150-carrying EVs displayed reduced capability to fully activate T cells at the site of immune synapse formation.¹⁵³ This is in line with previous studies suggesting that T suppressor factor does not act directly on effector T lymphocytes, but is bound by antigen-presenting macrophages, which then transmit the suppressive signal to the target cell population, mainly by releasing the so-called macrophage suppressor factor.¹⁴⁷ Indeed, these observations were recently confirmed by our studies,^{152,153} strongly implying a crucial role for APCs in the antigen-specific action of miRNA-150-carrying EVs. Firstly, we have demonstrated that Ts cell-derived EVs in fact target APCs, but not effector T cells themselves, as they failed to suppress the macrophage-depleted CHS effector cell population, and inhibited adoptively transferred CHS effector T lymphocytes only in the presence of antigen-presenting macrophages.¹⁵² Secondly, we have observed that the suppressive action of Ts cell-derived EVs is blocked by pre-incubation with antigenic peptides derived from the DTH-inducing antigen.¹⁵¹ This strongly suggests that LCs coating the Ts cell-derived EVs bind antigenic determinants complexed with MHC on the surface of APCs, which greatly facilitates their precise targeting and final engulfment by APCs presenting the particular antigen. Thirdly, we have shown that treatment of macrophages with Ts cell-derived EVs stimulates these cells to release secondary EVs that are able to inhibit adoptively transferred DTH effector T cells.¹⁵³ Moreover, APC-derived,

secondary EVs could be accumulated by T cells, which were facilitated by immune synapse formation.¹⁵³ This, in turn, suggests that the final step of suppression of effector T cells takes place during antigen presentation at the immune synapse and requires the release of secondary EVs by APCs, which target T cells, likely expressing TCR of corresponding antigen-specificities.

4.3 | Role of B1 cells in antigen-specific suppression mediated by miRNA-150-carrying EVs

Experiments with B cell- and immunoglobulin-deficient mice allowed us to prove that B1 cell-secreted, antigen-specific LCs detected on Ts cell-derived EVs by flow cytometry indeed ensure the specificity of EV-mediated suppression. It was shown that EVs from tolerized, pan immunoglobulin-deficient mice are non-suppressive, unless they are coated with LCs of the same specificity as the CHS effector cells (Figure 2).¹⁴⁰ Our preliminary results also suggest that LCs may bind to EVs via surface membrane lipids.^{118,140}

B1 cells were also preliminarily found to provide EVs originally coated with antibodies or LCs for transmission of regulatory miRNA-150.^{139,140,154} Analogously to the above described ability of Ts cell-derived EVs to bind or absorb miRNA-150, we observed that mere incubation of miRNA-150 with B1 cell-derived EVs is enough to transmit it to target cells to induce miRNA-150-mediated immune suppression.¹³⁹ These findings allowed us to propose an alternate transmission pathway of freely circulating, extracellular miRNAs targeting acceptor cells after *in vivo* association with EVs.¹³⁹ Accordingly, we suggest that miRNAs could be associated with or transfected into a subpopulation of EVs, which we termed 'activated EVs',¹¹⁸ in an as yet undefined process.¹³⁹ Our initial data suggest that association of miRNAs with EVs is dose-dependent and that miRNAs might be actively incorporated by EVs. However, this still requires detailed investigation, and resulting observations would greatly contribute to an understanding of the mechanism underlying the passage of circulating miRNAs between cells.¹⁵⁵

4.4 | Role of EVs and miRNA-150 in self-tolerance

Simultaneously, our studies revealed a novel concept for induction of self-tolerance. Initially, we intended to include a negative control for tolerization comprising EVs released by lymph nodes and spleens of mice intravenously administered with a high dose of syngeneic red blood cells, which were not conjugated with antigen. While those EVs very weakly suppressed active CHS,¹⁴⁸ they were found to impair macrophage

activity in humoral immune responses to a similar extent as EVs from mice tolerized with haptenated syngeneic red blood cells.¹⁵² These surprising observations inspired us to further investigate the putative regulatory function of EVs induced by intravenous administration of a high dose of intact syngeneic red blood cells. Subsequent research revealed that these EVs gain full ability to suppress CHS effector cells after coating with specific LCs, and that they also act in a miRNA-150-dependent manner.¹⁵⁶ Thus, we assumed that they might be involved in tolerance to self-antigens of red blood cells. To verify this hypothesis, we have developed and tested a new mouse model of DTH to syngeneic red blood cells, which was demonstrated to be suppressed by both a high dose of intravenously administered red blood cells and by released EVs, which depended on miRNA-150 promoting apoptosis of DTH effector T cells.¹⁵⁶

4.5 | Clinical relevance of studies on the APC-EV-miRNA-150 circuit

Our research findings allowed us to understand the biological mechanism of antigen-specific immune tolerance mediated by miRNA-150-carrying EVs that target APCs, further inhibiting effector T cells through released secondary EVs. We also concluded that initial generation of miRNA-150-transmitting EVs prevents the eventual immunization of mice to self-antigens of syngeneic red blood cells systemically administered in a high dose, while, after coating with LCs, EVs become suppressive of DTH responses to non-self-antigens.¹⁵⁶ Furthermore, tolerogenic EVs could be administered orally to induce a therapeutic effect. In addition, our observations allowed us to speculate that, in some instances, extracellular miRNAs may directly bind to or drop into a particular subset of EVs to be further transmitted. Finally, our studies brought the idea to direct EVs towards a desired cellular target by coating with antigen-specific antibody LCs of sufficient avidity.

It is worth noting that recent studies revealed the transmission of miRNA-150 in EVs released by Treg lymphocytes to DCs to polarize the latter towards an anti-inflammatory phenotype.⁸⁰ However, in the case of autoimmunity, miRNA-150 was suggested to contribute to both an up- and downregulation of immune responses to self-antigens.^{157,158} Along these lines, miRNA-150 was found abundantly expressed in Treg cell-derived EVs, while analysis of sera of psoriatic patients showed its levels to be increased in the case of active disease.¹⁵⁹ Overall, these findings reveal the role of miRNA-150 in the regulation of autoimmunity, and our current studies uncover miRNA-150 delivered by Ts cell-released EVs as a mediator of antigen-specific immune tolerance mechanisms, which was found to be induced by intravenous administration of a high dose of syngeneic red blood cells in mice. The complexity

of our research findings initiates the studies on the molecular mechanisms and interactions underlying the determined biological effects, and supports their additional translation to human biology. Currently, our studies focus on the molecular aspects of miRNA-150-induced immune suppression, in particular on identifying mRNA targets of miRNA-150.

5 | ANTIGEN-SPECIFIC TOLERANCE IN ALLERGIES, HYPERSENSITIVITIES AND AUTOIMMUNITY—FUTURE PERSPECTIVES

Extracellular vesicles may achieve insufficient delivery efficiency when lacking the ability of specific cell targeting, as it promotes their rapid and non-selective clearance by phagocytes,¹⁶⁰ macrophages especially.¹⁶¹ In such cases, EV-contained miRNAs may modify the phenotype of macrophages, and, as a consequence, may induce opposite effects to those intended. Instead, induction of CD47 expression on EVs, which provides a 'don't eat me' signal, may avoid rapid phagocytosis and clearance of EVs.¹⁶² Thus, from a clinical point of view, for mediating EV-based immune tolerance, the most challenging issues that have to be addressed concern the means of specific targeting of desired cells by EVs and the contained cargo to ensure the stable and long-lasting effects.¹⁶³ Simultaneously, research findings on the possibilities of induction of persistent immune tolerance to a particular antigen greatly contribute to current clinical approaches that are undertaken to eliminate the need to use immune suppressive drugs for non-specifically impairing immune responses.

According to the above considerations, antigen-specific antibodies or LCs bound to the EV surface membrane appear to constitute promising candidates for directing EVs towards selected acceptor cells (Figure 2). Such an approach requires the identification of a specific antigen present mostly on desired cells to avoid non-specific cell targeting. Thus, antigenic determinants presented by APCs in the MHC groove occur to be an interesting target for antibodies decorating the EV surface. On the other hand, equipping EVs with antigenic determinants complexed with MHC should direct them towards T cells-expressing TCRs of corresponding specificities. Beyond that, covalent binding of cytokines or growth factors, like GM-CSF,⁵⁵ and immune checkpoint molecules, like cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), or their ligands, programmed death-1 ligand-1 (PD-L1) especially,⁹⁵ to EV membranes may both increase the capacity of EV binding to desired cells via selective receptor ligation and greatly support the tolerogenic effect of EVs and their cargo. In such instances, the predicted antigen-specificity of EV-induced immune tolerance would be conferred by reprogramming, inhibiting and/or deleting the cells specifically

targeted for mediating the immune response against a particular antigen, that is, APCs and antigen-specific T cells. In the case of directed targeting of antigen-specific B cells, one can speculate that EVs could also be armed with antigen in a conformation allowing for binding by BCRs of corresponding specificities (Figure 1).

Apart from controlling cell targeting by EVs, efficient induction of immune tolerance requires tolerogenic reprogramming of target cell phenotype and function, or deletion of self-reactive or sensitized cells. This could be conferred by EV-derived cargo, including miRNAs,^{164,165} or transcripts of regulatory genes,¹⁶⁶ and additionally facilitated by cytokines or inhibitory molecules interacting with inhibitory receptors. Accordingly, the genetically encoded information for interfering with the cellular transcriptome and activation of downstream inhibitory signalling pathways should cause long-lasting and stable effects.¹⁶⁷ The tolerogenic effects could then be provided by the tolerized APCs and activated antigen-specific Treg and Breg cells, inducing clonal anergy and apoptosis of antigen-specific effector T and B lymphocytes due to the blockage of costimulatory signalling and the activity of Fas ligand and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) respectively.⁹⁵ Importantly, the same effects are likely to be successfully induced by EVs originating from regulatory and tolerized cells (Figure 1). Besides, EVs may also be considered as natural nanocarriers for selective drug delivery.^{34,168}

It should be stressed that some research data imply that nanoparticles suggested for substituting EVs in therapeutic utilization may induce generation of endogenous EVs that target APCs, which leads to their maturation and polarization towards the DC-1 subset and classically activated macrophages. Then, polarized APCs promote Th1 cell activation and differentiation to effector cells of DTH, which may contribute to inflammatory side effects of nanoparticle administration.¹⁶⁹ Hence, artificially generated nanoparticles may not meet the expected function *in vivo*, in contrast to EVs.

Naturally generated EVs are originally destined to be internalized by target cells. The current perspective is to load EVs with specific and functional regulatory molecules ensuring stable and long-lasting effects and to specifically direct EVs towards a selected cell target. For these purposes, it should be evaluated whether artificially produced liposomes could efficiently substitute natural EVs,¹⁷⁰ which are characterized by much lower immunogenicity and may likely cross biological barriers.¹⁷¹ Simultaneously, isolation of EVs from non-invasively collected body fluids, like saliva, human breast milk or presumably even bovine milk could be considered as easily accessible means of obtaining EVs for therapeutic purposes. Circulating blood could also be anticipated as a source of naturally produced EVs. Then, EV subsets may be isolated from collected serum or plasma, but one can speculate that EVs might

also be collected directly from circulating blood, analogously to CD34+ haematopoietic stem cells via magnetic sorting, affinity chromatography or related methods.^{93,172} However, MHC restriction of EV-mediated actions has to be well characterized, as it can influence treatment efficacy, and selective markers allowing for isolation of functional EV subsets have to be identified. Since isolation of EVs from body fluids is still fraught with many challenges,¹⁷³ well-recognized and standardized means of inducing tolerogenic EV generation in vivo may instead turn out to be safer and more efficient. For those purposes, the process of EV formation has to be characterized in all its aspects. However, this raises another crucial question of the mechanism of sorting and packaging of cargo into EVs, which very likely is not a random process.^{18,174} Addressing these questions will undoubtedly play a major part in advancing EV-based therapeutic approaches.

To conclude, our overall considerations summarize the current state-of-art in terms of induction of antigen-specific immune tolerance mediated by EVs and APCs, deliberate on the as yet undefined aspects and highlight the emerging perspectives of their future application in clinical practice.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

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