Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance


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Current treatments to control pathological or unwanted immune responses often use broadly immunosuppressive drugs. New approaches to induce antigen-specific immunological tolerance that control both cellular and humoral immune responses are desirable. Here we describe the use of synthetic, biodegradable nanoparticles carrying either protein or peptide antigens and a tolerogenic immunomodulator, rapamycin, to induce durable and antigen-specific immune tolerance, even in the presence of potent Toll-like receptor agonists. Treatment with tolerogenic nanoparticles results in the inhibition of CD4+ and CD8+ T-cell activation, an increase in regulatory cells, durable B-cell tolerance resistant to multiple immunogenic challenges, and the inhibition of antigen-specific hypersensitivity reactions, relaxing experimental autoimmune encephalomyelitis, and antibody responses against coagulation factor VIII in hemophilia A mice, even in animals previously sensitized to antigen. Only encapsulated rapamycin, not the free form, could induce immunological tolerance. Tolerogenic nanoparticle therapy represents a potential novel approach for the treatment of allergies, autoimmune diseases, and prevention of antidrug antibodies against biologic therapies.

Significance

Synthetic nanoparticles containing either protein or peptide antigen and the immunosuppressant rapamycin are capable of inducing durable and specific resistance to mounting immune responses toward the antigen. This immunological tolerance operates on lymphocytes even after multiple immunogenic challenges with the antigen and adding enhancers of immune responses (adjuvants). As a result, the animals treated with these tolerogenic nanoparticles (tNPs) show reduced allergic hypersensitivity disorders, protection from disease relapse in a model of multiple sclerosis, and prevention of inhibitory antidrug antibody responses in an animal model of hemophilia A. These results show the potential for nanocarriers to modify the immunoreactivity of a given molecule by providing tolerogenic instructions to the immune system, thereby preventing or reversing pathological and neutralizing immune responses.

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to secure self-tolerance (21–25). Thomson and colleagues (26, 27) demonstrated that treating DCs with rapamycin, an inhibitor of the mTOR pathway, induces a tolerogenic DC phenotype capable of inducing Treg differentiation and antigen-specific immune tolerance that is resistant to the proinflammatory cascade triggered by TLR signaling. However, conventional therapy with free rapamycin requires chronic systemic administration, resulting in broad immunosuppression due to its direct effect on lymphocytes (28), whereas low doses of rapamycin may paradoxically augment effector T-cell memory (29). Thus, it would be desirable to transiently target rapamycin’s effects to DCs and other antigen-presenting cells (APCs) at the time of antigen encounter. Nanoparticles (NPs) are an ideal mechanism to deliver antigen (16, 30, 31) and drugs (32) to APCs, as these cells are keyed to capture and internalize nanoparticles such as viruses.

Here we describe the development of tolerogenic NPs (tNPs) using materials and compounds that have been well validated in the clinic. These self-assembling, biodegradable poly(lactide-co-glycolide) (PLGA) tNPs containing either protein or peptide antigens and rapamycin are capable of inducing durable antigen-specific tolerance that control adaptive immune responses and withstand multiple immunogenic challenges with antigen. We demonstrate that either s.c. or i.v. administration of tNPs inhibits the activation of antigen-specific CD4+ and CD8+ T cells and B cells while inducing antigen-specific Tregs and Bregs. Swiss Jack Lambert (SIL) mice immunized with the myelin protein oligodendrocyte protein 139–151 peptide in complete Freund’s adjuvant (PLP139-151/CFA) and treated therapeutically with a single dose of NPs at the peak of disease are completely protected from developing relapsing paralysis. In hemophilia A animals, administration of tNP before or after the establishment of an anti-factor VIII (FVIII) antibody response led to a significant reduction of the neutralizing antibody response against FVIII. Treatment of mice with tNP prevents both cellular and humoral immunity even in the presence of potent TLR agonists. These effects are dependent on the presence of the encapsulated rapamycin (not free in solution).

Results

Induction of CD4+ T-Cell Tolerance Using tNPs. PLGA tNPs containing rapamycin and chicken ovalbumin (OVA) or rapamycin and hen egg lysozyme (HEL) were covalently linked to the emulsion process (Fig. 1 D). To investigate whether tNPs could inhibit CD4+ T-cell activation, T cells specific for the OVA323–339 peptide (OTII T cells) were adoptively transferred into naïve animals. Mice were then immunized s.c. with OVA323–339 admixed with a TLR7/8 agonist, R848, and concomitantly treated i.v. with tNP or control NPs. Four days postimmunization, the number of OTII T cells in the draining popliteal lymph node (popLN) of animals that were immunized but not treated was ~6.5-fold greater than that of their naive, unimmunized counterpart (Fig. 1B). The i.v. administration of tNPs completely abrogated the proliferation of OTII T cells, whereas empty NPs admixed with OVA323–339 peptide had little effect. Of note, in these experiments, the tNP treatment was administered i.v., whereas the immunization was provided locally by s.c. administration. To test the direct, local effect of tNP treatment, subsequent experiments evaluated s.c. administration of tNPs (Fig. 1C). Control NPs containing OVA323–339 only (NP[OVA323-339]) induced the activation and proliferation of OTII T cells in the draining popLN, whereas no expansion was observed after treatment with tNP containing rapamycin and OVA323–339. However, an increase in the percentage of Foxp3+CD25+ OTII T cells was evident after tNP treatment, indicating induction of T regulatory cells. Unexpectedly, the opposite trends were observed when an equivalent amount of free rapamycin (10 μg) was admixed with NP [OVA323–339] (Fig. 1C). These results suggest that administration of tNP led to the formation of MHC class II-restricted regulatory T cells that inhibited the activation of CD4 T-cell immunity and that codelivery of antigen with encapsulated rapamycin was critical. To avoid the possible direct suppressive activity of residual encapsulated rapamycin on CD4 T-cell activation, mice were treated i.v. with tNP 6 d before the OTII T-cell transfer to allow time for the inhibitory effect of rapamycin to dissipate (Fig. 1D). Immunized animals without previous treatment showed robust accumulation of the OTII T cells following immunization with free OVA323–339+R848 s.c. (Fig. 1D). Treatment of mice with tNP before OTII T-cell transfer and immunization inhibited the subsequent expansion of OTII T cells, whereas injections of control NPs containing rapamycin only (NP[Rapa]) had little to no effect. These results indicate that administration of tNP can suppress CD4+OTII T-cell responses through the induction of endogenous MHC class II-restricted suppressive cells.

Inhibition of Humoral Immunity by tNP. We next investigated the effect of tNP treatment during the development of a humoral response. Mice immunized s.c. with an admix of OVA and a TLR9 agonist, CpG oligonucleotides (OVA+CpG), showed a mounting antibody response with increasing anti-OVA titers with each successive boost (Fig. 2 A and B). Concomitant treatment with tNP administered either s.c. (Fig. 2C) or i.v. (Fig. 2B) inhibited the anti-OVA antibody response, even when the tNPs were admixed with CpG (Fig. 2A). The inhibition of the humoral response was dependent on the encapsulation of rapamycin, as providing the OVA323–339 peptide alone in free or encapsulated form (NP[OVA323-339]) or just free rapamycin (all admixed to OVA+CpG) did not affect the anti-OVA response (Fig. 2A). Importantly, both peptide- and protein-containing tNPs were similarly efficacious (Fig. 2B, Left and Middle panels, respectively). In contrast, control NP[OVA] administered s.c. or i.v. enhanced the humoral response (Fig. 2B, Right).

We next evaluated splenic B-cell activation and function in mice immunized i.v. with an immunogenic particulate form of OVA (pOVA). Approximately 10–12% of B cells (B220+) found in the local draining LN acquired a typical germinal center phenotype, expressing low levels of IgD, high levels of GL7, and the proliferation marker Ki67 (Fig. 2A). Importantly, both peptide- and protein-containing tNPs were similarly efficacious (Fig. 2B, Left and Middle panels, respectively). In contrast, control NP[OVA] administered s.c. or i.v. enhanced the humoral response (Fig. 2B, Right).

Durability of the tNP Treatment Effect. Mice were injected s.c. with NP or saline admixed with OVA+CpG (days 0, 14, and 28) and OVA (days 42, 56, 70, 84, and 98; Fig. 3A). Animals treated concurrently with tNPs showed a substantial delay in the development of anti-OVA antibodies, and the titers remained ~37-fold lower than those observed in the immunized controls. Of the 53 animals that were treated with tNP (nine independent experiments), 15 showed no detectable titers and 38 showed low titers at days 91–119 compared with animals in the untreated group (Inset table in Fig. 3A). Only three animals (5%) of tNP-treated animals showed titers greater than 2,000, compared with 59 animals (92%) in the control group. To test the durability of
tNPs in the CD4+ T-cell response. (A) tNP platform. Modular, synthetic, self-assembling tolerogenic PLGA NPs contain antigen and rapamycin. (B) NPs containing antigen and rapamycin (but not antigen alone) are tolerogenic. OTII T cells were transferred into CD45.1 mice on day 0. On day 4, popLNs were harvested and analyzed as in (A). Data are representative of two independent experiments (n = 4 per group). Data are cumulative of two independent experiments (n = 5). (D) Induction of antigen-specific T-cell responses by tNP. OTII T cells were transferred into CD45.1 mice on day 0. Animals received s.c. immunizations with vehicle (no immunization) or OVA223–339 admixed with R848. At the same time, animals were injected i.v. with saline (no treatment), empty NPs admixed with OVA223–339 (NP[Empty]+OVA223–339) or tNPs (Rapap+NP[OVA223–339]). Four days later, popLNs were harvested and the total number of OTII T cells was quantified by flow cytometry (n = 4 per group). Data are representative of two independent experiments (n = 5).

Antigen-Specific Tolerance Induction by tNP Administration. Animals from Fig. 3B that had shown a long-lasting inhibition of anti-OVA responses were immunized and boosted with keyhole limpet hemocyanin (KLH). The tNP-treated mice showed a robust anti-KLH response (Fig. 4A) similar to that of the control animals, indicating they were not chronically immunosuppressed. To further test the specificity of the effect and show that the immunosuppressive effects of rapamycin are not broad or systemic, animals were injected s.c. with OVA+CpG ± tNP in the right limbs and KLH+CpG in the left limbs (Fig. 4B). Mixing tNP (containing OVA223–339 and rapamycin) with OVA+CpG completely blocked the development of anti-OVA responses but had no effect on the anti-KLH response initiated in the contralateral limb (Fig. 4B). Moreover, a single injection of tNP containing OVA and rapamycin administered 14 d before repeated immunization with pOVA and KLH selectively inhibited the antibody response to OVA, but not KLH (Fig. 4C). In contrast, pretreatment at day -14 with control NP[Rapa] had no significant effect on the response to either OVA or KLH. In addition, animals that were treated three times every 2 wk with tNP+pOVA showed no detectable antibody response even at day 111 after five additional injections of pOVA in the absence of additional tNP treatment (Fig. 4D). Naive animals in which pOVA immunizations were delayed until day 42 developed a robust anti-OVA response by day 84 after three injections of pOVA. These results further support the notion that tNP treatment did not lead to systemic immunosuppression and induced durable tolerance rather than merely delaying the immune response.

tNP Trafficking. To determine the fate of tNP after injection in vivo, cyanine 7 (Cy7) and Cy5 fluorescently labeled NPs were developed. Whole-body imaging by 3D fluorescence-based tomography (34) showed that fluorescent NPs rapidly and selectively accumulated in the liver and the spleen following i.v. administration (Fig. 4E, Left and Fig. S1 A and B). In contrast, fluorescent NPs administered s.c. in the hind limb showed rapid and selective accumulation in the draining LNs (Fig. 4E, Right). Immunohistochemical analysis of the spleen 2 h after i.v. administration (Fig. 3B, Right) showed that fluorescent NPs were captured in the spleen by different cell types. Moreover, a single injection of tNP containing OVA and rapamycin administered 14 d before repeated immunization with pOVA and KLH selectively inhibited the antibody response to OVA, but not KLH (Fig. 4C). In contrast, pretreatment at day -14 with control NP[Rapa] had no significant effect on the response to either OVA or KLH. In addition, animals that were treated three times every 2 wk with tNP+pOVA showed no detectable antibody response even at day 111 after five additional injections of pOVA in the absence of additional tNP treatment (Fig. 4D). Naive animals in which pOVA immunizations were delayed until day 42 developed a robust anti-OVA response by day 84 after three injections of pOVA. These results further support the notion that tNP treatment did not lead to systemic immunosuppression and induced durable tolerance rather than merely delaying the immune response.

### Notes

- **Fig. 1.** tNPs in the CD4+ T-cell response. (A) tNP platform. Modular, synthetic, self-assembling tolerogenic PLGA NPs contain antigen and rapamycin. (B) NPs containing antigen and rapamycin (but not antigen alone) are tolerogenic. OTII T cells were transferred into CD45.1 mice on day -2. At day 0, animals received s.c. immunizations with vehicle (no immunization) or OVA223–339 admixed with R848. At the same time, animals were injected i.v. with saline (no treatment), empty NPs admixed with OVA223–339 (NP[Empty]+OVA223–339), or tNPs (rapamycin+OVA223–339). Four days later, popLNs were harvested and the total number of OTII T cells was quantified by flow cytometry (n = 4 per group). Data are representative of two independent experiments (C). Encapsulation of rapamycin is critical for the control of CD4+ T-cell responses by tNP. OTII T cells were transferred into CD45.1 mice 1 d before s.c. injection of tNPs (containing rapamycin and OVA223–339) or NP[OVA223–339] alone or NP[OVA232–339] admixed with free rapamycin (Rapam+NP[OVA232–339]). Seven days later, popLNs were harvested, and total number of OTII cells was enumerated (Left) and the percentage of Foxp3+CD25+ OTII T cells was determined (Right). Data are cumulative of two independent experiments (n = 5). (D) Induction of antigen-specific MHC class II-restricted CD4 T-cell tolerance using tNP. C57BL/6 mice were treated i.v. on day -7 with NP[Rapa] or tNPs (rapamycin+OVA223–339). On day -1, purified naive CFSE-labeled CD4+OTII T cells were administered i.v. and then mice were immunized with OVA223–339+R848 s.c. in the hind limbs on day 0. On day 4, popLNs were harvested and analyzed as in B. Data are representative of two independent experiments (n = 3 or 4 per group). All statistical analyses were performed using a one-way ANOVA with a Bonferroni posttest (**p ≤ 0.001).
The injection of NP–Cy5 showed a typical distribution for a particulate antigen, colocalizing with the macrophages and DCs of the red pulp and the marginal zone that delimits the white pulp (Fig. 4F). FACS analysis confirmed that most myeloid cells, including...
macrophages, conventional DC (cDC), and plasmacytoid DC (pDC), contained particles (Fig. S1C) (16, 30, 31). These results show that our NPs efficiently deliver their payload to APCs in the lymphoid organs.

Effects of tNP in Animal Models of Hypersensitivity. Animals repeatedly immunized i.v. with pOVA developed anaphylactoid responses after three weekly i.v. infusions of pOVA, reminiscent of the antibody-mediated hypersensitivity reactions observed in some patients who develop antidrug antibodies (35–37). Un-treated animals with high titers against OVA from Fig. 4C exhibited strong hypersensitivity to pOVA administered i.v., whereas animals that received a single prophylactic treatment of tNP showed no anti-OVA titers and were largely protected from anaphylaxis (Fig. 5A). Similarly, animals immunized with OVA+CpG and challenged with OVA s.c. experienced a significant delayed-type hypersensitivity (DTH) response on the day after challenge, whereas animals that received concomitant tNP treatment during immunization showed no inflammation (between day 91 and day 119) (Fig. 5B). The table shows the number of animals in each group with the indicated level of anti-OVA titers at the last bleed day (between day 91 and day 119).

Immunization with OVA and aluminum hydroxide gel (Alum) i.p. followed by intragastric (i.g.) or intranasal (i.n.) challenge with OVA induced the development of oral allergy or allergic airway inflammation, respectively. Sensitized animals treated therapeutically with tNP i.v. at the time of oral challenge showed a reduction in anaphylaxis (sickness score, diarrhea, and temperature drop; Fig. 5E). Treatment with tNP also resulted in decreased numbers of splenic T cells with an activated phenotype (CXCR5+CCR4+CD45R0+) and an increased proportion of cells with a B regulatory phenotype (CD19+CD45R+IL-10+) compared with saline-treated animals. Similar to animals with oral allergies, no significant effect was observed on the IgE levels using this therapeutic protocol.

Fig. 3. Sustained and robust control of antibody responses with tNP. (A) Long-term effect of tNP. C57BL/6 animals were immunized s.c. with OVA+CpG on days 0, 14, and 28 and boosted with OVA every 14 days until at least day 77 with or without tNP (containing rapamycin+OVA23-35). The figure depicts the anti-OVA IgG titers for the indicated range of days. Each dot represents the average titer for each group taken from nine independent experiments (n = 51 and 53 animals per group). The table shows the number of animals in each group with the indicated level of anti-OVA titers at the last bleed day (between day 91 and day 119). (B) Durability of tNP therapy. Mice were immunized s.c. with OVA+CpG and treated i.v. with vehicle (no treatment) or tNP (rapamycin+OVA23-35) on days 0, 14, and 28. Animals were boosted with OVA every 14 d from day 42 to day 224 without any further tNP treatment. The treated group received another injection of tNP on day 234. The animals were subsequently challenged with OVA+CpG s.c. on days 239 and 253, followed by a boost of OVA s.c. on day 267 (n = 5). (C) Therapeutic tNP treatment inhibits further boosting of antigen titers. Animals were immunized s.c. with OVA+CpG on days 0 and 14 without any treatment. At day 21, anti-OVA titers were measurable in all animals. The untreated control group continued to receive injections of OVA+CpG every 2 wk from days 28-213, whereas the treated group received biweekly OVA+CpG supplemented with tNP (rapamycin+OVA23-35) starting at day 21. EC50 was determined by ELISA (n = 5). The symbols represent the geometric mean ± 95% confidence interval. Statistics were performed using a two-way ANOVA with a Bonferroni posttest (**P < 0.01).
treatments with tNP during or after sensitization are safe and can significantly reduce inflammatory processes associated with hypersensitivity reactions.

**Fig. 4.** Antigen specificity of tolerance induction by tNP. (A) Antigen specificity of tNP therapy. Animals from Fig. 3B were immunized s.c. with 50 μg of KLH on both lateral flanks at the base of the tail (b.t.) on days 239, 253, and 267. Anti-KLH IgG and anti-OVA IgG titers from each animal on day 280 are shown. (B) Treatment with tNP does not result in broad immunosuppression. C57BL/6 mice were immunized with OVA+CpG in the right (front and hind) limbs and with KLH+CpG in the left limbs, whereas treated animals received OVA+CpG+tNP injections in the right limbs and KLH+CpG in the left limbs every 2 wk for 8 wk. Anti-OVA and anti-KLH titers were determined by ELISA (n = 5). (C) Prophylactic induction of antigen-specific tolerance. C57BL/6 mice were treated with a single injection i.v. of tNPs (rapamycin+OVA), NP[Rapa], or vehicle (no treatment) on day −14. Mice were immunized with pOVA i.v. and KLH s.c. on days 0, 14, and 28. Anti-OVA and anti-KLH titers were assessed on day 41. The results summarize three independent experiments (n = 5). (D) Durability of prophylactic induction of antigen-specific tolerance. Two groups of C57BL/6 animals (no treatment and tNP-treated) were immunized biweekly with eight injections with pOVA i.v. from day 0 to day 98. The start of immunization for a third group of animals was delayed until day 42 (delayed immunization control). In all figures and panels, the bars and the symbols represent the geometric mean ± 95% confidence interval (n = 5). All P values were calculated using a Bonferroni posttest of a regular one-way or two-way ANOVA test (***P ≤ 0.001). (E) In vivo trafficking of tNP. (Left) (i.v. injection) Balb/C animals were immunized with pOVA on days −27 and −13. On day 0, fluorescently tagged Cy7–tNP containing OVA and rapamycin were injected i.v., and the animals were imaged by 3D FMT at 6 h after injection. (Right) (s.c. injection) Naïve Balb/C mice were injected with fluorescently tagged Cy7–tNP s.c. and then imaged 1 h after injection (n = 3). I, iliac; P, popLNs; R, renal. (F) Cellular localization of tNP. Fluorescently tagged NP–Cy5 (red) NPs were injected i.v., and spleens were harvested 24 h later for cryosectioning and immunohistochemical analysis. Shown are representative low and high (Lower Right panel) magnification (10x and 100x, respectively) photomicrographs of the distribution of the NPs among the red pulp (RP), the white pulp (WP), and the marginal zone (MZ) delineated by the localization of macrophages (MPs, F4/80, green), DCs (CD11c, dark blue), and B cells (B220, cyan).

**Prophylactic and Therapeutic Efficacy of tNP in a Relapsing–Remitting Model of Experimental Autoimmune Encephalomyelitis.** Empty NP or tNP containing rapamycin and PLP<sub>139–151</sub> were administered
Fig. 5. Prevention and amelioration of hypersensitivity disorders by tNP. (A) Inhibition of anaphylactic response to i.v. antigen. Anaphylactic reactions in response to the third i.v. injection of OVA in animals described in Fig. 4C were assessed by three blinded scorers (0, no symptom; 1, lethargy; 2, lethargy and inability to right; 3, moribund). The results summarize three independent experiments (n = 5). (B) Inhibition of anti-OVA DTH response. C57BL/6 mice were immunized s.c. with OVA + CpG in the front limbs and treated i.v. with tNP or saline on days 0, 14, and 28. Animals were challenged s.c. on day 34 with 25 μg of OVA in one hind paw and saline in the other. The next day the difference in thickness between the two limbs was determined using a caliper. Data are representative of two independent experiments (n = 5). (C) In vivo cytotoxic T-lymphocyte (CTL) assay. Animals were treated with tNP or PBS on days 0 and 5 and then immunized with NP-encapsulated OVA and CpG on day 9. On day 14, animals received a mixture of CFSE-labeled target cells loaded with the MHC class I-restricted OVA peptide OVA257-264 (SIINFEKL) and labeled control cells. The next day, spleens were harvested and specific lysis of target cells was determined by flow cytometry (n = 3). (D) Inhibition of orally induced IgG and IgE responses using tNP. BALB/c mice were sensitized i.g. with OVA (5 mg) and cholera toxin (CTx, 10 μg) weekly for 10 wk. Mice were treated i.v. with saline (no treatment) or tNP (rapamycin+OVA). Anti-OVA titers were assessed on day 83 (n = 5). (E) Inhibition of anaphylactic response to an oral allergen. BALB/c mice were immunized with OVA + Alum on days 0 and 14 and challenged six times with OVA by i.g. gavage every 2 d from day 28 until day 38. Animals were treated i.v. with saline (no treatment) or tNP (rapamycin+OVA) during each oral challenge. Mock immunized animals received Alum. Animals were assessed by three blinded scorers for anaphylaxis, and the percent of animals with diarrhea was determined by flow cytometry (n = 5). (F) Inhibition of mMCP1 release. BALB/c mice were immunized with OVA + Alum and then challenged orally with OVA while receiving treatment with tNPs. Serum mMCP1 levels were assessed 1 h after the last (sixth) challenge by ELISA (n = 5). (G) Effect of therapeutic treatment with tNP on allergic airway inflammation. BALB/c animals were injected with OVA + Alum on days 0 and 5 and then challenged with OVA i.n. on days 12, 13, and 14. A sentinel group of animals killed on day 15 were confirmed to have allergic airways. At days 18, 25, and 32, animals received i.v. treatments of saline, NP[OVA232-339], or tNPs (rapamycin+OVA232-339). BAL fluid was collected after challenge and assessed for T cells, B cells, and eosinophils. Splenocytes were restimulated overnight with OVA and assessed for TCRβ+CXCR5+ T cells and CD19+B220+IL-10+ cells (Bregs). (H) Inhibition of IgE responses and airway reactivity using tNP. BALB/c animals were injected with OVA+Alum on days 0, 7, and 14 and then challenged daily with OVA i.n. from day 21–24. Animals were treated i.v. with saline (no treatment), NP[OVA232-339], or tNP (rapamycin+OVA232-339) on days 0, 7, and 14. The results are representative of two independent experiments (n = 5). The bars in all figures and panels represent the geometric mean (± 95% confidence interval). All P values were calculated using a Bonferroni posttest of a regular one-way or two-way ANOVA test (***P ≤ 0.001).
that block the procoagulant function of the therapeutic peptide showed partial protection. Importantly, animals treated therapeutically with a single i.v. injection of tNP administered prophylactically in SJL mice by s.c. injection 21 and 14 d before immunization with PLP139-151/CFA to induce relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE). Treatment with tNP inhibited the onset and severity of paralysis typical of this model of autoimmunity (Fig. 6A and Fig. S2A) and inhibited weight loss (Fig. S2B). The animals were followed for 3 wk to exclude the possibility of a delayed response. Mice treated with NP[Rapa] showed only a modest delay in disease onset, whereas animals treated with NP containing only the PLP139-151 peptide showed partial protection. Importantly, animals treated therapeutically with a single i.v. injection of tNP administered near the peak of disease showed complete inhibition of disease relapse (Fig. 6B and Fig. 52B). Therapeutic treatment with tNP by s.c. injection resulted in complete inhibition of relapse in 12 of 13 mice (Fig. 6B).

**Induction of Tolerance to FVIII in Hemophilia A Mice.** Hemophilia A mice deficient in the expression of FVIII (E16) present with similar defects in blood clotting and propensity to develop neutralizing antibodies against recombinant human FVIII (rhFVIII) as severe hemophilia A patients. Administration of rhFVIII led to the development of neutralizing antibody responses or “inhibitors” that block the procoagulant function of the therapeutic rhFVIII, similar to previous studies (38). Prophylactic administration of tNPs given at the same time as rhFVIII administration in naïve mice (Fig. 6C) or therapeutic administration of tNPs in mice previously primed with three injections of rhFVIII led to a significant reduction of anti-FVIII antibodies, including neutralizing antibodies (Fig. 6D).

**Discussion.** We were guided by the following design criteria in developing tNPs: (i) use of a polymer (PLGA) that is biocompatible, biodegradable, and used in multiple products licensed for clinical use by regulatory agencies, (ii) a small-molecule immunomodulatory agent (rapamycin) that has been validated in humans and is capable of securing immune tolerance programming even in the face of inflammatory signals, (iii) nano-sized particles to allow for efficient transport to lymphoid organs and capture by APCs, and (iv) concomitant delivery of either protein or peptide antigens with the small-molecule immunomodulator, thus enabling coordinated delivery of immunological instructions to APCs. Importantly, we have previously validated the manufacturing process for PLGA NPs through the successful production of multiple GMP lots that were released and used in a phase 1 vaccine clinical trial (39).

We show here that the tNPs are capable of inducing antigen-specific B-cell tolerance that is maintained for at least 200 d in the face of repeated challenges with antigen (Fig. 3B). Treatment with tNPs through a variety of routes (i.v. and s.c.) and schedules of treatment efficiently inhibited B-cell activation and antibody responses against antigens administered locally or systemically (i.v., s.c., i.g., and i.p.; Figs. 2B–D, 4C, and 5) as well as local and systemic hypersensitivity reactions (Fig. 5A, B, and E). Importantly, tNPs were capable of inducing tolerance even when coadministered with free antigen in the presence of potent TLR agonists, such as CpG or R848, and adjuvants, such as Alum and CTx (Figs. 1B, 3A and B, and 5). Prophylactic administration of tNPs inhibited anti-OVA IgE antibody production in allergy models (Fig. 5D and H). Therapeutic treatment had no effect on anti-IgE levels but attenuated the pathological effects of antigen administration in a model of oral allergy (Fig. 5E) and allergic airway inflammation (Fig. 5G). These results suggest that the beneficial effects mediated by tNP treatment after immunization could be exerted on the cellular compartment responding to the antigen rather than on the antibody levels per se. It is possible that therapeutic reduction of preexisting antibody levels would require more time due to the presence of long-lived plasma cells. However, therapeutic administration of tNPs applied on preexisting anti-OVA IgG responses and inhibited further boosting with the antigen even in the presence of CpG (Fig. 3C), suggesting an inhibitory effect on the memory cells responsible for the secondary or boost response. We believe that there are both temporal and spatial aspects to the induction of antigen-specific immune tolerance with tNPs. The temporal aspect of tolerance induction is illustrated in Fig. 4C, where tNPs are administered 14 d before repeated immunization with OVA and KLH. In this experiment, the tNP containing OVA and rapamycin selectively inhibits the antibody response to OVA but not KLH, whereas
NPs containing only rapamycin have no effect on either response. In contrast, Fig. 4B illustrates the spatial aspect of antigen-specific tolerance induction, where tNP and OVA+CpG are administered in the left flank and KLH+CpG is administered at the same time in the right flank. Because the tNP biodistribution following s.c. administration is localized to the draining LNs (Fig. 4E, Right), the tNP selectively inhibits the immune reaction against OVA in the LNs, draining the left flank without affecting the immune response to KLH in the right flank.

The precise mechanism of action of our tNPs remains to be determined. Nanoparticulates have been shown to be selectively endocytosed by APCs, such as macrophages and DCs (16, 30, 31). The codelivery of antigen and rapamycin provide an instruction set that could allow for the conditioning of tolerogenic DCs capable of inducing CD4+ Treg (22, 23). In support of this notion, prophylactic treatment with OVA323-339−loaded tNPs 7 d before transfer of OTII T cells resulted in complete inhibition of OTII T-cell expansion in response to immunization with OVA323-339+R848, whereas treatment with control NPs containing only rapamycin had no effect (Fig. 1D). These experiments show that the inhibitory effect induced by the tNPs is more durable than that expected from transient immunosuppression by rapamycin and is dependent on the presence of antigen, indicating the formation of OVA323-339−specific regulatory cells that can control the activation of the subsequently transferred OTII T cells. Indeed, tNP treatment induced an increase in the percentage of FoxP3+ antigen-specific T cells (Fig. 1B). Importantly, we found that admixing free rapamycin with NP-encapsulated OVA (or OVA323-339) did not result in immune tolerance. In fact, free rapamycin, administered at an equivalent dose as that used in tNP treatment, showed the opposite trend with respect to inhibition of OVA-specific T-cell expansion and induction of antigen-specific Tregs, perhaps due to the direct effect of free rapamycin on enhancing effector memory T-cell formation (40). Moreover, free rapamycin showed a consistent trend toward enhancing anti-OVA−specific antibody responses. Similarly, NPs loaded with protein antigen in the absence of rapamycin were very immunogenic. These results indicate that the biology of both the rapamycin and the antigen can be profoundly influenced by the context in which they are delivered.

Tolerance induction using NPs was also demonstrated by Yeste et al. (19). In this work, NPs loaded with the CD22 ligand described by Macauley et al. (18), which induce B-cell, but not T-cell, tolerance. Finally, it is notable that our tNPs are capable of inhibiting immune responses when administered either i.v. or s.c., whereas other NP approaches appear to be limited to systemic administration (13, 19, 32). More experimentation is necessary to compare these technologies and determine whether they can be used in similar applications.

The emergence of NP-based therapies for the induction of antigen-specific immune tolerance holds considerable promise for the future of immunotherapy. Tolerogenic therapies could be beneficial for the treatment of allergic asthma, life-threatening food allergies, and autoimmune disease (8). A single therapeutic dose of tNP administered at the peak of disease completely prevented disease relapse in EAE, a model of human multiple sclerosis (Fig. 6B). Antigen-specific tolerance could be useful in preventing antidiug antibodies, which can compromise efficacy and safety of biologics. A prominent example is the neutralizing antibody responses to FVIII, which results in major complications for up to 30% of hemophilia A patients (42). Currently, patients that develop inhibitors are treated with large frequent doses of FVIII, which is extremely costly. Here we show that adding tNP to the therapeutic regimen of FVIII for a limited number of injections significantly increases the likelihood of establishment of tolerance (Fig. 6C and D). Protein therapies are the fastest growing segment of new drug approvals, and a proliferation of novel engineered proteins, such as multivalent antibodies and antibody-drug conjugates, may be at increased risk for immunogenicity (43). NPs could potentially also be applied to prevent immunogenicity associated with vectors used for gene therapy. The ability to translate antigen-specific therapy to human clinical practice would represent a breakthrough that could potentially benefit patients across a wide spectrum of clinical indications.

Materials and Methods

Complete details of materials are provided in SI Materials and Methods.

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tNP and NP Manufacturing. tNPs containing both antigen and rapamycin were prepared using a water−in-oil−in-water double emulsion solvent evaporation method (44, 45). Briefly, PLGA, pegylated polyacetic acid (PLA-PEG), and rapamycin were dissolved in dichloromethane to form the oil phase. An aqueous solution of antigen (OVA protein, OVA323-339 peptide, FVIII35-89 peptide, FVIII319-337 peptide, FVIII2191-2210 peptide, or PLP139-151 peptide) was then added to the oil phase and emulsified by sonication (Branson Digital Sonifier 250A). Following sonication, the emulsion was allowed to stand at 4 °C for 2 h, and a double emulsion was created by adding an aqueous solution of polyvinylalcohol and sonication for a second time. The double emulsion was added to a beaker containing phosphate buffer solution and stirred at room temperature for 2 h to allow the dichloromethane to evaporate. When creating NPs containing rapamycin but no antigen, or NPs without any encapsulated agents, a similar oil-in-water single emulsion process was used. The resulting NPs were washed twice by centrifuging at 75,600 × g and 4 °C followed by resuspension of the pellet in PBS. Fluorescent Cy5- and Cy7-containing NPs were manufactured as described above using PLGA-Cy5 or PLGA-Cy7 conjugate, respectively. PLGA with a butyl amine end group was prepared from PLGA−acid, which was then treated with Cy7−acid or Cy5−acid in the presence of a coupling agent (O-Benzotriazol-1-yloxy-N,N,N′,N′-tetramethyluronium tetrafluoroborate) to afford the conjugates.

Animal Models. Animal procedures involving hemophilia A mice, EAE, and in vivo imaging were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, Hooke Laboratories, and Molecular Research Imaging, respectively. All other mouse experiments were approved by the Institutional Animal Care and Use Committee of Avastus Preclinical Services, following local and national guidelines and regulations.

Releasing EAE model. EAE was induced by injection of SJL mice s.c. at four sites in the back with PLP139-151 emulsified in CFA (Hooke Laboratories) followed 2 h later by i.p. injection of 154 ng of pertussis toxin. Blinded EAE scores were determined using EAE scores

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were assessed daily starting from day 7, and body weight was measured three times per week. EAE was scored on a 0–5 scale as follows: 0, no obvious changes; 1, moribund; 2, limp tail and weakness of hind legs; 3, limp tail and complete paralysis of hind legs (most common) or limp with paralysis of one front and one hind leg; 4, complete hind leg and partial front leg paralysis; 5, death or euthanized because of severe paralysis.

**Allergic airway model.** BALB/c female mice aged 8–10 wk were sensitized by i.p. injections of 10 μg OVA adsorbed to 4 mg of Alum on days 0 and 5. On days 7, 8, and 9, mice were administered PBS for the control group or 25 μg OVA i.n. Mice were treated with tNP or PBS on days 25, 32, and 39. Finally, mice were administered PBS for the control group or 25 μg OVA i.n. for 3 consecutive days from days 46–48 and were killed 1 h after the last challenge by a lethal dose of isofluorane. Lungs were lavaged three times with 1 mL of ice-cold PBS containing 3 mM EDTA. Oral allergy model. BALB/c female mice were sensitized by i.p. injections of 10 μg OVA adsorbed to 4 mg of Alum on days 0 and 5. On days 7, 8, and 9, mice were administered PBS for the control group or 25 μg OVA i.n. for 3 consecutive days from days 46–48 and were killed 1 h after the last challenge by a lethal dose of isofluorane. Lungs were lavaged three times with 1 mL of ice-cold PBS containing 3 mM EDTA.

**Fluorescence Imaging.** Frozen sections were fixed with cold acetone and stained for the indicated markers and visualized at room temperature using an upright epifluorescence microscope Leica DMi6000B system equipped with a Leica DFC340FX. Images were processed using ImageJ software.

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