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Hydrogen-Bonded Multilayers of Tannic Acid as Mediators of T-Cell Immunity

Veronika Kozlovskaya, Bing Xue, Weiqi Lei, Lindsey E. Padgett, Hubert M. Tse, and Eugenia Kharlampieva**

Type 1 diabetes is an autoimmune-mediated disease resulting in the destruction of insulin-secreting pancreatic β-cells. Transplantation of insulin-producing islets is a viable treatment to restore euglycemia in Type 1 diabetics; however, the clinical application remains limited due to the use of toxic immunosuppressive therapies to prevent immune-mediated rejection. A nanothin polymer material with dual antioxidant and immunosuppressive properties capable of modulating both innate and adaptive immune responses crucial for transplantation outcome is presented. Through the use of hollow microparticles (capsules) composed of hydrogen-bonded multilayers of natural polyphenol (tannic acid) with poly(*N*-vinylpyrrolidone) (TA/PVPON) and with poly(*N*-vinylcaprolactam) (TA/PVCL), proinflammatory reactive oxygen and nitrogen species are efficiently dissipated and the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α proinflammatory cytokines is attenuated by cognate antigen-stimulated autoreactive CD4+ T cells. These results provide evidence that TA-containing capsules are efficacious in immunomodulation and may provide physical transplant protection and prevent diabetogenic autoreactive T-cell responses. Future studies will determine if xeno- and allotransplantation with (TA/PVPON)- or (TA/PVCL)-coated pancreatic islets will decrease the risk of graft rejection due to attenuation of oxidative stress and IFN-γ, and restore euglycemia in Type 1 diabetics.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune-mediated life-threatening disease resulting in the destruction of insulin-secreting pancreatic β-cells by islet-infiltrating leukocytes, including both direct (cytotoxic T-cell-mediated) and indirect (cytokine and reactive oxygen species (ROS)-mediated) mechanisms. While these causes of β-cell destruction are fundamentally different, both involve the generation of cytotoxic ROS.[1–4] In T1D, self-tolerance against insulin-producing β-cells is compromised, as autoreactive CD4+ and CD8+ T cells synthesize proinflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α) and cytotoxic mechanisms including Fas and perforin to destroy insulin-producing β-cells.[5,6] Blocking IFN-γ by a soluble nonimmunogenic form of the IFN-γ receptor has been shown to improve the disease conditions while in presence of IL-12p70, a promoter of T helper 1 (Th1) cell differentiation, the disease conditions are aggravated.[7,8] In addition, autoreactive CD4+ T cells may recruit macrophages to the islet and contribute to pancreatic β-cell destruction by releasing high levels of IL-1β, TNF-α, and ROS.[9–12] In turn, ROS can further exacerbate β-cell damage by functioning as a signaling molecule to induce the activation of proinflammatory cytokine signaling pathways in macrophages and dendritic cells.[3]

As a result of insulin deficiency in T1D, numerous complications including cardiovascular disease, nephropathy, and retinopathy may ensue and diminish the quality of life.[13] In this case, the use of exogenous insulin is vital for maintaining normal glucose levels in blood. Transplantation of insulin-producing cells or pancreatic islets is considered the most reliable strategy to achieve in vivo glucose control and prevent the devastating complications associated with T1D.[14] However, despite early successes, the clinical application of islet transplantation remains limited due to challenges associated with islet integrity and function after isolation from the pancreata of cadaveric donors and the use of toxic immunosuppressive therapies to protect donor islets from immune-mediated rejection.[15] These challenges have inspired the development of islet encapsulation[16–19] and islet surface modification[20–23] strategies to stabilize islet morphology, functionality, and ensure transplant survival without or with minimal immunosuppressive therapy.[24] Recently, we have reported on a nanoscale protective coating of individual islets using a hydrogen-bonded layer-by-layer (LbL) assembly of tannic acid (TA) and poly(*N*-vinylpyrrolidone) (PVPON).[25] The (TA/PVPON) multilayer-coated islets

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were stable for 7 d in vitro, nontoxic, and displayed an elevated insulin stimulation index in vitro culture for 96 h as compared to uncoated islets.[25]

TA is a natural polyphenolic antioxidant and has been used in fabrication of multilayer films, capsules, and cell modification coatings of biomedical relevance.[26–35] The antioxidant activities of TA[36–40] may be beneficial for TA-containing protective coatings since most inflammatory processes are associated with oxidative stress due to enhanced ROS synthesis and/or a decrease in antioxidant activities. ROS are highly reactive oxygen derivatives, which include the superoxide radical ($O_2^{\cdot -}$), the hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2); those along with reactive nitrogen species such as nitric oxide (NO) can function as signaling molecules for immune activation. Lvov and co-workers[41] showed that antioxidant activity of TA is preserved in TA/polycation multilayers. Cao and He[42] reported that antioxidant stability of a drug formulation was improved when poly(triethylene glycol methyl acrylate-*co*-tocopheryl acrylate) complexed with TA was used as a potential antioxidant prodrug to enable localized neuroprotection. Recently, De Geest and co-workers[43] encapsulated tumor-associated antigen by conjugating cancer cells with (TA/PVPON) multilayers to be used for anticancer immunotherapy. The same group showed that (TA/PVPON) microparticles with encapsulated vaccine protein antigens were effectively internalized by dendritic cells in vitro and promoted the antigen-specific humoral and cellular immune responses in vivo as potential vaccine carriers.[44]

Despite the intriguing potential of TA, little is known about immunomodulatory and antioxidant capability of TA-based materials to provide islet protection and prevent autoreactive T-cell responses, crucial for successful transplantation. In the process of human islet isolation, β-cells in isolated pancreatic islets can be impaired by oxidative stress[45] because of the reduced levels of their inherent antioxidant protection, which can lead to imbalanced levels of ROS. Activation of macrophages and T cells by ROS followed by the secretion of proinflammatory cytokines can significantly contribute to the progression of immune system activation and inflammation[46] and potentially lead to functional impairment of the islet transplant. Typically, immunomodulatory compounds employed in different models of oxidative stress injuries are directly injected into tissues. In one example, isolated islets demonstrated higher survival after their transplantation in mice when cultured in the presence of Mn (III) porphyrin-based catalytic antioxidants suggesting that the modulation of oxidative stress can help in better islet protection.[47] There are only a few studies on functionalization of coatings with the active compounds, mostly limited to conjugation to islets or copolymerization into gels on the islet surfaces.[48,49]

Our group recently demonstrated that (TA/PVPON) multilayer coatings suppressed immune cell-mediated proinflammatory cytokine synthesis.[25] However, the mechanism through which TA-based coating exhibits immunomodulatory activity remains unclear. In the current study, we explore the capability of TA-containing multilayer capsules (also known as shells) as immune mediators capable of modulating both innate and adaptive immune responses important for transplantation outcome. We determined the efficacy of various types of hydrogen-bonded multilayers of TA to scavenge reactive oxygen and nitrogen species, as well as the potential of TA-containing (PVPON/TA) or (poly(*N*-vinylcaprolactam)/TA) (PVCL/TA) hydrogen-bonded protective shells to dampen proinflammatory cytokine production from diabetogenic autoreactive T cells. The effects of capsule thickness, composition, and outer layer are studied under various stimulation type and time. To the best of our knowledge, this is the only example of a nanothin material with dual immunomodulatory and antioxidant functionality capable of modulating both innate and adaptive immune responses, crucial for allo- and xenotransplantation.

2. Results and Discussion

2.1. Scavenging of ROS by the TA-Containing Shells

We explored the antioxidant capability of the hydrogen-bonded coatings of TA by determining the ability of PVPON/TA or PVCL/TA hydrogen-bonded shells to dissipate ROS synthesis. For that, the certain concentrations of the corresponding hydrogen-bonded multilayer capsules of (neutral polymer/TA) were prepared as hollow replicas of 4-μm silica particles coated with the corresponding multilayers (**Figure 1**a) as reported previously[25] and tested using respiratory burst assay. As control, TA-free hollow multilayer capsules made from either PVPON or PVCL multilayers were used (Figure 1b).[50]

Using a luminol oxidation assay, we observed total suppression of ROS synthesis in the presence of TA-containing shells (**Figure 2**). The experiments were based on the production of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) after stimulation of OT-II splenic T cells (splenocytes) with phorbol 12-myristate 13-acetate (PMA) and ionomycin (In). PMA/In-stimulated splenocytes rapidly released $O_2^{\cdot -}$ and H_2O_2 to facilitate the oxidation of luminol in the presence of horseradish peroxidase (HRP) and emission of chemiluminescence (Figure 2a).[51] The resultant luminol-dependent chemiluminescence was measured in the presence (+) or absence (–) of TA-containing, $(PVPON/TA)_4$ and $(PVCL/TA)_4$, or control TA-free, $(PVPON)_4$ and $(PVCL)_4$ multilayer shells. Chemiluminescence increased almost threefold compared to background when splenocytes were stimulated (Figure 2b). In drastic contrast, ROS production was suppressed in the presence of $(PVPON/TA)_4$ and $(PVCL/TA)_4$ shells as chemiluminescence did not increase after PMA/In stimulation of the T cells. Importantly, splenocytes incubated with TA-free $(PVPON)_4$ or $(PVCL)_4$ multilayer shells did not exhibit a significant decrease in chemiluminescence compared to the control group. These data suggest that the high antioxidant activity of (PVPON/TA) and (PVCL/TA) capsules is due to the presence of TA within the shells. Apparently, despite TA H-bonding with PVPON or PVCL, there are still phenolic groups available, which are liberated from H-bonding at pH 7.4 and capable of scavenging ROS without compromising stability of the multilayer shell.[52] We found that the synthesis of free radicals detected by the luminol oxidation at 20 min post-treatment with TA-containing multilayer shells and beyond an hour was undetectable. As we have demonstrated previously, scavenging of innate immune-derived free radicals at these early time points will have a profound inhibitory effect on the differentiation and maturation of antigen-specific T cells.[4,9]

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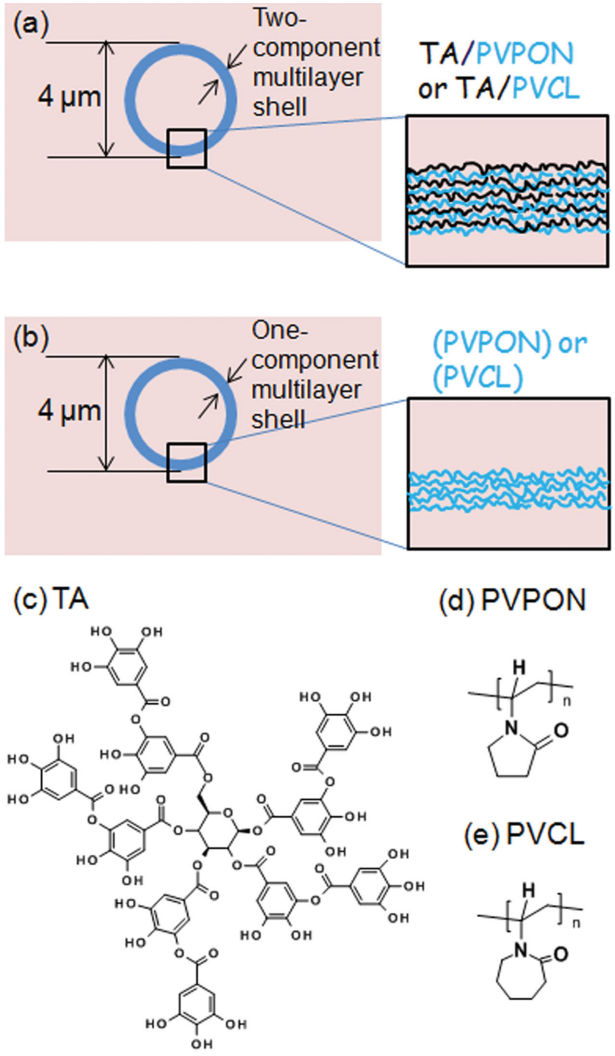

Figure 1. a) TA-containing multilayer capsules of $(TA/PVPON)_n$, or $(TA/PVCL)_n$ were prepared via LbL assembly of TA and PVPON (or PCVCL) on spherical silica particles of 4 μm; b) TA-free capsules of $(PVPON)_n$ or $(PVCL)_n$ multilayers. c–e) Chemical structures of tannic acid (TA), poly(*N*-vinylpyrrolidone) (PVPON), and poly(*N*-vinylcaprolactam) (PVCL).

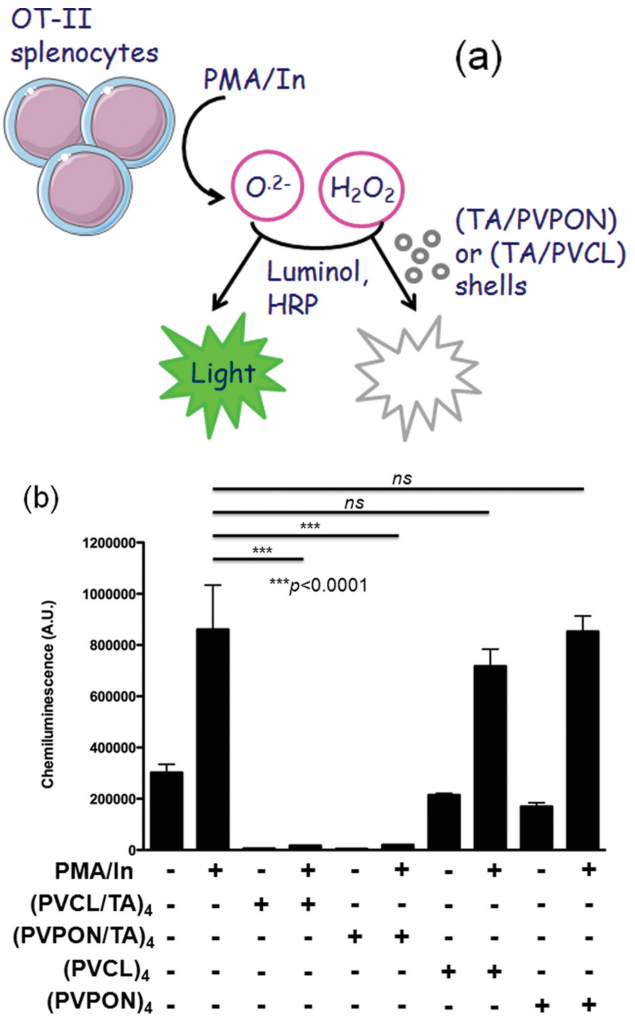

Figure 2. a) Scavenging of ROS synthesis by the TA-based hydrogen-bonded coatings. b) Luminol oxidation assay with OT-II splenocytes stimulated for 20 min with 100 ng mL^{-1} PMA and 1 μg mL^{-1} In in the presence (+) or absence (–) of TA-containing hydrogen-bonded shells $(PVCL/TA)_4$, $(PVPON/TA)_4$, or TA-free $(PVCL)_4$, and $(PVPON)_4$ multilayer shells. Data shown are representative of three independent experiments performed with at least triplicates for each sample. *** $p < 0.001$, ns not significant.

We also found that neither the TA-containing nor TA-free multilayer shells compromised the viability of OT-II splenocytes. For that, MTT cell viability assay was performed with OT-II splenocytes stimulated with 1×10^{-6} M $OVA_{323–339}$ in the presence or absence of TA-containing hydrogen-bonded $(PVCL/TA)_4$ and $(PVPON/TA)_4$ capsules or TA-free multilayer shells of $(PVCL)_4$, $(PVPON)_4$ for 24 h (**Figure 3**). We did not observe any significant decrease in absorbance from the cells in the presence of shells compared to that in the case of the activated splenocytes alone. These results suggest that the cell viability was not compromised, and the decrease in chemiluminescence resulted from the antioxidant activity of the TA-containing hydrogen-bonded shells (Figure 2). The increase in absorbance by splenocytes treated with TA-based shells may be attributed to an increase in overall cell viability as compared to shells lacking TA. Since TA is an antioxidant, TA-containing shells may alter the redox state of antigen-stimulated T cells and enhance proliferation and survival (Figure 3).

2.2. Diminishing of Nitrite Synthesis by TA-Containing Shells

Nitric oxide has been established to be the primary mediator for cytokine-induced islet damage as well as it can lead to inhibition of insulin release from islet β-cells.[53] To mediate physiological signaling, nitric oxide and other reactive nitrogen species can be produced from NO_2^- through its reduction by all mammalian reductases under hypoxia and acidosis.[54] Thus, in addition to dissipating ROS synthesis, the ability of TA-containing hydrogen-bonded shells to scavenge reactive nitrogen species was examined. BDC-2.5 splenocytes (5×10^5) were stimulated with 1×10^{-6} M cognate BDC-2.5 mimotope for 96 h and the nitrite levels in tissue culture supernatants were assayed by the Greiss assay.

Figure 4a demonstrates that a threefold increase in NO_2^- concentration from 0.02×10^{-9} to 0.067×10^{-9} M was observed in the culture supernatants of the splenocytes alone

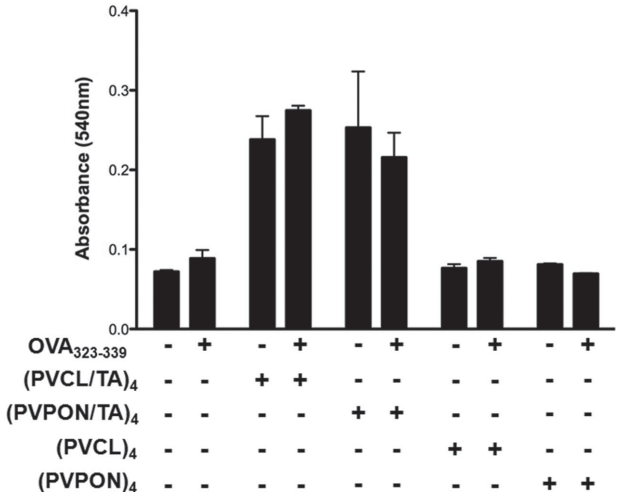

Figure 3. MTT cell viability assay was performed with OT-II splenocytes stimulated with 1×10^{-6} M $OVA_{323-339}$ in the presence (+) or absence (–) of TA-containing hydrogen-bonded $(PVCL/TA)_4$, and $(PVPON/TA)_4$, or TA-free $(PVCL)_4$ and $(PVPON)_4$ multilayer capsules for 24 h. Data shown are representative of three independent experiments performed with at least triplicates for each sample.

in comparison to unstimulated controls. Remarkably, the stimulation of BDC-2.5 splenocytes with their cognate mimotope in the presence of 5×10^6 $(PVCL/TA)_5$ and $(PVPON/TA)_5$ hydrogen-bonded shells for 96 h resulted in diminished nitrite levels by twofold, from 0.067×10^{-9} to 0.029×10^{-9} M for $(PVCL/TA)_5$, and by 1.2-fold, from 0.067×10^{-9} to 0.057×10^{-9} M, for $(PVPON/TA)_5$ after the 96-h co-treatment (Figure 4a). The more efficient, almost twofold, dissipation of nitrite levels in culture solutions by (PVCL/TA) shells may be attributed to a larger shell thickness of the system compared to that of (PVPON/TA). PVCL is a hydrophobic homologue of PVPON with two additional methylene groups in the lactam ring,[55] therefore, the levels of TA deposited within one bilayer can be controlled by varying hydrophobicity of the neutral polymer assembled with TA allowing for obtaining thicker PVCL/TA bilayers compared to PVPON/TA bilayers. Thus, as reported previously, the average bilayer thickness of (PVPON/TA) and (PVCL/TA) capsule walls prepared under similar conditions were 2.2 ± 0.2 and 4.2 ± 0.1 nm, respectively.[56] Subsequently, our results on nitrite dissipation by TA-containing hydrogen-bonded shells are in good agreement with the thickness data for those capsule systems implying that twofold increased amount of TA in PVCL/TA capsules compared to that in PVPON/TA resulted in a twofold decrease of nitrite levels (Figure 4a). Based on the results, we calculated the amount of TA assembled within the hydrogen-bonded coating, which is necessary to dissipate 1×10^{-9} M of nitrite to be ≈69 µg. Given 1 mg m^{-2} adsorbed amount of polymer per 1 nm film thickness,[57] total amount of TA adsorbed within a spherical $(TA/polymer)_n$ shell was obtained as $[TA], 10^{-3} g = \pi n t_{TA} d^2$, in which n is the number of bilayers adsorbed within the multilayer; t_{TA} is an average TA thickness (nm, taken as 50% of the average bilayer thickness,[52] measured by AFM for the shells; d (m) is the size of the sacrificial spherical template used for capsule preparation (4 µm).

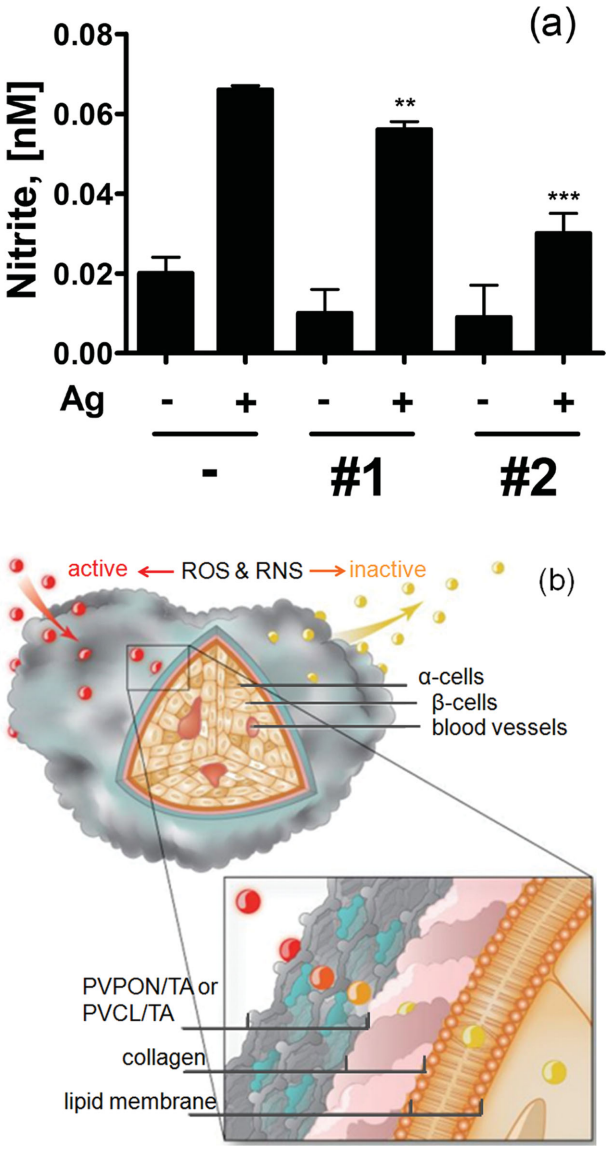

Figure 4. a) Tissue culture supernatants from BDC-2.5 splenocytes (5×10^5) stimulated with 1×10^{-6} M BDC-2.5 mimotope in the presence (+) or absence (–) of 5×10^6 $(PVPON/TA)_5$ (#1), $(PVCL/TA)_5$ (#2) capsules for 96 h were assayed for nitrite levels by the Greiss assay. Data shown are representative of three independent experiments performed with at least triplicates for each sample . *** $p < 0.001$, ** $p < 0.005$ in comparison to BDC-2.5 mimotope-stimulated samples alone. b) Schematics of ROS and RNS protection of islets coated by (PVCL/TA) or (PVPON/TA) hydrogen-bonded coatings.

In Figure 4b, the protective properties of PVCL/TA and PVPON/TA hydrogen-bonded multilayer coatings against reactive oxygen and nitrogen species are schematically summarized. The scavenging of free radicals by the coating because of the antioxidant properties of TA component can lead to suppression of proinflammatory cytokine synthesis via dampening redox-dependent signaling pathways and islet protection. Our results provide evidence that by dissipation of ROS synthesis, TA-based hydrogen-bonded coatings should be able to diminish diabetogenic T-cell effector responses as we have

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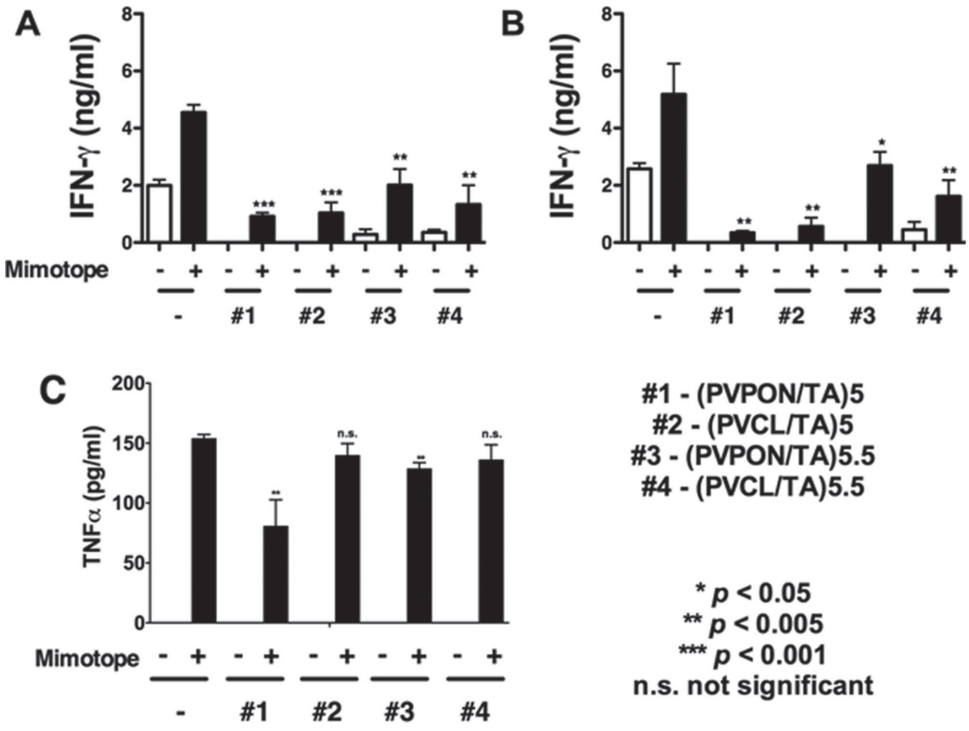

Figure 5. BDC-2.5 primary recall assay with BDC-2.5 mimotope and TA-containing capsules. Supernatants from BDC-2.5 splenocytes (5×10^5) stimulated with 1×10^{-6} M BDC-2.5 mimotope in the presence or absence of 5×10^6 $(PVPON/TA)_5$ (#1), $(PVCL/TA)_5$ (#2), $(PVPON/TA)_{5.5}$ (#3), and $(PVCL/TA)_{5.5}$ (#4) capsules for a) 24 and b) 48 h were assayed for IFN-γ and for TNF-α c) by ELISA. Data shown are representative of three independent experiments performed with at least triplicates for each sample. *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$, ns – not significant in comparison to BDC-2.5 mimotope-stimulated samples alone.

previously demonstrated with a superoxide dismutase mimetic compound.[48,58]

2.3. Inhibition of Adaptive Immune Effector Responses with TA-Containing Shells

We have previously shown that modulating redox status during innate immune activation is efficient in attenuating the synthesis of proinflammatory cytokines that can contribute to adaptive immune maturation and rejection of transplanted islets.[9,59,60] Here, we have studied whether TA-containing hydrogen-bonded coatings could produce a similar modulating effect on the adaptive immune response of autoreactive T cells. The immunomodulatory properties were investigated using three types of proinflammatory cytokines (IFN-γ, TNF-α, and IL-2) synthesized by stimulated diabetogenic autoreactive CD4+ T cells.

The effect of the coatings on suppression of autoreactive BDC-2.5 CD4+ T-cell cytokine responses was examined by measuring IFN-γ released by BDC-2.5 splenocytes after stimulation with their cognate BDC-2.5 mimotope antigenic peptide in the presence (+) or absence (–) of the hollow capsules for 24 and 48 h. To determine the effect of the coating top layer, the multilayer capsules with TA-capping layer labeled as $(PVPON/TA)_5$ or $(PVCL/TA)_5$, and with a neutral capping layer labeled as $(PVPON/TA)_{5.5}$ or $(PVCL/TA)_{5.5}$, were prepared and tested.

Figure 5 demonstrates that all TA-based shells were effective in significantly dampening IFN-γ synthesis. For instance, capsule-free BDC-2.5 mimotope stimulation for 24 h resulted in 4.7 ng mL^{-1} IFN-γ synthesis, while a fourfold decrease in the cytokine levels was observed for the T-cell stimulation in the presence of $(PVPON/TA)_5$ and $(PVCL/TA)_5$ capsules (1 ng mL^{-1}), with a 2.3-fold and 3.4-fold decrease in IFN-γ synthesis in co-culture with $(PVPON/TA)_{5.5}$ and $(PVCL/TA)_{5.5}$ capsules, respectively (Figure 5a). The cytokine synthesis attenuation was even more pronounced after 48-h stimulation of the T cells in the presence of TA-containing hydrogen-bonded shells resulting in 14- and eightfold decrease in synthesis of IFN-γ in the presence of $(PVPON/TA)_5$ and $(PVCL/TA)_5$ capsules, respectively (Figure 5b). In comparison to TA-capped capsules, the neutral polymer-capped $(PVPON/TA)_{5.5}$ and $(PVCL/TA)_{5.5}$ shells were less effective in dampening IFN-γ production showing a two- and threefold decrease in the cytokine synthesis, respectively (Figure 5b). In addition to a decrease in IFN-γ production, TNF-α levels from BDC-2.5 splenocytes stimulated with the BDC-2.5 mimotope in the presence of TA-based hydrogen-bonded capsules were also decreased in the presence of $(PVPON/TA)_5$ and $(PVPON/TA)_{5.5}$ capsules (Figure 5c).

To determine if TA-based shells affect Th1 cytokine responses due to stimulation with the BDC-2.5 mimotope alone, BDC-2.5 splenocytes were stimulated with the mitogen, concanavalin A (ConA), a nonspecific inducer of T-cell responses, in the presence (+) or absence (–) of the capsules for 24 and 48 h. The ability of TA-based hydrogen-bonded shells to decrease autoreactive effector T-cell responses upon stimulation with ConA or the BDC-2.5 mimotope provides additional evidence that islet

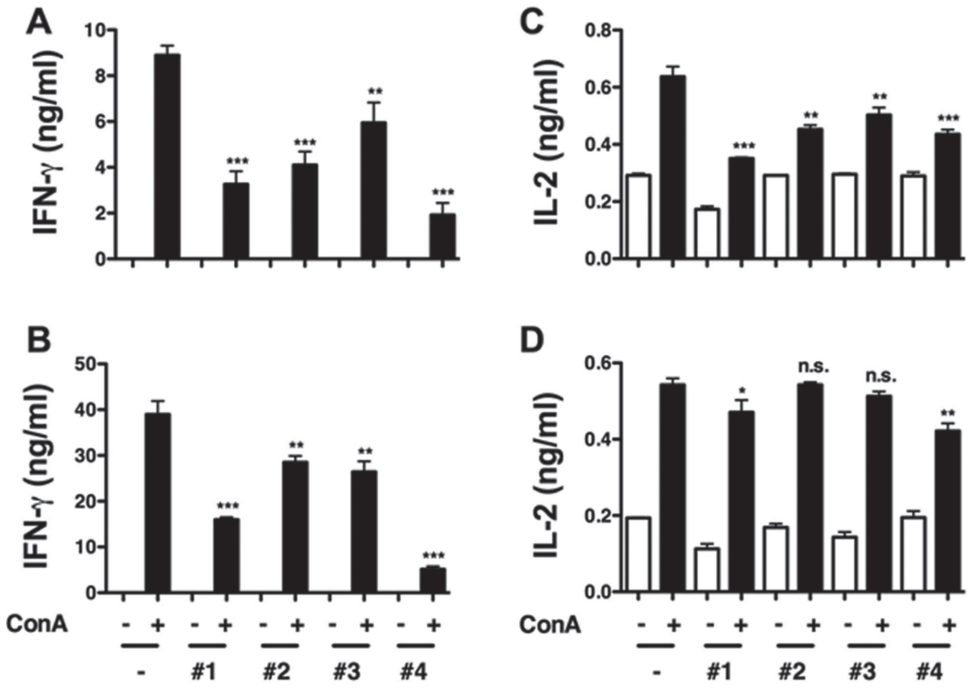

Figure 6. Concanavalin A (ConA)-stimulated BDC-2.5 splenocytes treated with TA-containing shells. Supernatants from BDC-2.5 splenocytes (5×10^5) stimulated with 2.5 μg mL^{-1} ConA in the presence or absence of 5×10^6 $(PVPON/TA)_5$ (#1), $(PVCL/TA)_5$ (#2), $(PVPON/TA)_{5.5}$ (#3), and $(PVCL/TA)_{5.5}$ (#4) capsules for a,c) 24 and b,d) 48 h were assayed for a,b) IFN-γ and c,d) IL-2 synthesis by ELISA. Data shown are representative of three independent experiments performed with at least triplicates for each sample. *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$, ns not significant in comparison to BDC-2.5 ConA-stimulated samples alone.

encapsulation with the TA-based coating may elicit an immunoprotective effect in the context of islet allo- and xenotransplantation. ConA-stimulation for 24 and 48 h resulted in a decreased IFN-γ synthesis, similar to what was observed with BDC-2.5 mimotope-stimulated splenocytes. For TA-capped five-bilayer capsules, (PVPON/TA) system seemed to be more effective than (PVCL/TA) in suppression of the proinflammatory cytokine production with 61% versus 53% decrease of IFN-γ levels, respectively, compared to that for the capsule-free stimulation (**Figure 6**a). The opposite was observed in the case of the capsules with TA shielded by a layer of neutral polymer with 33% versus 88% decrease in the cytokine synthesis for $(PVPON/TA)_{5.5}$ and $(PVCL/TA)_{5.5}$, respectively. Similar results were obtained after 48-h stimulation of the T cells in the presence of the capsules (Figure 6b).

Next, the synthesis of interleukin-2 (IL-2), a cytokine involved in T-cell proliferation, was monitored for 24 and 48 h. The IL-2 levels decreased from 0.64 to 0.36 ng mL^{-1}, to 0.45 ng mL^{-1}, to 0.5 ng mL^{-1}, and to 0.44 ng mL^{-1} when ConA-stimulated BDC-2.5 splenocytes were co-treated with $(PVPON/TA)_5$, $(PVCL/TA)_5$, $(PVPON/TA)_{5.5}$, and $(PVCL/TA)_{5.5}$, respectively for 24 h (Figure 6c). However, the suppressive effects were transient as IL-2 synthesis was restored to nearly control levels after 48 h of stimulation (Figure 6d). This result agrees well with our previous work where no significant change in IL-2 synthesis was observed after 96 h of T-cell stimulation.[25]

An important question is if the suppressive effects, observed in co-culture of T cells with TA-based shells, were due to an increase in synthesis of anti-inflammatory IL-10 cytokine, a major suppressor of Th1 cytokine synthesis.[61] To address the question, co-treatment of mimotope-stimulated BDC-2.5 splenocytes with $(PVPON/TA)_5$, $(PVCL/TA)_5$ shells for 72 h was performed and no enhancement of IL-10 production was observed (**Figure 7**). These results confirmed that the suppression of Th1 effector responses discussed above was indeed because of the TA-based hydrogen-bonded coatings. The mechanisms through which TA-based capsules affect T-cell cytokine synthesis remain to be yet fully elucidated. However, as reported previously, green tee polyphenols, e.g., epigallocatechin-3-gallate, were shown to inhibit both IFN-γ and IL-2 production by B6 spleen

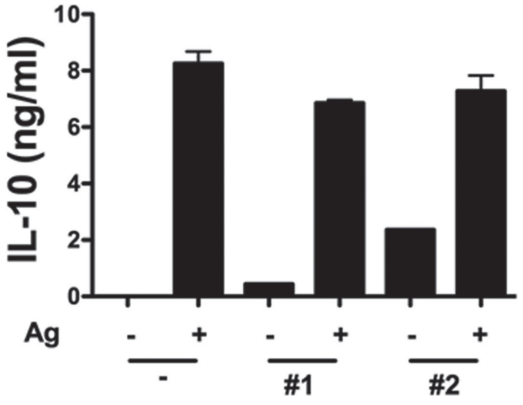

Figure 7. Production of IL-10 in tissue culture supernatants after 72-h stimulation in the absence (–) or presence (+) of 5×10^6 $(PVPON/TA)_5$ (#1), $(PVCL/TA)_5$ (#2) capsules as detected by ELISA. Data shown are representative of three independent experiments performed with at least triplicates for each sample.

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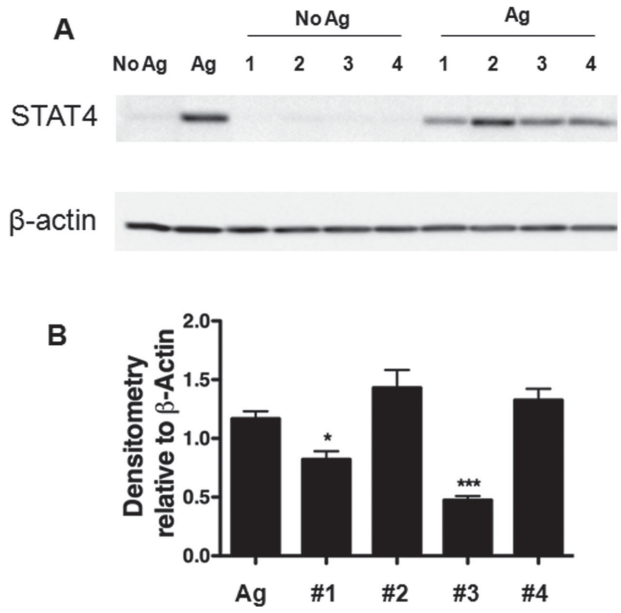

Figure 8. a) BDC-2.5 primary recall assay with BDC-2.5 mimotope and 5×10^6 $(PVPON/TA)_5$ (#1), $(PVCL/TA)_5$ (#2), $(PVPON/TA)_{5.5}$ (#3), and $(PVCL/TA)_{5.5}$ (#4) capsules. b) Densitometry statistics for Western blotting represent the average of three experiments. $*p < 0.05$ and $***p < 0.001$ in comparison to BDC-2.5 mimotope-stimulated samples alone.

cells beginning at concentration >10 μg mL^{-1} through inhibition of intracellular signals involved in T-cell activation and differentiation into Th1 effector cells.[62,63] In our case, the total concentration of TA assembled within the shells at which the inhibition of IFN-γ was observed for (PVPON/TA) shells was 13 μg mL^{-1}, which is similar to the reported value.

The STAT4 signaling pathway is important in the differentiation of CD4+ T cells producing Th1 cytokine (IFN-γ) responses. STAT4 functions as a transcription factor to induce *Ifng* mRNA accumulation. Thus, to define the mechanism for the observed decrease in Th1 cytokine synthesis (IFN-γ) mediated by TA-based shells on autoreactive CD4+ T cells, the expression of the STAT4 signaling pathway for IFN-γ synthesis was examined by Western blot analysis using β-actin as a loading control. Whole cell lysates from 5×10^5 BDC-2.5 splenocytes stimulated with 1×10^{-6} M BDC-2.5 mimotope in the presence or absence of 5×10^6 shells for 48 h were probed in a Western blot for STAT4 and β-actin expression. As shown in **Figure 8**a, mimotope stimulation of BDC-2.5 splenocytes alone resulted in an increase in STAT4 protein expression while no STAT4 expression was observed without T-cell stimulation in the presence of TA-based hydrogen-bonded capsules. The costimulation of the splenocytes with $(PVPON/TA)_5$ and $(PVPON/TA)_{5.5}$ capsules elicited a significant decrease in STAT4 protein expression as demonstrated by densitometry analysis with β-actin expression (Figure 8) while no inhibition on the STAT4 expression was seen in the case of PVCL/TA capsules. Our data demonstrate that the inhibition of the STAT4 signaling pathway (Figure 8) that is involved in transcriptional regulation of *Ifng*[64–66] is mediated by $(PVPON/TA)_5$ and $(PVPON/TA)_{5.5}$ capsules, but not by PVCL/TA capsules. We did not observe a decrease in the levels of IL-10 synthesis with TA-containing capsules (Figure 7) and subsequently, this provides evidence that inhibition of proinflammatory IFN-γ secretion is occurring at the transcriptional level. These results suggest that there is an ensemble of various paths for IFN-γ production suppression observed in Figures 5 and 6 that are affected by TA-based hydrogen-bonded capsules and modulating STAT4 expression is among those. Previously described, polyphenolic antioxidants quercitin and epigallocathechin-3-gallate inhibited the STAT4 signaling pathway in purified T lymphocytes, and ameliorated the autoimmune disease, experimental autoimmune encephalomyelitis.[67] The suppression of IL-2 by quercitin was also demonstrated in Th1 cells; however, the mechanism of such repression was found to be different from IFN-γ suppression.[68]

3. Conclusion

We showed that (TA/PVPON) and (TA/PVCL) multilayers display efficient antioxidant and immunomodulatory properties by scavenging free radicals and suppressing the synthesis of IFN-γ and TNF-α proinflammatory cytokines, respectively. IFN-γ synthesis from autoreactive CD4+ T cells was attenuated 14-fold in the presence $(TA/PVPON)_5$ capsules and eightfold in the presence of $(TA/PVCL)_5$ capsules. The total concentration of TA assembled within the shells at which the inhibition of IFN-γ was observed for (PVPON/TA) shells was 13 μg mL^{-1}. In comparison to TA-capped capsules, the neutral polymer-capped $(PVPON/TA)_{5.5}$ and $(PVCL/TA)_{5.5}$ shells were less effective in dampening IFN-γ production showing a two- and threefold decrease in the cytokine synthesis, respectively. We suggest that the free radical scavenging activity of the capsules is because of the antioxidant properties of TA component. Dissipation of ROS synthesis diminishes diabetogenic T-cell effector responses via dampening the redox-dependent STAT4 signaling pathway involved in IFN-γ synthesis. Overall, our study demonstrates that hydrogen-bonded LbL technology when applied for coating individual, living pancreatic islets have the potential to control and regulate the production of proinflammatory cytokines in vicinity of the islet graft coated with TA-containing films. Transplantation of (PVPON/TA)- or (PVCL/TA)-coated islets may decrease the risk of xeno- and allograft rejection due to the attenuation of oxidative stress and proinflammatory cytokine synthesis, and ultimately, enable Type 1 diabetics a lifestyle independent of exogenous insulin administration.

4. Experimental Section

Materials: PVPON, (average M_w = 1 300 000 g mol^{-1}), TA, (M_w = 1700 g mol^{-1}), and poly(methacrylic acid) (PMAA, M_w = 21 800 g mol^{-1}, $Đ$ = 1.32), mono- and dibasic sodium phosphate, glutaric aldehyde (GA), were purchased from Sigma–Aldrich. PVCL (M_w = 20 800 g mol^{-1}, $Đ$ = 1.19) was synthesized as described elsewhere.[69] Ultrapure (Siemens) filtered water with a resistivity of 18.2 MΩ cm was used for preparation of buffered solutions. Silica microparticles of 4 μm in diameter were purchased from Polysciences Inc. Chicken $OVA_{323–339}$ peptide sequence (ISQAVHAAHAEINEAGR) and the BDC-2.5 mimotope (EKAHRPIWARMDAKK) were synthesized by Sigma Genosys. Western blot antibodies to STAT4 (C-20) and β-actin were purchased from Santa Cruz and Sigma, respectively. Anti-mouse IgG and anti-rabbit

IgG secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson Immunoresearch.

Mice: OT-II and NOD.BDC-2.5 TCR transgenic mice were bred and housed under specific pathogen-free conditions at the Research Support Building animal facility at the University of Alabama-Birmingham. Female mice at 8 weeks of age were used in all experiments. All animal studies were performed in accordance with the UAB Institutional Animal Use and Care Committee in compliance with the laws of the United States of America.

Preparation of TA-Containing Capsules: Hollow hydrogen-bonded shells (capsules) were prepared by coating 4.0 ± 0.1 μm silica particles with $(TA/PVPON)_n$ or $(TA/PVCL)_n$ multilayer film followed by particle dissolution in 8% aqueous solution of hydrofluoric acid as established previously.[30] Specifically, 1.5 mL of 10%-aqueous suspension of particles was exposed to PVPON (0.5 mg mL^{-1}) or to PVCL (0.5 mg mL^{-1}) solutions in 0.01 M sodium phosphate at pH 3.5 for 10 min. After that, the silica particle suspension was pelleted in a 1.5 mL Eppendorf centrifuge tube and washed two times with 0.01 M sodium phosphate at pH 3.5 to remove unbound excess of polymers. Then, TA was allowed to adsorb onto particle surfaces from 0.5 mg mL^{-1} solution for 10 min. After each deposited layer, particles were centrifuged for 2 min at 2000 rpm and washed two times with the rinsing solution. Alternating coating of particles with the polymers was continued until the desired number of layers was achieved. Multilayer capsules were obtained by dissolving silica cores in aqueous hydrofluoric acid (8% wt) followed by their dialysis in deionized water for 3 d in the dark.[30] Capsules were vortexed and sonicated (15 s each) for three times before use in any experiments. Concentration of the capsule suspensions, [shells/microliter], was measured by capsule counting under an optical microscope using a hemocytometer.

Preparation of TA-Free $(PVPON)_4$ or $(PVCL)_4$ Capsules: The TA-free capsules were prepared as described in our previous work.[70,71] Hydrogen-bonded multilayers of PMAA with amino-containing poly(*N*-vinylpyrrolidone) (PVPON-NH_2–7, M_w = 143884 g mol^{-1}, $Đ$ = 1.55) or poly(*N*-vinylcaprolactam) (PVCL-NH_2-5, 23800, $Đ$ = 1.5) copolymers with a certain molar percentage of amino group-containing polymer units were deposited on the silica particles. Assembly of the hydrogen-bonded layers was performed at pH 3.5, starting from PVPON-NH_2 or PVCL-NH_2 copolymer followed by PMAA. Each deposition cycle was followed by rinsing three times with a buffer solution at pH 3.5 to remove excess polymer, followed by centrifugation of suspensions at 2000 rpm for 2 min to remove supernatant. After four bilayers of (PVPON-NH_2/PMAA) or (PVCL-NH_2/PMAA) were deposited, chemical cross-linking of PVPON-NH_2 or PVCL-NH_2 layers was performed. For that, the core–shell particles were exposed to GA solution (5 wt%) at pH 5 for $(PVPON\text{-}NH_2/PMAA)_4$ or pH 6.5 for $(PVCL\text{-}NH_2/PMAA)_4$ for 12 h. After that, coated core–shell particles were exposed to pH 8.5 for 4 h, followed by rinsing at pH 4. The core dissolution was performed as described above and resultant $(PVPON)_4$ or $(PVCL)_4$ capsules were purified by dialysis in deionized water for 3 d.

Primary Recall Assays and Cytokine Measurements by ELISA: NOD.BDC-2.5 or OT-II splenocyte single-cell suspensions (5×10^5 cells) were seeded in a 96-well flat bottom plate and stimulated with 0.1×10^{-6} M or 1×10^{-6} M BDC-2.5 mimotope or 1×10^{-6} M $OVA_{323–339}$, respectively, in the presence or absence of 10^8–10^3 shells in 200 μL total volume of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 10×10^{-3} M HEPES buffer, 4×10^{-3} M L-glutamine, 2× non-essential amino acids, 1×10^{-3} M sodium pyruvate, 61.5×10^{-6} M 2-mercaptoethanol, and 100 μg mL^{-1} Gentamicin (Invitrogen) (complete DMEM). After incubation at 37 °C in a 5% CO_2 humid air chamber for 2, 3, or 4 d, supernatants were collected to examine cytokine synthesis. IFN-γ, IL-10, and IL-2 production was measured using antibody pairs from BD Biosciences as described previously.[60] ELISA plates were read on a BioTek Synergy2 microplate reader (BioTek) and analyzed using Gen5 v.1.10 software (BioTek). MTT assay to assess cell viability was performed according to the manufacturer's protocol (Sigma–Aldrich).

Oxidation of Luminol to Detect ROS Synthesis: Luminol is cell permeable and readily oxidized by superoxide and hydrogen peroxide resulting in chemiluminescence and can be used to detect ROS synthesis with OT-II splenocytes. Briefly, OT-II single-cell suspensions were plated onto a 96-well round-bottom plate at 5×10^5 cells in the presence or absence of 2.5×10^6 polymer shells in phenol red-free HBSS with 200×10^{-6} M luminol (Sigma) and 0.32 Units mL^{-1} of horseradish peroxidase (Sigma) in a total volume of 200 μL. Luminescence was quantified using a SpectraMax L Luminescence microplate reader and was recorded every 2 min for 1 h after stimulation with 100 ng mL^{-1} PMA and 1μg mL^{-1} In.

Western Immunoblotting: Whole cell lysates were prepared as described previously,[72] separated on a 4%–20% gradient SDS-PAGE gel, and transferred onto 0.45 μm charged PVDF membranes. The membranes were incubated overnight at 4 °C with antibodies against STAT4 or β-actin and exposed to the appropriate secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch). Chemiluminescence was detected with ECL plus (Amersham Pharmacia) and analyzed with Image Lab software v5.1 (Bio-Rad) to generate densitometry data.

Nitrite Assay: NO_2^- production was measured by adding 50 μL of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 50 μL of tissue culture supernatant from primary recall assays. The NO_2^- concentration was determined by comparing the optical density at 550 nm with a standard curve generated from various known concentrations of sodium nitrite dissolved in culture medium.

Statistical Analysis: Data were analyzed using GraphPad Prism Version 5.0 statistical software. Determination of the difference between mean values for each experimental group was assessed using the two-tailed Student's *t* test, with $p < 0.05$ considered significant. All experiments were performed at least three separate times with data obtained in triplicate wells in each experiment.

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