Published in final edited form as:

Biomaterials. 2020 February ; 230: 119626. doi:10.1016/j.biomaterials.2019.119626.

An Injectable Cytokine Trap for Local Treatment of Autoimmune Disease

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Abstract

Systemic cytokine therapy is limited by toxicity due to activation of unwanted immune cells in off-target tissues. Injectable nanomaterials that interact with the immune system have potential to offer improved pharmacokinetics and cell specificity compared to systemic cytokine therapy by instead capturing and potentiating endogenous cytokine. Here we demonstrate the use of high aspect ratio polycaprolactone nanowires conjugated to cytokine-binding antibodies that assemble into porous matrices when injected into the subcutaneous space. Nanowires are well tolerated *in vivo* over several weeks, incite minimal foreign body response and resist clearance. Nanowires conjugated with JES6-1, an anti-interleukin-2 (IL-2) antibody, were designed to capture endogenous IL-2 and selectively activate tissue resident regulatory T cells (Tregs). Together these nanowire-antibody matrices were capable of sequestering endogenous IL-2 in the skin and were successful in rebalancing local immune compartments to a more suppressive, Treg-mediated phenotype in both wild type and transgenic murine autoimmune disease models.

Graphical Abstract

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Disclosures

The authors declare no competing financial interests.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Keywords

Immunomodulation; Nanomaterials; Cytokines; Autoimmunity

Introduction

Injectable matrices that interface with host immune populations are a promising platform for local immunomodulation. These have been shown to be highly effective cancer vaccines, [1,2] artificial antigen presenting cells[3,4] and chemotherapeutic delivery vehicles combined with immune cell adjuvants.[5,6] However, comparatively little has been demonstrated in the context of autoimmune disease, where cytokine profiles and their signaling between various T helper cell populations are often responsible for driving or ameliorating progression.[7,8]

For autoimmune diseases of the skin, such as vitiligo, alopecia areata, pemphigus and psoriasis, T lymphocytes have been shown to play a key role.[9,10] While autoimmune diseases rarely occur along a single immune axis, regulatory T cell (Treg) dysregulation has been particularly implicated in psoriasis, where Tregs contribute to disease through ineffective control of pro-inflammatory adaptive immune compartments such as effector T (Teff) cells. [9] There is evidence that Tregs isolated from healthy individuals, however, have the capability to suppress inflammatory effector T cells from tissue derived from psoriatic patients, suggesting in diseased patients - at least at the time of a flare or in tissue where a lesion is present - tissue resident Tregs lack the ability to mitigate autoreactive, effector T cell populations that promote disease pathogenesis. [11]

While cytokines remain a popular mechanistic target in the pharmaceutical industry for autoimmune diseases such as psoriasis,[12-14] the use of recombinant cytokines themselves as a standalone therapy to bias a patient's immune response has run into multiple clinical roadblocks.[15,16] Their lack of bioavailability, narrow therapeutic window, short half-life and often severe off-target cellular and tissue level side effects such as cytokine release syndrome have made systemic cytokine therapy an unattractive and expensive candidate for immunotherapy.[17] Cytokines are a challenging drug target as they are often pleiotropic, [18,19] i.e. having multiple binding mechanisms across distinct immune subsets, which may

not be conserved across all tissues, particularly in a disease setting. An ideal immunomodulatory drug delivery approach that takes advantage of cytokines' pleiotropic nature would allow us to tailor the immune response locally while controlling off-target effects and increasing residence time in tissue.

IL-2 is a particularly interesting target for this approach as it is still used in systemic cytokine therapy today[20,21] and transduces T cell activity through two orthogonal axes. [22] IL-2 preferentially signals via a trimeric receptor complex composed of the IL-2R α chain (CD25), the IL-2R β chain (CD122), and the common gamma chain, IL-2R γ (CD132).[23] Tregs express the complete receptor complex, which binds IL-2 with high affinity, while resting effector CD4 $_{+}$ T cells (Teff), CD8 $_{+}$ T cells and natural killer (NK) cells express only the β and γ chains, which bind IL-2 with only intermediate affinity. As Tregs are unique suppressor cells that are key to tolerance and regulating autoimmunity by balancing effector T cell function, bolstering their response in diseases such as psoriasis by selective cytokine signalling could prove therapeutically promising.

Both receptor complexes have a distinct ligand binding site, and as such antibodies and Fc fusions have been developed to selectively sequester the ligand binding region of IL-2 and preferentially activate suppressive (Treg) or inflammatory (Teff, CD8) populations. [19,22,24] Such biologics can not only tip the balance to immunosuppression or to immunoreactivity, but also enhance function beyond the standalone cytokine.[25,26] Instead of dosing with exogenous cytokine, our approach was to locally inject a matrix conjugated to one of these antibodies to passively trap the cytokine that naturally flows through the tissue, concentrate it, and by judicious choice of antibody structure, potentiate a local, cell specific immune response in both healthy and inflammatory murine disease models (Fig. 1).

We have previously reported on a fabrication technique of high aspect ratio polycaprolactone (PCL) nanowires that inherently address strict requirements for background inflammation due to low immunogenicity of PCL[27] and the resistance to phagocytic clearance conferred by their asymmetric structure.[28,29] We developed a stable conjugation chemistry that can present antibodies to the surrounding microenvironment for more than 1 week and incited minimal inflammatory infiltrate. In this study we have conjugated nanowires with the JES6-1A12 antibody clone (JNWs) which, when bound to IL-2, not only potentiates CD25 $^{+}$ cell interaction but precludes CD25 $^{-}$ cells from doing so.[22,30-32] Since regulatory T cells typically express high levels of CD25, our biomaterial-based strategy is geared towards augmentation of local Treg activity after injection of nanowires by restricting both the pleiotropism and clearance of endogenous cytokine from the target tissue using this unique matrix.

Inflammatory diseases in the skin are often characterized by transient flares that are accessible by injection of a biomaterial intervention strategy and would benefit from short-term, targeted local immunosuppression without the off-target side effects of systemic therapy. These nanowires depots act as hotspots for localized immunosuppression that naturally degrade over time and do not require removal. We tested the hypothesis that a matrix that captures endogenous IL-2 over the course of a highly inflammatory autoimmune

disease event would shift the local immune compartment to a more suppressive phenotype and locally abrogate disease.

Materials and Methods

Mouse experiments were performed in compliance with the University of California, San Francisco Institutional Animal Care and Use Committee guidelines with protocols approved for this study (Protocol AN110246).

Fabrication of PCL Nanowires.

Synthesis of MP-PCL and nanowire fabrication was performed as previously described.[28] All reagents and solvents were purchased from commercial sources and used as received without further purification. Briefly, a 125mg/mL solution of either pure 45kDa PCL or a 30% blend of MP-PCL and 45kDa PCL was generated in trifluoroethanol and spun cast onto a clean wafer at 1000rpm for 30s. Nanowires used for in vitro or in vivo tracking studies had 1mg/mL of Nile Red incorporated in solvent mixture. Anodized alumina membranes (37mm, 200nm pore diameter) were then placed in contact with the polymer film as it was heated above its melting temperature (100°C) while applying sparing pressure to membranes to initiate capillary action into the pores. The templating process was deemed complete when no polymer film remained underneath, typically after 3h. The membranes were allowed to cool, then removed from substrate and etched in 5M NaOH for 30min at 4°C and briefly sonicated to aid dispersion. Etchant solution was passed through a 0.22µm PES filter and retentate rinsed in 5x volume of cold distilled water. NWs were then rinsed off filter with a solution of 1% polyvinyl alcohol (Mowiol 5-88, Sigma) and sonicated again to disperse. NW solution was then passed through a 40µm filter and retentate was discarded. They are then centrifuged at 10krf for 15min to further wash 3x in distilled water, concentrated and stored in 1x D-PBS with 0.04% (w/v) of EDTA without calcium or magnesium (reducing buffer).

To conjugate to JES6-1 anti-IL-2 antibodies, first protein was reduced using freshly dissolved aliquots of tris(2-carboxyethyl)phosphine (TCEP) in reducing buffer at a 4.5 molar excess for 1 hour at 37°C under gentle shaking. Reduced antibody solution at 0.1mg/mL was added to the nanowires and thiol-maleimide reaction proceeded at room temperature for 2h under gentle shaking to produce JES6-1 conjugated nanowires or JNWs. JNWs were then washed thrice in d-PBS to remove excess antibody by discarding flow-through on a 0.2µm centrifuge filter spun down at 2000rcf for 2 min, then resuspended in sterile saline and subsequently stored at 4°C until use. JNWs were always injected into animals or used for in vitro experiments within 24h of conjugation.

Nanowires produced via this method were measured to be 18 ± 4.3 µm in length and are structurally identical as those outlined previously.[28] Diameter was estimated by scanning electron micrograph images to be 200 nm. Amount of conjugated antibody was normalized by incubating nanowires with fixed concentrations of target cytokine, centrifuging and quantifying amount of remaining unbound cytokine in supernatant by ELISA. Doses are outlined in each specific *in vitro* and *in vivo* experiment but concentrations were normalized to 100ng of active antibody per 1mL of JNW suspension unless otherwise noted.

SEM images were taken on a Carl Zeiss Ultra 55 FE-SEM at 5kV using a secondary electron detector after drying nanowire suspension on metal stub and coating with ~4nm of gold by vacuum sputtering.

In vitro experiments.

For T cell *in vitro* experiments, wild type C57BL/6 mice were sacrificed and their inguinal, axillary and brachial skin draining lymph nodes were pooled. Lymph nodes were harvested, mechanically disrupted, and passed through a 100µm filter in complete media and washed and plated in 24 well plates at 10⁵ cells/well. Cells were supplemented with 40 units/mL of recombinant mouse IL-2 (Tonbo Biosciences) and Dynabeads (Mouse T-Activator CD3/CD28 beads, Cat. #11453D Gibco) at a 1:10 bead:cell ratio and cultured with either control nanowires with no conjugated antibody (nanowires alone) or JNWs at a concentration of 100ng active antibody per mL for 48h. Cells were then fixed and stained for flow cytometry. One experimental replicate constitutes pooled lymphocytes from one mouse.

In Vivo Experiments.

For macrophage tissue staining and immunofluorescence, dorsal skin surrounding injection sites of JNWs with Nile Red dye was harvested at 2-, 4- and 6-weeks post injection into wild type C57BL/6 mice, placed into OCT and frozen on cold isopentane in liquid nitrogen. 15µm sections cut via cryotome were then fixed in 10% formalin and stained with anti-F4/80 antibody in 5% goat serum. They were then rinsed in DI water and mounted with ProLong Gold Antifade Reagent (Life Technologies) with DAPI.

For FOXP3-GFP albino C57BL/6 mice, we injected two mice per group with 6 injection sites per mouse at the same concentration of JNWs or unconjugated wires only as used in T cell activation experiments. Each mouse was then sacrificed at day 5 post injection. Approximately 2mm × 2mm skin explants were then harvested as described above and placed in 2% PFA in normal saline and submerged overnight at 4°C. Sections were then washed thrice in PBS and then again submerged overnight in a 30% sucrose solution and gently agitated at 4°C. Skin was then blotted dry and subsequently frozen in OCT on isopentane, sectioned and stained as described above with an anti-GFP AlexaFluor 488 antibody (Life Technologies) and imaged after 12h in mounting media. Each experimental replicate represents one section from a different explant in the respective group. Image analysis was done with ImageJ software drawn within ROI of each 10x image but due to spectral overlap, area within nanowire injection site excluded from analysis.

Slides were imaged on a Zeiss Axio Imager M2 microscope using a 10x objective.

T cell activation studies.

Female BALB/C mice (Jackson Labs) of 6-8 weeks of age were treated with either nanowires alone (n=6), nanowires conjugated with anti-IL2 (n=6) (clone JES6-1A12, Bio X Cell) or equivalent dose of soluble JES6-1A12 (n=6). Each mouse was shaved and injected subcutaneously into the dorsal skin over ten separate sites (50µL per site) with a 29-gauge needle. Nanowire dose for each mouse was standardized to 100ng of active anti-IL2 as measured by ELISA. At the respective time point, mice were sacrificed, whole mouse dorsal

skin was harvested which included all 10 injection sites for that respective mouse, digested and stained. Skin was finely minced and digested in RPMI media with collagenase XI (2mg/mL, Sigma), hyaluronidase (0.5 mg/mL, Sigma) and DNase (0.1 mg/mL, Sigma) for 45 minutes while being shaken at 200rpm at 37°C. The skin suspension was diluted in 10mL of RPMI media, vortexed and filtered through a 100µm filter. Six skin draining lymph nodes were harvested and pooled for each respective mouse. Lymph nodes were mashed through a 100µm filter. Single cell suspensions from lymph node and skin were then subjected to flow cytometry staining and analysis (see Supplemental Information S1 through S3 for gating strategies). Wild type T cell activation data is representative of two experimental replicates.

K5-TGO-DO11 Autoimmune model.

Mixed gender mice, which were colony mates from several litters, carrying the K5-TGO-DO11.10 transgene were administered chow containing 1g/kg doxycycline at Day 1 as previously described.[33] 24h later, either nanowires alone or JNWs were injected as described above. Mice were then allowed to continue on doxycycline chow until Day 6 when they were sacrificed and processed as above. N=9 for JNW treated group, n=8 for blank NW only group, and n=4 for the no-disease control group which did not receive doxycycline chow.

Intracellular cytokine staining was performed on T cells isolated from the K5-TGO-DO11.10 mice skin and lymph nodes. Following isolation, cells were plated in 1x Tonbo T cell stimulation cocktail containing PMA, Ionomycin and Brefeldin-A for four hours. Two samples were lost due to instrument error.

For H&E experimental analysis, approximately 2mm × 2mm skin explants were fixed for a week in 10% formalin in normal saline and then dehydrated overnight in 70% ethanol in water. Explants were then fixed in paraffin, cut in 4µm sections, deparaffinized and subsequently stained with H&E and imaged on a Zeiss Axio Imager M2 microscope. For plaque analysis, five representative thicknesses were blindly applied and averaged on each section, representing one experimental replicate, with each replicate from a different mouse. Only samples with intact sections were included in analysis.

Flow Cytometry Panels and Staining.

For T cell activation at baseline, $1-2 \times 10^6$ cells were stained with anti-CD45-Alexa700 (30-F11, eBioscience), anti-CD4-FITC (RM4-5, eBioscience), anti-CD3-APC-eFluor780 (145-2C11, eBioscience), anti-CD8-BV605 (53-6.7, BioLegend), anti-CD122-PE (TM-b1, eBioscience), anti-CD25-BV650 (PC61, BioLegend), anti-TCRγδ-PerCPeFluor710 (GL-3, eBioscience), anti-CD49b-BV711 (HMa2, BioLegend), Ghost Violet 510 viability dye (Tonbo), then fixed and permeabilized with the eBioscience FOXP3/Transcription Factor Staining Buffer kit. Samples were then stained intracellularly with anti-Ki-67-PeCy7 (B56, BD), anti-CTLA-4-APC (UC10- 4B9, eBioscience), and anti-FOXP3-eFluor450 (FJK-16s, eBioscience).

For T cell cytokine studies in the K5-TGO-DO11 transgenic mice disease model, $1-2 \times 10^6$ cells were stained with anti-CD45-Alexa700 (30-F11, eBioscience), anti-CD4-BV650 (RM4-5, BD Biosciences), anti-CD3-BV711 (145-2C11, BD Biosciences), anti-TCR-

DO11.10-APC (KJ1-26, eBioscience), and Ghost Violet 510 viability dye (Tonbo), then fixed and permeabilized with the eBioscience FOXP3/Transcription Factor Staining Buffer kit. Samples were then stained intracellularly with anti-IL17A-PeCy7 (TC11-18H10, Biolegend), anti-IL2-PE (JES6-5H4, Tonbo), anti-IFN γ -FITC(XMG1.2, eBioscience), and anti-FOXP3-eFluor450 (FJK-16s, eBioscience).

For T cell activation studies in the K5-TGO-DO11 transgenic mice disease model, $1-2 \times 10^6$ cells were stained with anti-CD45-Alexa700 (30-F11, eBioscience), anti-CD4-BV650 (RM4-5, BD Biosciences), anti-CD3-BV711 (145-2C11, BD Biosciences), anti-ICOS-FITC (C398.4A, eBioscience), anti-Ly6G-PerCP-Cy5.5 (1A8, Biolegend), anti-CD11b-APC-eFluor780 (M1/70, eBioscience), anti-F4/80-BV785 (BM8, Biolegend), anti-TCR-DO11.10-APC (KJ1-26, eBioscience), Ghost Violet 510 viability dye (Tonbo), then fixed and permeabilized with the eBioscience FOXP3/Transcription Factor Staining Buffer kit. Samples were then stained intracellularly with anti-Ki-67-PeCy7 (B56, BD), anti-CTLA-4-APC (UC10-4B9, eBioscience), and anti-FOXP3-eFluor450 (FJK-16s, eBioscience). Samples were acquired on an LSRFortessa flow cytometer (Becton Dickinson).

Statistics and Software.

Statistical analysis was performed via one-way ANOVA to determine significance between groups with Tukey's multiple comparisons correction in GraphPad Prism v8.0. Two group analysis used two-tailed t-test. All plots show standard deviation, * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$. All flow cytometry gating and analysis done in FlowJo v10.

Data Statement.

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

Results

To address whether subcutaneously injected wires give rise to a longer-term inflammatory response, we longitudinally observed healthy 6-8 week female wild type C57BL/6 mice injected subcutaneously with PCL nanowires (NWs) labelled with Nile Red. Mice were sacrificed at 2, 4 and 6 weeks where nanowire nodules were cryosectioned in OCT and stained with anti-F4/80 antibody to label macrophages surrounding the nodule (Fig. 2a-f) and minimal inflammatory infiltrate was observed.

Next, we tested the activation of particular immune subsets *in vitro* when cultured with the nanowires. Freshly harvested lymphocytes were plated with JES6-1 conjugated nanowires (JNWs) or unconjugated PCL nanowires in media spiked with recombinant mIL-2. We saw a significant increase in the amount of CD45⁺ CD3⁺ CD4⁺ FOXP3⁺ Tregs as a proportion of all CD4⁺ cells present in the wells, and a concurrent decrease in CD45⁺ CD3⁺ CD4⁺ FOXP3⁻ effector cells in cultures with JNWs compared to nanowires alone (Fig. 2g,h), indicating that the JNWs were selectively stimulating the CD25⁺ Tregs.

We then sought to examine how JNWs influence local immune cells over time without being pre-complexed to exogenous IL-2 in healthy and immune competent BALB/C mice. Conjugated JNWs were injected subcutaneously across ten sites in the dorsal skin of healthy female 6-8-week old mice, where endogenous cytokine was allowed to passively accumulate in the nanowire matrix for a period of 4 days. Mice were then sacrificed and the dorsal skin was harvested for digestion to stain resident T cell population for flow cytometry. We observed Treg activation and proliferation as evidenced by increased Cytotoxic T Cell Antigen 4 (CTLA-4) and Ki67 staining in the skin resident cells of the JNW treated group compared to mice treated with nanowires alone (Fig. 3c,d) but preceded any change in number of either CD4⁺ cell population in the skin (Fig. 3a,b).

In a subsequent experiment where JNW matrices were allowed to persist for 5 days before tissue harvest, we saw a significant increase in the proportion of Tregs by flow cytometry after whole skin digestion in the JNW treated group compared to nanowires alone (Fig. 3e) as well as increased CD25 staining (Fig. 3i), a known downstream effect of IL-2 signaling. [34] Interestingly, we see a concomitant decrease in the numbers of FOXP3⁻ CD4⁺ effector T cells in mice treated with JNWs, which we attribute to a decrease in available IL-2 for binding (Fig. 3f). These effects were transient and skin returned to homeostatic levels by Day 10 post-administration (Supplemental Information S4).

Single cell suspensions from pooled skin draining lymph nodes from each mouse were similarly stained for flow cytometry, where these differences between JNWs and nanowires alone were not recapitulated, showing a highly localized immune response within the tissue. Surprisingly, we observed the opposite trend in mice that were treated with soluble antibody - a minor decrease in Treg and increase in effector T cells in these downstream lymph nodes that was not seen with the JNWs, suggesting the conjugation is stable and mitigates off-target effects even in immediately adjacent tissue (Fig. 3g,h).

Next, we labelled the JNWs with Nile Red and injected them similarly into FOXP3-GFP mice to observe local differences in Treg numbers around the matrix. Indeed, when stained by immunofluorescence with an anti-GFP reporter we observed greater than four-fold average increase in Treg nuclei local to a JNW injection site compared to blank vehicle control (i.e. within sections containing injection sites) (Fig. 3j-l).

Given increased FOXP3⁺ Treg numbers proximal to the injection site, we asked whether this immunosuppressive JNW nanomaterial matrix could abrogate a tissue-specific autoimmune response in a T cell driven murine model of transient skin inflammation. We chose to use a transgenic mouse model of skin-specific autoimmunity, in which disease results from a tetracycline-inducible expression of ovalbumin (OVA) specifically expressed in keratinocytes through the keratin 5 (K5) promoter.[33] Mice also carrying the MHC class II restricted DO11.10 T cell receptor which recognizes OVA, i.e. which are K5-TGO-DO11.10 transgenic, will generate a CD4⁺ effector T cell mediated inflammatory response when subject to this inducible autoantigen expression. Thymically derived antigen-specific Tregs in turn respond to autoantigen expression to reduce, but not eliminate, subsequent skin inflammation. Mice are healthy and maintain immune homeostasis until antigen is induced by tetracycline administration, where rapid infiltration of thymus derived CD4⁺ T cells into

the skin generate a fulminant, skin resident Th1 type driven autoimmune disease. Antigen generation in the skin results in pronounced inflammatory dermatitis, as well as marked scaling, alopecia and weight loss. Disease is apparent at 24h and peaks at 8-10 days post-induction. The skin infiltrate is characterized by antigen specific DO11.10⁺ T cells that primarily produce interferon gamma (IFN γ) as well as interleukin-17 (IL-17). Subsequent antigen reduction results in less severe disease due to the expansion of the memory Treg compartment within the skin; hence this model is appropriate to understand whether JNW nanomaterials are capable of augmenting Treg capacity to affect tissue-specific autoimmunity.

We induced antigen in homozygous K5-TGO-DO11.10 transgenic mice at Day 1 and administered ten subcutaneous injections into their shaved dorsal skin similarly to prior wild type experiments at day 2. Mice were sacrificed at day 6 and subject to whole dorsal skin digestion, with results outlined in Figures 4 and 5.

Immune repertoire analysis showed similar results to wild type models. Antigen-specific regulatory T cells (CD45⁺ CD3⁺ CD4⁺ FOXP3⁺ DO11.10⁺ subset) were increased within the skin both as a fraction of all CD4⁺ cells and in number in animals dosed with JNWs vs a blank, nanowire only control (Fig. 4a-f). We also saw a significant decrease in antigen specific effector T cells (CD45⁺ CD3⁺ CD4⁺ FOXP3⁻ DO11.10⁺ subset) when mice were dosed with JNW. Similar to wild type models, this proliferation was restricted to the skin and was not recapitulated in the skin draining lymph nodes (Fig. 4g,h).

We phenotyped both populations of cells via CTLA-4 and Ki67 staining (Fig. 5). We saw skin from mice dosed with JNWs had significantly more antigen specific CTLA-4⁺ Tregs as well as an increase in number of these cells expressing proliferation marker Ki67 (Fig. 5a,c). Concurrently, we observed a decrease in the number of CTLA-4⁺ stained effector T cells (Fig. 5b). Effectors trended towards being more proliferative though this result was not significant (Fig. 5d). Overall, we saw a significant increase in Treg to effector T cell ratio within dorsal skin of animals treated with JNWs as compared to blank controls (Fig. 5e).

We subjected the skin cell digest to stimulation with a PMA-ionomycin-BFA cocktail to non-specifically activate T cells and observe their capability of producing cytokines. We then stained these stimulated cells after 4h for IFN γ cytokine production and observed that antigen specific effector T cells, which are predominant cytokine generators in this model, had a modest reduction of IFN γ production in mice treated with JNWs (Fig. 5f). While this result wasn't significant, in a subsequent experiment we did observe a recovery of IFN γ expression to a fraction of CD4⁺ T cells seen in a healthy cohort (Supplemental Information S4). We saw no difference in IL-17 production (Fig. 5g).

Lastly, 4 μ m skin sections within 1mm of the nanowire injection site were taken from mice on day 6 of disease, then paraffin embedded, sectioned, and stained for H&E (Fig. 5i,j). In general, we observed a decrease in infiltrate in sections with mice treated with JNWs though overall architecture remained the same. However, this disease is characterized by a neutrophilic crust or plaque that forms on the surface of the skin. While these were still present to some degree in all skin samples their thickness and coverage were significantly

decreased compared to mice that received the vehicle only control, which was quantified by blinded assessment of skin sections (Fig. 5h).

Discussion

Relatively long-lived nanomaterial strategies that interface with the host immune system remain elusive for several reasons. First, materials used for drug delivery often have inflammatory byproducts that may exacerbate autoimmune disease already present in the tissue.[35,36] In addition, the longer the nanomaterial resides in this area the more likely there will be deleterious pharmacokinetic effects such as foreign body response[37,38], which has prompted the biomaterials field to investigate materials that naturally degrade in tissue. This has the added benefit of eliminating need for material removal in a clinical setting, reducing cost and care burden.

However, attempting to influence the immune microenvironment during an inflammatory event requires material design that itself incites little background immune activity, in addition to stability and biocompatibility. Post-injection, we observed that the PCL nanowires resist clearance by macrophages for the first 4 weeks as there is minimal colocalization with macrophage staining. In addition to injection sites being well tolerated longitudinally, we see that by week 4 the sites have little foreign body response as evidenced by the lack of macrophages surrounding the site as compared to week 2. By week 6 the site has become notably more macroporous, as well as fully cell permeable given DAPI staining across the entire nodule cross section.

This agrees with prior data which showed the majority of depots are cleared by week 6;[28] given the propensity of PCL for bulk degradation (as compared to surface degradation experienced by other polyesters) this is likely the final stage in tissue persistence.[39-41] Previous work has shown that the low protein adsorption potential of PCL inhibits macrophage mediated foreign body response in long term PCL implants,[27] in addition to the M2 phenotype shift of local macrophages that surround porous scaffolds.[42-44]

Broadly, tissue-resident Tregs play a critical role in establishing and maintaining peripheral immune tolerance.[10] These cells have been shown to have a unique surface marker repertoire and cytokine-generating capabilities, and respond by becoming highly migratory during an inflammatory event to counterbalance effector Th1 populations and maintain immune homeostasis.[45] While the use of exogenous cytokines are attractive candidates for systemic immunomodulation, their poor pharmacokinetics and short-half lives result in nonspecific activation of central T cell subsets and lack the ability to activate these key tissue-resident lymphocyte populations.

The pathology of skin-based autoimmune disease such as psoriasis is often characterized by transient flares which present as visible inflammatory lesions or plaques.[9] These can be either treated locally by corticosteroid creams, which non-specifically downregulate immune activity; or systemically by anti-cytokine therapy, which lacks tissue targeting characteristics and carries a significant side effect profile. Here, we demonstrate a more attractive materials-based strategy that allows both localized suppression of tissue resident T cells that drive

inflammation and activate regulatory T cells that ameliorate it. These could be injected at or near an epidermal lesion and then biodegrade naturally after the flare has abated.

Once conjugated to the JES6-1 antibody, nanowire matrices were not only able to trap and concentrate endogenous IL-2 in the skin, but also able to bias the local immune compartment towards a more suppressive phenotype. Treg numbers are increased in skin adjacent to injection sites, to such an extent that we observed a significant increase in the fraction of Tregs found in the tissue as a whole via our flow cytometry studies. Furthermore, in our disease model, Tregs were functionally suppressive across the whole back skin, as histological observation of the skin showed widespread amelioration of disease. Thus, while Tregs are capable of being activated at injection sites, they functionally suppress disease within the whole tissue, which may be attributed to prior observations that IL-2 signaling works in concert with key migratory pathways such as CCR7.[46]

We also observed a concomitant decrease in resting cutaneous effector T cell populations, which typically express relatively little CD25. Given FOXP3⁺ cells typically express high levels of CD25 in the skin, it's likely they are better able to bind to the JES6-1 clone which restricts IL-2 binding to T cells that express the CD25⁺ variant of the IL-2 receptor and are therefore preferentially activated. Furthermore, increased CD25 staining in skin-resident Tregs along with their prior activation shown by CTLA-4 expression implies they are highly activated after proliferation when exposed to the JNWs and are not simply increasing in number.

There was no significant difference in skin-resident CD4⁺ T cells in mice dosed with an equivalent amount of soluble JES6-1 antibody compared to the blank nanowire-only control, likely due to the short half-life of these IgG proteins when administered subcutaneously. The downstream changes in CD4⁺ T cells in the lymph nodes were only seen with soluble JES6-1, suggesting that the antibody is not rapidly dissociating from the JNW depot and causing off-target activation via downstream lymphatic drainage.

In these K5-TGO-DO11 disease model experiments, we observed a similar trend towards a more Treg driven phenotype in the dorsal skin 5 days post injection and nearing peak of disease. Marked increases in CTLA-4 and Ki67 expression in antigen specific, skin resident Tregs mirrored wild type experimental results. Even though the antigen specific resident effector T cells had been activated by ovalbumin produced by keratinocytes, we saw no such change in CTLA-4 staining when compared to nanowire-only controls.

Interestingly, we saw Ki67⁺ staining in Teffs trending upwards at Day 5; it's likely that as these cells become activated, they upregulate CD25, which allows them access to IL-2 sequestered in the JNW matrix. However, their numbers remained significantly lower than in the control group. This indicates that the JNW matrix predominantly promotes a Treg-driven microenvironment when used in a treatment setting even in a highly inflammatory disease state where both CD4⁺ populations are already stimulated by their cognate antigen. As off target immunomodulation is a concern for therapeutic use, we again assessed the adjacent lymph node T cell activation levels and saw no difference between control and JNW, suggesting that activity is restricted to the skin.

Once antigen is induced by doxycycline administration, the immunologic and tissue level hallmarks of disease in this model include increased IFN γ production by effector T cells as well as a nuclei rich, neutrophilic plaque that forms particularly on dorsal skin. We observed modest abrogation of these key disease-specific metrics, which suggests that this tissue and cell specific augmentation of Tregs suppresses this plaque formation locally.

Overall, sustained IL-2 capture and presentation in the skin can promote Treg function and moderate downstream characteristics of skin-resident autoimmune disease in this CD4⁺ T cell driven murine model. We believe that passive capture of endogenous signalling molecules via a porous matrix such as these PCL nanowires demonstrates a promising platform to target key tissue-resident populations in peripheral tissue. Further specificity of immunomodulation would entail generating an antigen-specific immune response by presenting both antigen and cell-restricted costimulatory molecules on the nanowire surface, which is an area for future research.

Conclusion

In summary, we have demonstrated that this injectable cytokine trap can selectively neutralize specific T cell subsets and influence the local immune repertoire. These depots do not incite long term rejection or foreign body response and incite minimal myeloid infiltrate. This platform's unique ability to amplify local immune response from tailored antibodies has a highly local effect in a transgenic skin resident autoimmune model by shifting the immune compartment in favor of a more suppressive phenotype. This results in tissue level responses that minimize plaque formation adjacent to injection sites in this model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by AbbVie (#A123942) and the NIH (#R01EB018842). Authors are grateful to the Electron Microscopy Facility at SFSU and Clive Owen for help with SEM imaging and acknowledge the SCAC Flow Cytometry Core at UCSF (NIH #P30 DK063720). Confocal images were taken at UCSF Nikon Imaging Center. CRZ would also like to thank Priscila Muñoz Sandoval for help with animal husbandry and Nicole Repina for graphic design assistance.

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Highlights

- Polymeric nanowires conjugated to anti-cytokine antibodies can sequester endogenous cytokines *in vivo* and induce local proliferation of cognate cell type
- Nanowires are stable *in vivo* and do not induce bystander inflammation until degradation up to 6 weeks post injection into the skin
- Immunomodulation is restricted to the skin by passive accumulation of cytokine without need for dosing systemically
- When injected into animals with skin-based autoimmune disease, nanowires suppress immune response and reduce lesions local to injection sites

Figure 1. An Injectable Cytokine Trap.

(a) Schematic of selective cytokine accumulation and subsequent immune cell activation. (b) typical nanowire injection site in mouse skin, scale bar 1mm. (c) close up of resected nodule, scale bar 0.5mm. (d) SEM of PCL nanowires, scale bar 1µm. (e) Confocal immunofluorescence image of JNWs and regulatory T cells in the skin, scale bar 10µm. Treg nuclei in green channel, nuclei in blue, JNWs in red.

Figure 2. PCL Nanowire clearance *in vivo* and JNW activation *in vitro*.

Nanowire injection sites at 2 (a,b), 4 (c,d) and 6 (e,f) weeks post injection. Scale bar top 100 μ m, bottom 200 μ m. (g,h) In vitro culture of JNWs with lymphocytes from pooled skin draining lymph nodes after 48h of culture in 1nM IL-2 spiked media. Each data point represents pooled node sample from one animal. Two-way t-test, ** denotes $p < 0.01$. Macrophages in green channel, nuclei in blue and Nile Red labeled nanowires in red.

Figure 3. Immune profiling of JNWs in wild type mice.

Regulatory and effector T cell population (a,b) and median fluorescence of skin resident Tregs (c,d) at 4 days post injection. (e,f) Regulatory and effector T cell populations at 5 days post injection. (g,h) T cell populations in pooled skin draining lymph nodes. (i) CD25 MFI in skin resident Tregs at day 5. (j) quantification of Treg foci in stained cryosections of FOXP3-GFP mice that were injected with either (k) JNWs or (l) nanowires alone. Scale bar 200 μ m. Nanowire injection sites outlined in white dotted line, arrow indicates a single Treg nuclei in green, nuclei in blue. Each data point in panels (a-j) consist of one animal. 1-way ANOVA with multiple corrections for (a-i), two-way t-test for (j), * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Selective expansion of Tregs in K5-TGO-DO11 Autoimmune Model.

(a,b) Quantification of DO11⁺CD3⁺ T cell populations as a fraction of all CD4⁺ T cells in skin. (c,d) Quantification of DO11⁺CD3⁺ T cell populations in absolute cell numbers per 100,000 CD3⁺ T cells. (e,f) Flow cytometry of DO11⁺ CD3⁺ T cell populations in mice given doxycycline chow (+disease). (g,h) Quantification of DO11⁺ CD3⁺ T cell populations as a fraction of all CD4⁺ T cells in SDLNs. One-way ANOVA, * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Immune profiling of JNW activity in K5-TGO-DO11 Autoimmune Model shows JNWs suppress tissue-specific autoimmune disease.

K5 TGO DO11.10 mice injected with NWs or blank nanowires only, diseased groups in red, all plots show cells from mouse skin. (a,b) Number of CTLA-4⁺ DO11.10⁺ CD3⁺ CD4⁺ effector T cells and FOXP3⁺ regulatory T cells per 100k CD3⁺ cells. (c,d) Number of Ki67⁺ DO11.10⁺ CD3⁺ CD4⁺ effector T cells and FOXP3⁺ regulatory T cells per 100k CD3⁺ cells. (e) DO11⁺ Treg:Teff cell ratio from dorsal skin of K5-TGO-DO11 animals. (f,g) IFN γ and IL-17 expressing DO11⁺ effector T cells by intracellular cytokine staining. (i,j) Representative H&E stained skin sections from diseased mice treated with blank control (left) and JNW (right), arrow showing plaque on surface of skin in diseased animals. (k) Quantification of plaque thickness across cohorts. (l) Treg to effector T cell ratio in each animal. Each data point represents a single animal, one-way ANOVA with multiple comparisons correction. * denotes $p < 0.05$, ** $p < 0.01$. Scale bar 200 μ m.