

1 ***Nano- and micro-based systems for immunotolerance induction in Multiple Sclerosis***

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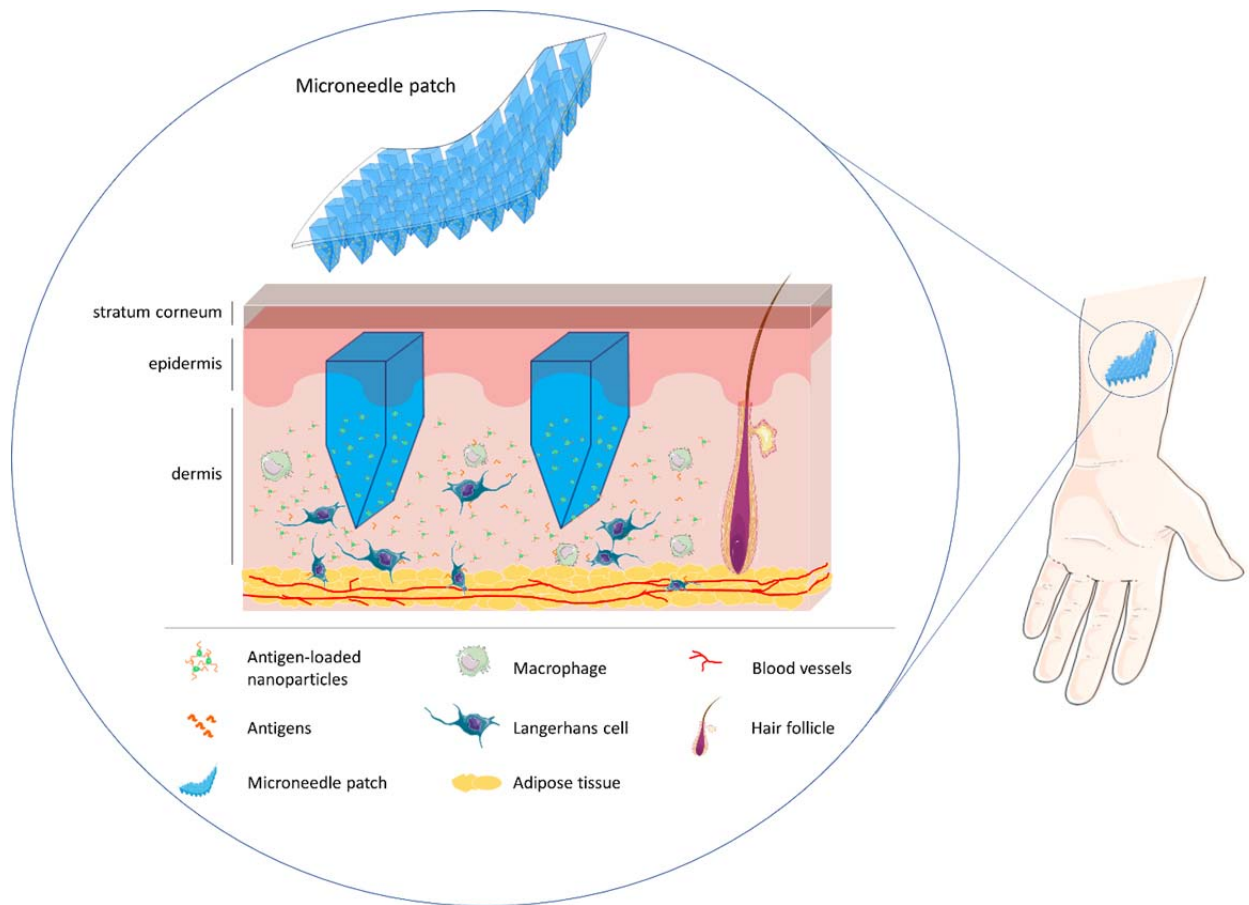
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10 **Abstract**

11 It is estimated that more than 2.5 million individuals worldwide have multiple sclerosis (MS). MS is an
12 autoimmune neurodegenerative disease resulting from the destruction of the myelin sheath that enwraps
13 axons driven by an immune cell attack to the central nervous system. Current therapeutic programs for
14 MS focus in immunosuppression and more recently in the use of immunomodulatory molecules. These
15 therapeutic approaches provide significant improvements in the management of the disease, but are
16 frequently associated with an increased susceptibility of opportunistic infection. In this commentary, we
17 highlight the application of nano and micro-technologies as emerging and innovative solutions for MS
18 therapy with the potential to restore immune homeostasis via antigen-specific interactions. Furthermore,
19 we propose and discuss the usage of a minimally invasive approach, namely microneedle patches, as a
20 new therapeutic route.

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24 **Microneedle patches for the delivery of specific antigens to restore immunotolerance in the**
 25 **context of Multiple Sclerosis.**

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28 **Keywords (5-10)**

29 Multiple Sclerosis, Microneedles, Nanoparticles, Tolerance, immunomodulation

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31 **1. Multiple Sclerosis: etiology and current therapeutics**

32 Multiple sclerosis (MS) is a chronic immune mediated demyelinating disease of the central nervous
 33 system (CNS) caused by a strong T cell attack directed towards proteins of the myelin sheath wrapping
 34 CNS axons; this ultimately culminates in demyelination and neuronal degeneration [1, 2]. In developed
 35 countries it is the second cause of neurological disability in young adults, with high burden for the patient,
 36 the family and the resources of the health system [3]. It is a complex disease and its underlying
 37 mechanisms are only partially understood. Most patients initially present with a clinically isolated
 38 syndrome (CIS). These CIS patients experience an acute episode, which typically affects one brain
 39 region, being the clinical symptoms variable depending on the involvement of motor, sensory, visual or
 40 autonomic systems [4]. Some CIS patients will evolve to definite MS disease, while others won't.
 41 Nowadays, the diagnosis of definite MS is based on recognized clinical criteria, with the support of
 42 magnetic resonance imaging (MRI) data and cerebrospinal fluid (CSF) analysis [2], and can only be done

43 when there is dissemination of neurologic dysfunction in space and time [2, 4, 5], and after differential
44 diagnosis is excluded [5]. Concerning the MRI findings, the presence of multifocal demyelinating lesions
45 at different timepoints involving preferentially the periventricular white matter, the brain stem, the
46 cerebellum and the spinal cord are indicative of MS [2]. Furthermore, the presence of oligoclonal bands or
47 increased concentration of immunoglobulin (Ig)G in the patients' CSF are widely used to support MS
48 diagnosis, but are not MS- specific [2, 6].

49 Patients with definite MS can develop different profiles of the disease, being classified as relapse-
50 remitting (RR)-MS, primary progressive (PP)-MS or secondary progressive (SP)-MS. RR-MS represents
51 about 80-85% of MS cases [7] and is characterized by transient symptoms (relapse) that often improve
52 within weeks (remission). However, the ability to fully recover from relapse episodes diminishes with time,
53 and irreversible damage accumulates in the CNS, giving rise to SP-MS. The remaining 15-20% of
54 patients has PP-MS, and does not show this relapse-remitting pattern; rather, their symptoms become
55 gradually worst along the course of the disease.

56 MS is nowadays a treatable, although not curable, disease. The first proven MS treatments were
57 approved in the nineties and consisted in different formulations of interferon-1 administered
58 intramuscularly or subcutaneously. Although a major breakthrough at the time and still an important part
59 of treatment options today, given their excellent safety, interferon-1 based treatments are only moderately
60 efficacious, leading to full control of the disease in only a small percentage of patients [8]. Interferons
61 have pleiotropic effects, including a reduced T-cell entry into the CNS [9]. Glatiramer acetate is a mixture
62 of oligopeptides designed to mimic the aminoacid composition of myelin that induces a skew towards a
63 regulatory response. Also administered subcutaneously, it has an efficacy similar to interferons and an
64 excellent safety record that make it still an important player in MS treatment options [8].

65 Recently the portfolio of approved MS treatments was enriched by more options that encircle two major
66 therapeutic approaches targeting either T-cells or B-cells. Concerning T-cells modulation a collection of
67 drugs is currently being applied in the clinical setting, with moderate success, as next described.
68 Teriflunomide is an oral medication that interferes with the fast expansion of recently activated
69 lymphocytes, preserving the basal proliferation of memory cells. It has a moderate efficacy, similar to
70 interferons, but some safety concerns, including teratogenic potential [10]. Dymethylfumarate is an oral
71 treatment with a putative dual mechanism of action, including immunosuppression and neuroprotection.
72 In clinical trials it demonstrated a good efficacy in controlling the disease but concerns regarding its long-
73 term safety, particularly the profound lymphopenia and the risk of a serious opportunistic CNS infection,
74 progressive multifocal leukoencephalopathy (PML), might limit its use [11]. Fingolimod, a functional
75 antagonist of S1P receptors that blocks lymphocyte egress from lymph nodes [12], has also a good
76 efficacy but similar concerns over lymphopenia and PML [8]. Natalizumab is a highly efficacious
77 monoclonal antibody that blocks lymphocyte entry into the CNS; however, it is associated with a high risk
78 of PML in patients which have antibodies against the causing organism, JC virus, which almost limits its
79 use to seronegative patients representing less than half of MS patients [13].

80 While all the above-mentioned drugs interfere with T-cell function, alternatively, ocrelizumab is a
81 monoclonal antibody that destroys B-cells and, surprisingly, was shown to have a good efficacy in MS
82 patients [14]. Although not yet approved, it has forced a major revision of MS pathogenesis to take into
83 account the role of B-cells, which are not only seen as antibody producers but also as antigen presenting
84 and cytokine releasing cells, able to activate Th1 and Th17 responses and induce pathology [15].
85 Although data are still preliminary, ocrelizumab may increase the risk of serious infections, including PML,
86 which might limit its use. Targeting both B and T cells, alemtuzumab is a monoclonal antibody that
87 induces a severe depletion of circulating lymphocytes and is said to "reset" the immune system [16].
88 Upon immune reconstitution, alemtuzumab-treated patients are able to stay disease free for periods of up
89 to 5 years (longer follow-ups are still scarce), in what represents a first step towards an effective cure [16].
90 However, such efficacy comes at the cost of an increased risk of serious infections in the first weeks,

91 requiring antibiotic prophylaxis and precautions, and a significantly higher risk of autoimmune disturbances
92 in the following years [16].

93 As can be gleaned from the above, MS treatments are in an exciting era, with several new options being
94 approved, many more in the pipeline, and new drug targets and modes of action being available. More
95 importantly, some treatments have been shown to allow a reset of the immune system, which might make
96 a cure even more reachable than before. However, we weren't able, so far, of breaking the close ties
97 between efficacy and risk. These severely limit the use of more efficacious medications in all patients and
98 the extension of their benefits to all patients. Thus, the finding of a highly efficacious and safe therapeutic
99 is still an unmet medical need. This paves the way for nanotechnology-based approaches.

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101 **2. Nanoengineered systems for MS therapeutics**

102 The use of nanotechnology and in particular of nanoparticles has been actively investigated for the
103 development of new therapies for MS. Taking advantage of their size, nanoparticles are easily
104 internalized by the cells, being suitable carriers for drugs, immunomodulatory molecules or antigens. The
105 use of materials at the nanoscale is expected to provide unique opportunities to improve drug solubility
106 and bioavailability, allowing targeted delivery, controlled release and consequently more effective routes
107 of administration and lower toxicity [17]. Interestingly, not only nanoparticles can serve as carriers of
108 relevant molecules as they can also trigger an immunomodulatory effect. In fact, variations in the
109 chemical composition, size and shape of nanoparticles differentially impact the immune response [18]
110 [19], which might be even more significant in the context of autoimmune diseases [20].

111 In MS, the use of nanoparticles has been investigated within two major applications: 1) as drug delivery
112 systems, and 2) as vectors for antigen-specific immunomodulation. Nanoparticles can bring new solutions
113 for the delivery of drugs that specifically target the immune or the neurodegenerative aspects of MS.
114 Recent reports describe the encapsulation in liposomal formulations of immunomodulatory drugs
115 currently applied in MS therapeutics such as methylprednisolone [21] or fingolimod [22]. These nano-
116 sized formulations showed a higher efficiency in a MS animal model due to improved pharmacokinetics
117 and biodistribution when compared to the free drugs. Focusing in reducing neurodegeneration, polymeric
118 nanoparticles targeted to oligodendrocyte precursor cells were applied to deliver leukaemia inhibitory
119 factor (LIF) and to successfully promote remyelination [23].

120 Importantly, nanoparticles have also been explored as vectors for antigen-specific immunomodulation.
121 The delivery of autoantigens related with the autoimmune response in MS is expected to allow the
122 specific blockade of the damaging effects of self-reactive immune-cell function while maintaining the
123 ability of the immune system to clear non-self antigens, thus restoring immunotolerance. This "tolerant
124 approach" was firstly tested by the administration of soluble autoantigens, but the massive amounts of
125 antigen required [24] along with some reported cases of anaphylactic response [25] prompt the need for
126 new and safer solutions. The administration of peptides crosslinked to splenic leucocytes demonstrated
127 very promising results, inducing a robust antigen-specific tolerance [26, 27]. Nonetheless, in order to
128 circumvent the use of cellular components and the drawbacks associated, like cost and manipulation,
129 researchers have been focused in the development of alternative strategies based on nanoparticulate
130 systems. The premise is that nanoparticles crosslinked with disease-associated antigens and their
131 epitopes can target antigen-presenting cells (APCs) capable to regulate T cell function and
132 simultaneously support the induction and/or expansion of regulatory T cells (Tregs), restoring
133 immunological tolerance.

134 The intravenous administration of poly(D,L-lactide-co-glycolide) (PLGA) microparticles crosslinked with
135 proteolipid protein (PLP)₁₃₉₋₁₅₁ peptide (the immunodominant T cell myelin epitope in SJL mice from the

136 myelin most abundant protein - PLP) showed remarkable results, being able not only to reduce the
137 clinical score if administrated prophylactically, but also to treat ongoing disease [28]. Interestingly, PLP₁₃₉₋
138 ₁₅₁ administrated in the form of colloidal hydrogel demonstrated to be effective only if administrated before
139 the disease onset [29]. The use of poly(ethylene-co-maleic acid) (PEMA) as surfactant allowed the
140 preparation of PLGA nanoparticles and provided a reliable platform for different antigen crosslinking, as
141 demonstrated by the relevant results in the induction of immunological tolerance both in the context of
142 experimental autoimmune encephalomyelitis (EAE), the animal model for MS [30] and in a transplantation
143 model [31].

144 Alternatively to antigen crosslinking, nanoparticles can be loaded with the antigen of interest. This
145 concept can be extended to the development of multifunctional systems that combine the delivery of
146 antigens with other molecules/drugs as a mean to turn the immune response more specific and/or more
147 effective. Loading gold nanoparticles with the T-cell epitope from myelin oligodendrocyte glycoprotein
148 (MOG₃₅₋₅₅) and ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester - a tolerogenic
149 molecule) showed to induce functional regulatory T cells in an EAE animal model more efficiently than
150 MOG-loaded particles [32]. PLGA nanoparticles containing MOG₃₅₋₅₅ and interleukin-10 (IL-10) mediate a
151 sustained release of the molecules [33] and, although the results in terms of regulatory T cells expansion
152 were not as impressive as the obtained for crosslinked nanoparticles, it was shown that the severity of the
153 disease can be reduced via subcutaneous administration of the particles. In the PLP-associated EAE
154 model, the use of rapamycin, an immunosuppressant molecule, loaded in PLGA nanoparticles along with
155 PLP₁₃₉₋₁₅₁ peptide promoted complete inhibition of disease relapses after both intravenous and
156 subcutaneous administration [34].

157 The results achieved so far using nanoparticles to induce immunotolerance are promising and should
158 soon initiate clinical trials. A further step towards the use of nanoparticles to induce immunotolerance
159 might be achieved when combined with alternative and minimally invasive routes of administration, which
160 will next be explored.

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163 **3. A microneedle-based immunotolerance approach for MS**

164 Microneedles have been extensively investigated in the recent years as mean to mediate the delivery of
165 drugs and/or antigens to the epidermal and/or intradermal space, overcoming the skin stratum corneum
166 barrier. These devices hold the potential of allowing self-administration and painless application.
167 Moreover, microneedle devices can be designed to dissolve in the skin, eliminating the issue of
168 microneedle remaining and removal from the skin and allowing a safe disposal without biohazardous
169 waste [35].

170 Although microneedles show great promise for the delivery of drugs [36] and also as sensing devices
171 [37], it is in the research area of vaccines that they showed, so far, more advances. It was demonstrated
172 that vaccination using microneedles triggers stronger immune responses comparing to conventional
173 injection procedures, allowing sparing of antigens [38]. Indeed, the use of microneedle-based devices for
174 influenza vaccination is currently under clinical trials.

175 The improved antigen immunogenicity using microneedle devices is considered to be related to the
176 delivery of antigens at the epidermal and intradermal layer of the skin. The skin is highly rich in
177 immunologically active APCs, which deliver antigens to the proximal lymph nodes where T and B cells are
178 activated, triggering the immune response [39]. Also, in the skin (particularly at the epidermis and the
179 epithelium from the hair follicles) monocytes and Langerhans cells are abundant. Langerhans cells
180 display intrinsic tolerogenic properties *in vivo* [40]. Moreover, a small trial enrolling 14 MS patients

181 showed that the passive diffusion of myelin-related peptides through the skin induces some
182 improvements in the disease, namely reducing the incidence of relapses and the area of lesion (assessed
183 by MRI) [41]. These findings highlight the potential of this route of administration in immunotolerance-
184 based therapies.

185

186 Overall, it is clear that therapeutics based on immunotolerance will take remarkable benefit from the
187 application of micro and nano- technological knowledge. Nanoparticle design can assure antigen-specific
188 response, targeted delivery and controlled dosing; whereas microneedle devices, as we propose, open
189 the unique opportunity for sustained intradermal delivery, further contributing to an antigen-specific
190 response and a tolerogenic effect in a minimally invasive therapeutic approach for MS.

191

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200 **References**

201 1.Lassmann H,van Horssen J. The molecular basis of neurodegeneration in multiple sclerosis. *FEBS Lett*
202 2011; 585: 3715-23.

203 2.Noseworthy JH, Lucchinetti C, Rodriguez M,Weinshenker BG. Multiple sclerosis. *New England Journal*
204 *of Medicine* 2000; 343: 938-52.

205 3.Borreani C, Bianchi E, Pietrolongo E, Rossi I, Cilia S, Giuntoli M, Giordano A, Confalonieri P, Lugaresi
206 A, Patti F, et al. Unmet needs of people with severe multiple sclerosis and their carers: qualitative findings
207 for a home-based intervention. *PLoS ONE* 2014; 9: e109679.

208 4.Compston A,Coles A. Multiple sclerosis. *Lancet* 2008; 372: 1502-17.

209 5.Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdová E,
210 Hutchinson M, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald
211 criteria. *Ann Neurol* 2011; 69: 292-302.

212 6.Fossey SC, Vnencak-Jones CL, Olsen NJ, Sriram S, Garrison G, Deng X, Croke PS,Aune TM.
213 Identification of molecular biomarkers for multiple sclerosis. *J Mol Diagn* 2007; 9: 197-204.

214 7.Sospedra M,Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* 2005; 23: 683-747.

215 8.Oh J,O'Connor PW. Established disease-modifying treatments in relapsing-remitting multiple sclerosis.
216 *Current opinion in neurology* 2015; 28: 220-29.

217 9.Kieseier BC. The mechanism of action of interferon- β in relapsing multiple sclerosis. *CNS Drugs* 2011;
218 25: 491-502.

219 10.Miller AE. Teriflunomide: A Once-daily Oral Medication for the Treatment of Relapsing Forms of
220 Multiple Sclerosis. *Clin Ther* 2015; 37: 2366-80.

221 11.Bomprezzi R. Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview.
222 Ther Adv Neurol Disord 2015; 8: 20-30.

223 12.Subei AM,Cohen JA. Sphingosine 1-phosphate receptor modulators in multiple sclerosis. CNS Drugs
224 2015; 29: 565-75.

225 13.Derfuss T, Kuhle J, Lindberg R,Kappos L. Natalizumab therapy for multiple sclerosis. Semin Neurol
226 2013; 33: 26-36.

227 14.Kappos L, Li D, Calabresi PA, O'Connor P,Bar-Or A. Ocrelizumab in relapsing-remitting multiple
228 sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial. The Lancet 2011; 78: 1779-87.

229 15.Hauser SL. The Charcot Lecture | beating MS: a story of B cells, with twists and turns. Mult Scler
230 2015; 21: 8-21.

231 16.Hartung H-P, Aktas O,Boyko AN. Alemtuzumab: a new therapy for active relapsing-remitting multiple
232 sclerosis. Mult Scler 2015; 21: 22-34.

233 17.Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS,Farokhzad OC. Nanoparticles in medicine:
234 Therapeutic applications and developments. Clin Pharmacol Ther 2008; 83: 761-69.

235 18.Cruz LJ, Tacke PJ, Fokkink R, Joosten B, Stuart MC, Albericio F, Torensma R, Figdor CG. Targeted
236 PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN in
237 vitro. J Control Release 2010; 144: 118-26.

238 19.Beletskii A, Galloway A, Rele S, Stone M,Malinowski F. Engineered PRINT (R) nanoparticles for
239 controlled delivery of antigens and immunostimulants. Hum Vaccin Immunother 2014; 10: 1908-13.

240 20.Roberts RA, Eitas TK, Byrne JD, Johnson BM, Short PJ, McKinnon KP, Reisdorf S, Luft JC,
241 DeSimone JM,Ting JP. Towards programming immune tolerance through geometric manipulation of
242 phosphatidylserine. Biomaterials 2015; 72: 1-10.

243 21.Turjeman K, Bavli Y, Kizelsztein P, Schilt Y, Allon N, Katzir TB, Sasson E, Raviv U, Ovadia
244 H,Barenholz Y. Nano-Drugs Based on Nano Sterically Stabilized Liposomes for the Treatment of
245 Inflammatory Neurodegenerative Diseases. Plos One 2015; 10: e0130442.

246 22.Mao Y, Wang J, Zhao Y, Wu Y, Kwak KJ, Chen C-S, Byrd JC, Lee RJ, Phelps MA, Lee LJ, et al. A
247 novel liposomal formulation of FTY720 (Fingolimod) for promising enhanced targeted delivery.
248 Nanomedicine 2014; 10: 393-400.

249 23.Rittchen S, Boyd A, Burns A, Park J, Fahmy TM, Metcalfe S,Williams A. Myelin repair in vivo is
250 increased by targeting oligodendrocyte precursor cells with nanoparticles encapsulating leukaemia
251 inhibitory factor (LIF). Biomaterials 2015; 56: 78-85.

252 24.Karpus WJ, Kennedy KJ, Smith WS,Miller SD. Inhibition of relapsing experimental autoimmune
253 encephalomyelitis in SJL mice by feeding the immunodominant PLP139-151 peptide. J Neurosci Res
254 1996; 45: 410-23.

255 25.Smith CE, Eagar TN, Strominger JL,Miller SD. Differential induction of IgE-mediated anaphylaxis after
256 soluble vs. cell-bound tolerogenic peptide therapy of autoimmune encephalomyelitis. Proc Natl Acad Sci
257 USA 2005; 102: 9595-600.

258 26.Getts DR, Turley DM, Smith CE, Harp CT, McCarthy D, Feeney EM, Getts MT, Martin AJ, Luo X, Terry
259 RL, et al. Tolerance Induced by Apoptotic Antigen-Coupled Leukocytes Is Induced by PD-L1(+) and IL-
260 10-Producing Splenic Macrophages and Maintained by T Regulatory Cells. J Immunol 2011; 187: 2405-
261 17.

262 27.Luo X, Pothoven KL, McCarthy D, DeGutes M, Martin A, Getts DR, Xia G, He J, Zhang X, Kaufman
263 DB, et al. ECDI-fixed allogeneic splenocytes induce donor-specific tolerance for long-term survival of islet
264 transplants via two distinct mechanisms. Proc Natl Acad Sci USA 2008; 105: 14527-32.

265 28.Getts DR, Martin AJ, McCarthy DP, Terry RL, Hunter ZN, Yap WT, Getts MT, Pleiss M, Luo X, King
266 NJC, et al. Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate
267 experimental autoimmune encephalomyelitis. Nat Biotech 2012; 30: 1217-24.

268 29. Bueyuektimkin B, Wang Q, Kiptoo P, Stewart JM, Berkland C, Siahaan TJ. Vaccine-like Controlled-
269 Release Delivery of an Immunomodulating Peptide To Treat Experimental Autoimmune
270 Encephalomyelitis. *Mol Pharm* 2012; 9: 979-85.

271 30. Hunter Z, McCarthy DP, Yap WT, Harp CT, Getts DR, Shea LD, Miller SD. A Biodegradable
272 Nanoparticle Platform for the Induction of Antigen-Specific Immune Tolerance for Treatment of
273 Autoimmune Disease. *ACS Nano* 2014; 8: 2148-60.

274 31. Bryant J, Hlavaty KA, Zhang X, Yap W-T, Zhang L, Shea LD, Luo X. Nanoparticle delivery of donor
275 antigens for transplant tolerance in allogeneic islet transplantation. *Biomaterials* 2014; 35: 8887-94.

276 32. Yeste A, Nadeau M, Burns EJ, Weiner HL, Quintana FJ. Nanoparticle-mediated codelivery of myelin
277 antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis. *Proc*
278 *Natl Acad Sci USA* 2012; 109: 11270-75.

279 33. Cappellano G, Woldetsadik AD, Orilieri E, Shivakumar Y, Rizzi M, Carniato F, Gigliotti CL, Boggio E,
280 Clemente N, Comi C, et al. Subcutaneous inverse vaccination with PLGA particles loaded with a MOG
281 peptide and IL-10 decreases the severity of experimental autoimmune encephalomyelitis. *Vaccine* 2014;
282 32: 5681-89.

283 34. Maldonado RA, LaMothe RA, Ferrari JD, Zhang A-H, Rossi RJ, Kolte PN, Griset AP, O'Neil C,
284 Altreuter DH, Browning E, et al. Polymeric synthetic nanoparticles for the induction of antigen-specific
285 immunological tolerance. *Proc Natl Acad Sci USA* 2015; 112: E156-E65.

286 35. Lee JW, Park J-H, Prausnitz MR. Dissolving microneedles for transdermal drug delivery. *Biomaterials*
287 2008; 29: 2113-24.

288 36. Prausnitz MR. Microneedles for transdermal drug delivery. *Adv Drug Deliv Rev* 2004; 56: 581-87.

289 37. Yu J, Zhang Y, Ye Y, DiSanto R, Sun W, Ranson D, Ligler FS, Buse JB, Gu Z. Microneedle-array
290 patches loaded with hypoxia-sensitive vesicles provide fast glucose-responsive insulin delivery. *Proc Natl*
291 *Acad Sci USA* 2015; 112: 8260-65.

292 38. Koutsonanos DG, Vassilieva EV, Stavropoulou A, Zarnitsyn VG, Esser ES, Taherbhai MT, Prausnitz
293 MR, Compans RW, Skountzou I. Delivery of subunit influenza vaccine to skin with microneedles improves
294 immunogenicity and long-lived protection. *Sci Rep* 2012; 2: 357.

295 39. Koutsonanos DG, Martin MdP, Zarnitsyn VG, Sullivan SP, Compans RW, Prausnitz MR, Skountzou I.
296 Transdermal Influenza Immunization with Vaccine-Coated Microneedle Arrays. *Plos One* 2009; 4: e4773.

297 40. Shklovskaya E, O'Sullivan BJ, Lai Guan N, Roediger B, Thomas R, Weninger W, de St Groth BF.
298 Langerhans cells are precommitted to immune tolerance induction. *Proc Natl Acad Sci USA* 2011; 108:
299 18049-54.

300 41. Walczak A, Siger M, Ciach A, Szczepanik M, Selmaj K. Transdermal Application of Myelin Peptides in
301 Multiple Sclerosis Treatment. *JAMA Neurol* 2013; 70: 1105-09.

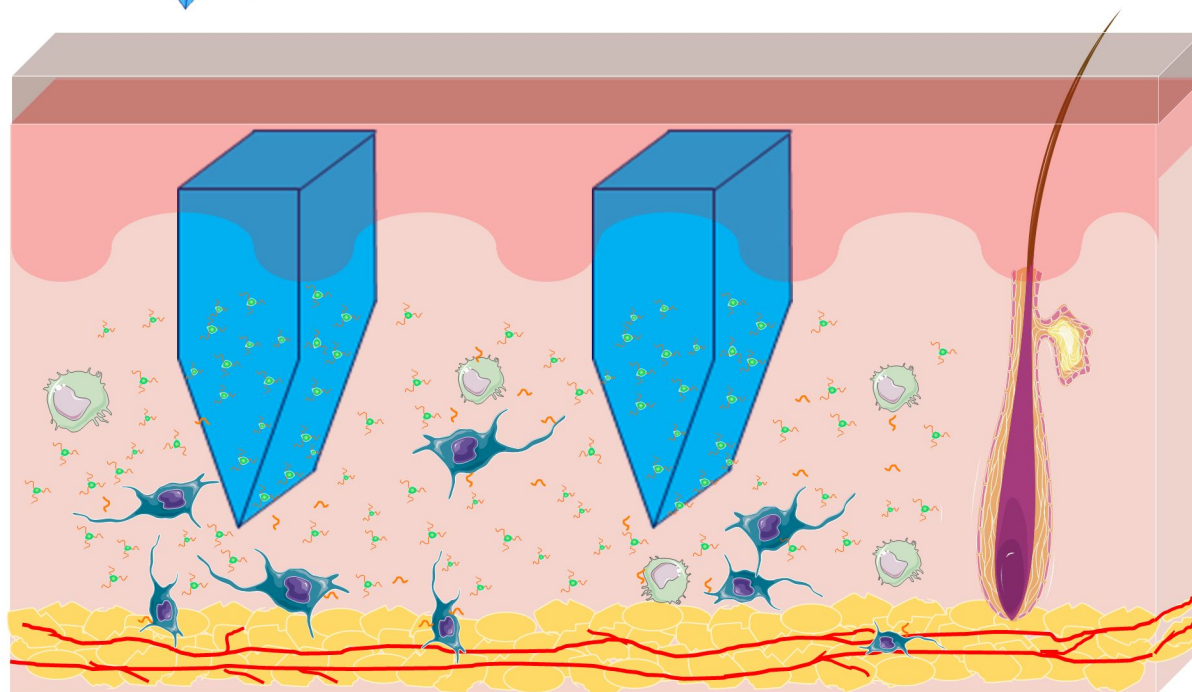
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Microneedle patch

stratum corneum

epidermis

dermis



Antigen-loaded nanoparticles



Antigens



Microneedle patch



Macrophage



Langerhans cell



Adipose tissue



Blood vessels



Hair follicle

