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Engineering an “Infectious” T_{reg} Biomimetic through Chemoselective Tethering of TGF-β1 to PEG Brush Surfaces

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Abstract

Modulation of immunological responses to allografts following transplantation is of pivotal importance to improving graft outcome and duration. Of the many approaches, harnessing the dominant tolerance induced by regulatory T cells (T_{reg}) holds tremendous promise. Recent studies have highlighted the unique potency of cell surface-bound TGF-β1 on T_{reg} for promoting infectious tolerance, i.e. to confer suppressive capacity from one cell to another. To mimic this characteristic, TGF-β1 was chemoselectively tethered to inert and viable polymer grafting platforms using Staudinger ligation. We report the synthesis and functional characterization of these engineered TGF-β1 surfaces. Inert beads tethered with TGF-β1 were capable of efficiently converting naïve CD4⁺ CD62L^{hi} T cells to functional T_{reg}. Concordantly, translation of conjugation scheme from inert surfaces to viable cells also led to efficient generation of functional T_{reg}. Further, the capacity of these platforms to generate antigen-specific T_{reg} was demonstrated. These findings illustrate the unique faculty of tethered TGF-β1 biomaterial platforms to function as an “infectious” T_{reg} and provide a compelling approach for generating tolerogenic microenvironments for allograft transplantation.

Keywords

biomaterials; immunomodulation; Staudinger ligation; regulatory T cells

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Introduction

Cellular transplant therapy using allogeneic cells is consistently hampered by graft rejection due to targeted immunological attack [1]. Allograft rejection is initiated when presentation of allogeneic antigens, either directly or indirectly, results in the clonal expansion and activation of alloantigen-driven T cells. Currently, allograft recipients rely on the administration of systemic immunosuppressant or anti-rejection drugs, which not only impart a montage of detrimental side effects but commonly fail over time [2, 3].

To address these challenges, current approaches seek to engineer a graft microenvironment that favors immunological or operational tolerance, i.e. a lack of reactivity to donor alloantigens with retention of generalized immune responses to other foreign antigens or insults [4]. Harnessing native regulatory pathways are of particular interest, such as downregulation of effector T cells (T_{eff}) and the generation of regulatory T cells (T_{reg}) specific to the offending antigen(s) [5]. T_{reg} , a subset of peripheral $CD4^+$ T cells classically identified as $CD4^+CD25^+FoxP3^+$, facilitate the downregulation of T_{eff} responses via immunosuppressive cytokines and cell surface receptors, as well as suppression of antigen presentation pathways [6, 7]. Increasing the number of antigen-specific T_{reg} has been strongly associated with allograft tolerance [8–10]. In this regard, T_{reg} are observed within tolerant grafts, indicating that alloreactive T_{reg} can be potentially recruited to and/or retained in sites where alloantigen is expressed and participate in tolerance induction [11–13]. This has been further supported by recent studies using biomaterials to co-deliver T_{regs} with the allograft, which has resulted in improved graft outcomes [14, 15].

Transforming Growth Factor- β 1 (TGF- β 1) has long been identified as a key player in immunosuppression, as elevated TGF- β 1 expression has been found in specialized tissues and cells where immune activation is dampened, such as tumor exosomes, placental tissue, and mesenchymal stem cells [16–20]. *In vitro*, soluble TGF- β 1 is highly effective at converting peripheral $CD4^+CD25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T_{reg} , thus serving as a popular inducer of T_{reg} *ex vivo* for subsequent *in vivo* delivery [21–24]. While infusion of soluble TGF- β 1 to dampen allograft responses may initially appear attractive, persistent delivery of TGF- β 1 has been shown to lead to fibrosis, a side effect that can prove to be highly pernicious for the survival of cellular transplants [25–27].

Of interest, recent studies have discovered a cell surface-bound form of TGF- β 1, which has been found, to date, only on platelets and activated T_{reg} [28–31]. The mechanism of immunomodulation of surface-bound TGF- β 1 is proposed to be unlike that of its soluble counterpart. While soluble TGF- β 1 has been found to suppress immune responses via inhibition of T cells proliferation and promotion of T_{reg} function [21, 22], surface-bound TGF- β 1 has been hypothesized to have a unique and specific potency where its major role on the T_{reg} is to convert responder T cells into T_{reg} via “infectious tolerance”, i.e. the transference of a tolerance-inducing state from one cell population to another [32–34]. Engineering a surface-bound TGF- β 1 biomaterial platform presents the opportunity to generate an interface that mimics the infectious properties of the T_{reg} . Such a platform provides a means to investigate the function exerted by surface-bound TGF- β 1 alone and

provides an appealing alternative for targeted local immunomodulation that circumvents the shortcomings of soluble release.

Of additional consideration in engineering an immunomodulatory biomaterial is not only the agent of interest, but also the context of presentation. While TGF- β 1 is a potent prerequisite for T_{reg} generation, the ubiquitous expansion of polyclonal T_{reg} (i.e. T_{reg} generated without a specific antigen stimulator) is not as effective as the selective expansion of monoclonal T_{reg} (i.e. T_{reg} generated in the presence of a specific antigen) [35, 36]. When T_{reg} are generated in the presence of the offending antigen, the effect exerted by the T_{reg} to dampen T_{eff} is more impressive [37–39]. As a corollary, the possibility of systemic tolerance to the specific antigen is elevated. Generating abundant antigen-specific T_{reg} is challenging, as they are only produced when both TGF- β 1 and antigen-specific effectors are present [40, 41]. A biomaterial platform that provides antigen presentation in close proximity to immobilized TGF- β 1 could synchronize these cues in a manner that results in the efficient and local generation of antigen-specific T_{reg}.

In this report, we evaluated this approach by tethering TGF- β 1, in a chemoselective manner, to a PEG-based polymeric brush grafted onto inert beads and viable cells (Figure 1). These immunomodulatory coatings were subsequently explored for their capacity to convert naïve T cells to T_{reg}, both in a generalized manner and in response to a specific antigen. The implications of these immunomodulatory biomaterials to direct host immune responses towards antigen tolerance are also discussed.

Methods

Materials

Silicon wafers (2 inch diameter $\times$ 0.5 mm thickness) were purchased from Sigma-Aldrich. TGF- β 1 (lyophilized) was purchased from Novoprotein. Culture buffers and RPMI media were purchased from Cellgro and Gibco, respectively. Complete RPMI medium was supplemented with 10% fetal bovine serum (HyClone from Thermo Scientific), 330 mg/L of L-glutamine, 96 U/mL of streptomycin, and 22 mL/L of 1 M HEPES buffer. All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

Mouse models

Transgenic mouse strains were employed to assist in cell characterization and to provide antigen specificity. To evaluate the generation of T_{reg}, reporter FoxP3 mice were used, which permit for ease in identification and live sorting of T_{reg} via fluorescent reporting. Two T_{reg} reporter strains were used in this study: FoxP3^{GFP} mice (B6.Cg-FoxP3^{tm2Tch}/J; Jackson Laboratory, Bar Harbor, ME, USA), which co-express GFP and FoxP3 [42]; and FoxP3^{RFP} mice (FoxP3^{RFP} bicistronic reporter on the C57BL/6 background; generously provided by Richard Flavell, Yale University, New Haven, CT [43]), which co-express RFP and FoxP3. For antigen specificity, OTII mice (B6.Cg-Tg(Tcr α Tcr β) 425Cbn/J, Jackson Laboratory), which express a transgenic TCR (V α 2, V β 5.1/5.2) specific for I-A^b restricted peptide (OVA) amino acids 323–339 derived from chicken OVA, were used [44]. To generate an OVA-antigen specific T_{reg} reporter strain, OTII mice were bred with FoxP3^{RFP}-B6 mice to

generate the OTII-FoxP3^{RFP} strain (verified via Vα2, Vβ5, and FoxP3-RFP in the CD3⁺CD4⁺ gate, as described previously [45]). All mice were maintained at the University of Miami Miller School Of Medicine. All animal studies were carried out under protocols approved by the University of Miami Institutional Animal Care and Use Committee.

MDT functionalization of TGF-β1 and BSA

MDT-NHS (26.8 mg, 1-methyl-2-diphenylphosphino-terephthalate N-hydroxysuccinimide ester, synthesized as previously reported [46]), 200 mg of H₂N-PEG-COOH (MW 3600, synthesized as previously reported [47]), and 800 μL of anhydrous N,N-dimethylformamide (DMF) were stirred for 10 min under argon atmosphere. Triethylamine (31.8 μL) was added to the solution, with stirring under Ar atmosphere. After 3 h, 10 mL of cold diethyl ether (Et₂O) was added and stirred. The precipitate, MDT-PEG-COOH, was collected by centrifugation, dissolved in 8 mL ethanol (EtOH) at 37 °C, and subsequently cooled via ice-water bath to re-precipitate the product. Precipitated MDT-PEG-COOH was collected by centrifugation, rinsed with 10 mL cold Et₂O, and dried under reduced pressure. Yield: 187 mg. Kaiser Test: negative. Selected characteristic ATR-FT-IR (PerkinElmer Spectrum 100, KBr beam splitter, FR-DTGS detector, 4 scans at 4 cm⁻¹ resolution) bands: 2884 (C-H of PEG), 1656 (amide I), 1101 (C-O of PEG), 1538 (amide II), 749 (MDT), and 699 (MDT) cm⁻¹. Further, 21.1 μL of N,N'-diisopropylcarbodiimide (DIC) was added to a solution of 180.0 mg of MDT-PEG-COOH, 15.8 mg of N-hydroxysuccinimide (NHS), and 700 μL of anhydrous DMF with stirring under Ar atmosphere. After 3 h, the product, MDT-PEG-NHS, was purified similarly to MDT-PEG-COOH and dried under reduced pressure. Yield: 155 mg. Selected characteristic ATR-FT-IR bands: 2884, 1812 (C=O of NHS), 1783 (C=O of NHS), 1730 (C=O of NHS ester), 1660, 1102, 1543, 750, and 700 cm⁻¹.

As a control linker, NHS-PEG-CH₃ was also synthesized. First, drop-wise addition of 50 mg glutaric anhydride in 400 μL DMF to a solution of H₂N-PEG-CH₃ (2.0 g, MW 5000, JenKem Technology) in 4 mL DMF at 37 °C under Ar atmosphere. Then, 112 μL triethylamine (TEA) in 400 μL DMF was added. After 25 min, the product (HOOC-PEG-CH₃) was purified similarly as MDT-PEG-COOH, except 60 mL Et₂O and 50 mL EtOH were used. Yield: 1.94 g. Kaiser Test: negative. Selected characteristic ATR-FT-IR bands: 2883, 1729, 1668, 1545, and 1093 cm⁻¹. Second, 373 μL DIC in 800 μL DMF was added to a solution of 137 mg NHS, 1.94 g HOOC-PEG-CH₃, and 3 mL DMF at 37 °C under Ar atmosphere. After stirring for 2 h at room temperature, NHS-PEG-CH₃ was purified as described for HOOC-PEG-CH₃. Yield: 1.8 g. Selected characteristic ATR-FT-IR bands: 2883, 1813, 1783, 1739, 1668, 1545, and 1096 cm⁻¹.

MDT-PEG-NHS, at 3×10^{-4} M was incubated with 3×10^{-7} M TGF-β1 in PBS pH 7.4 at 4°C overnight. The resulting product, TGF-β1-PEG-MDT (termed _{MDT}TGF-β1), was subsequently filter concentrated via centrifugation with a 10 kDa filter for 1 h, during which 400 μl of PBS was added every 10 min. The same process was conducted to generate BSA-PEG-MDT (termed _{MDT}BSA), with bovine serum albumin (BSA, Seracare). As an additional control to evaluate chemoselectivity, TGF-β1 was linked to NHS-PEG-CH₃ (MW 5000) via incubation of NHS-PEG-CH₃ at 3×10^{-4} M was incubated with 3×10^{-7} M TGF-β1 in PBS pH 7.4 at 4°C overnight. The resulting product, TGF-β1-PEG-CH₃

(termed $\text{CH}_3\text{TGF-}\beta 1$), was subsequently filter concentrated via centrifugation with a 10 kDa filter for 1 h, during which 400 μL of PBS was added every 10 min.

Tethering of TGF- $\beta 1$ to azido-functionalized silicon wafers

Azido-functionalization of silicon wafer was performed as previously described [47]. The resulting azido wafer surface was incubated with MDT-TGF- $\beta 1$ or $\text{CH}_3\text{TGF-}\beta 1$ (control) (15 ng/ml) in PBS overnight at 37°C in a Petri dish with a water reservoir and covered to prevent dehydration. Next, the wafers were rinsed 3 times with PBS, followed by H_2O , 50% H_2O in EtOH, and finally EtOH before drying with argon. The film thickness on silicon wafer surfaces was quantified with a J.A. Woollam Co. α -SE spectroscopic ellipsometer at a 70° angle in standard mode. Data were fitted to the “Si with transparent film” model.

Synthesis of MDT-PEG-carboxyfluorescein-TGF- $\beta 1$ and CH_3 -PEG-carboxyfluorescein-TGF- $\beta 1$

For confocal imaging, a TGF- $\beta 1$ PEG conjugate was generated, with a carboxyfluorescein label within the linker to permit fluorescent imaging. A TGF- $\beta 1$ PEG-carboxyfluorescein conjugate with a terminal MDT, termed MDT-PEG-carboxyfluorescein-TGF- $\beta 1$, permits covalent conjugation of the MDT group specific to N_3 surface via Staudinger ligation. This was generated in 4 steps. Step 1 (fabricate the fluorescent conjugate): Fmoc-Asp(OtBu)-OSu (60 mg, Bachem B-1075) was reacted with tBoc-PEG-NH₂ (400 mg, Laysan Bio, MW 3400) in 1 mL DMF at 37 °C under Ar atmosphere followed by drop-wise addition of 41 μL N,N-diisopropylethylamine in 500 μL DMF. After 15 min, 500 μL piperidine was added and stirred for 25 min. The product, tBoc-PEG-Asp(OtBu)-NH₂, was precipitated out with 10 mL cold diethyl ether and collected by centrifugation. The collected precipitate was dissolved in 10 mL EtOH at 37 °C and the solution was cooled down in ice-water bath to reprecipitate the product. The collected precipitate was washed with cold Et₂O and dried under reduced pressure. Yield 404.0 mg. Selected characteristic ATR-FT-IR bands: 3421, 2883, 1777, 1735, 1718, 1676, 1499, 1103, 763, and 740 cm^{-1} . Step 2: TEA (18 μL) in 400 μL anhydrous dichloromethane (DCM) was added to a solution of 262 mg tBoc-PEG-Asp(OtBu)-NH₂ (from step 1), 30 mg carboxyfluorescein-NHS (Thermo Scientific), and 500 μL DMF and stirred for 25 min. Cold Et₂O (10 mL) was added and stirred well. The precipitated that formed was collected by centrifugation and dried under reduced pressure. The precipitate was dissolved in 150 μL DCM containing 50 μL triisopropylsilane and 1.8 mL trifluoroacetic acid. After 2 h, the product, carboxyfluorescein-Asp(COOH)-PEG-NH₂, was collected and purified as in step 1. Yield: 191 mg. Selected characteristic ATR-FT-IR bands: 3482, 3096, 2885, 1980, 1761, 1741, 1675, 1613, 1543, and 1102 cm^{-1} . Step 3: carboxyfluorescein-Asp(COOH)-PEG-NH₂ (95 mg) was reacted with either 13.0 mg MDT-OPfp (Sigma Aldrich) or 2.3 μL acetic anhydride in 1 mL DMF and added 10.2 μL or 10.9 μL of TEA, respectively. After 25 min, both products were collected and purified as in Step 1, except only 7 mL Et₂O or 7 mL EtOH was used instead. Yield of carboxyfluorescein-Asp(COOH)-PEG-MDT: 86 mg. Selected characteristic ATR-FT-IR bands: 3504, 3325, 2885, 1760, 1720, 1661, 1615, 1538, 1101, 749, and 699 cm^{-1} . Yield of carboxyfluorescein-Asp(COOH)-PEG-Ac: 80 mg. Selected characteristic ATR-FT-IR bands: 3499, 3320, 2885, 1761, 1660, 1615, 1548, and 1098 cm^{-1} . Step 4: DIC (25.6 μL or 25.3 μL) was added to a solution of 9.4 mg NHS with 86 mg carboxyfluorescein-Asp(COOH)-PEG-MDT or 9.3 mg

NHS with 80 mg carboxyfluorescein-Asp(COOH)-PEG-Ac, respectively, in 500 μ L DMF. After 3.5 h, the products were purified as in step 1 except that 5 mL EtOH was used instead. Yield for carboxyfluorescein-Asp(NHS)-PEG-MDT: 76 mg. Selected characteristic ATR-FT-IR bands: 3506, 3321, 3062, 2885, 1816, 1781, 1761, 1738, 1661, 1539, 1100, 750, 728, and 699 cm^{-1} . Yield for carboxyfluorescein-Asp(NHS)-PEG-Ac: 70 mg. Selected characteristic ATR-FT-IR bands: 3510, 3324, 3072, 2885, 1815, 1781, 1762, 1740, 1659, 1541, and 1097 cm^{-1} .

Following fabrication of the carboxyfluorescein-Asp(NHS)-PEG-MDT and its control carboxyfluorescein-Asp(NHS)-PEG-Ac, these linkers were incubated with TGF- β 1 to form a conjugate, termed $\text{MDT-CF}^{\text{TGF-}\beta 1}$ and $\text{AC-CF}^{\text{TGF-}\beta 1}$, respectively. Unreacted label was removed from the solution via purification (as described for NHS-PEG-MDT) prior to incubation with azido-functionalized beads.

Tethering of TGF- β 1 to azido-functionalized glass beads

Glass beads (150–212 μm , Sigma Aldrich) were functionalized to generate a uniform azide surface in the same manner as that of the silicon wafers [47]. For TGF- β 1 functional studies, azido-functionalized glass beads were incubated overnight with 6 ng/ml, 30 ng/ml, or 60 ng/ml of $\text{MDT}^{\text{TGF-}\beta 1}$. Resulting glass beads conjugated with TGF- β 1 from these 3 doses were termed: $\text{G}^{\text{TGF-}\beta 1^{\text{LOW}}}$, $\text{G}^{\text{TGF-}\beta 1^{\text{MED}}}$, and $\text{G}^{\text{TGF-}\beta 1^{\text{HIGH}}}$, respectively. As controls, azido-functionalized glass beads were incubated overnight with 6 ng/ml, 30 ng/ml, or 60 ng/ml of MDT^{BSA} . Resulting glass beads conjugated with BSA from these 3 doses were termed: $\text{G}^{\text{BSA}^{\text{LOW}}}$, $\text{G}^{\text{BSA}^{\text{MED}}}$, and $\text{G}^{\text{BSA}^{\text{HIGH}}}$, respectively.

For imaging studies, the azido-functionalized glass beads were subsequently incubated for 1 h with 15 ng/ml of MDT-PEG-carboxyfluorescein-TGF- β 1. The coating uniformity and stability was assessed by the detection of fluorescently labeled TGF- β 1 using a Leica SP5 spectral confocal inverted microscope. Multi-slice images (2–4 μm thickness, 1024×1024 , $20 \times$ objective) were collected and compiled using the Leica three-dimensional projection function.

Tethering of TGF- β 1 to azido-modified Antigen Presenting Cells (APCs)

C57BL/6 splenocytes were isolated as previously described [48]. T cells were removed by incubation with CD4 (clone RL172) and CD8 (HO2.2) mAbs and Low-Tox-M rabbit complement (Accurate Chemical & Scientific) at 37°C for 45 min followed by 3 washes with complete medium. The resulting cell suspension was used as antigen presenting cells (APCs). Subsequently, APCs were mitomycin C treated at 37°C for 45 min. For tethering of TGF- β 1, APCs were first functionalized with azide using a bifunctional N_3 -PEG-NHS (Nanocs, MW 5000) at 1 mg/ml in PBS (pH 7.8) for 1 h at 37°C. Subsequently, MDT-PEG-TGF- β 1 (60 ng/ml) was linked to the APCs functionalized with azide via chemoselective ligation of the MDT to N_3 present on the APCs for 1 h at 37 °C. For controls, MDT-PEG-BSA at the same concentration was used. The resulting surface modified APCs, coated with either TGF- β 1 or BSA (control), were termed $\text{APC}^{\text{TGF-}\beta 1}$ and APC^{BSA} , respectively.

Conversion of splenic naïve T cells into FoxP3⁺ T_{reg} cells

Splenic naïve CD4⁺ CD62L^{hi} GFP⁻ (or RFP⁻) cells were sorted from FoxP3^{GFP}-B6 mice (or FoxP3^{RFP}-B6 mice), using BD FACSAria cell sorter. The purity of sorted CD4⁺ CD62L^{hi} GFP⁻ (or RFP⁻) cells was at least 98%. This population was termed “responder cells” as they respond to the surrounding stimuli, resulting in proliferation and conversion to T_{regs}. Responder cells were labeled with CellTrace Violet (Life Technologies) and the labeled cells (10⁵) were polyclonally stimulated with αCD3/CD28-conjugated DynaBeads, which provide primary and co-stimulatory signal to promote T cell activation and expansion, in 96 well plates in the presence or absence of the tested platform (see below paragraph for details on platforms tested). After 3 days, cell proliferation was monitored by dilution of CellTrace Violet and induced FoxP3 expression by conversion of GFP⁻ to GFP⁺ (or RFP⁻ to RFP⁺) cells, both via flow cytometry.

To evaluate the impact of MDT conjugation on TGF-β1 activity, soluble MDT-TGF-β1 (0.3, 3, or 6 ng/ml) was added to the cell culture outlined above and T_{reg} generation was assessed. As a control, unmodified TGF-β1 (3 ng/ml) was used. To evaluate the ability of *G*-TGF-β1 to induce T_{reg}, glass beads (1500 beads/ml) tethered with TGF-β1, i.e. *G*-TGF-β1^{LOW}, *G*-TGF-β1^{MED}, or *G*-TGF-β1^{HIGH}, were added to the culture. As controls, glass beads (1500 beads/ml) tethered with BSA, i.e. *G*-BSA^{LOW}, *G*-BSA^{MED}, or *G*-BSA^{HIGH}, were used. For studies exploring cell surface modification to generate polyclonal T_{regs}, PEGylated APCs expressing TGF-β1 (APC-TGF-β1) were added to the culture at ratios of 1:2, 1:1, and 2:1 of APC-TGF-β1:CD4⁺CD62L^{hi}RFP⁻ responder cells. As a control, APCs modified with BSA (APC-BSA) were used.

For studies evaluating the generation of monoclonal T_{reg} using cell surface modification, modifications in the co-culture system was made to clearly track T_{reg} generation and efficiently present antigen. PEGylated APCs modified to express TGF-β1 (APC-TGF-β1) were co-cultured with naïve OTII⁺CD4⁺CD62L^{hi}RFP⁻ responder cells (sorted from OTII-FoxP3^{RFP} mice using a BD FACSAria cell sorter) at ratios of 1:2, 1:1, and 2:1 of APC-TGF-β1 : OTII⁺CD4⁺CD62L^{hi}RFP⁻ responder cells. As with previous experiments, responder cells were labeled via CellTrace Violet. For specific antigen activation of the CD4⁺CD62L^{hi}RFP⁻ naïve cells, synthetic OVA 323–339 peptide (0.5 μM, Anaspec) was added, as well as 10⁵ APCs (isolated from C57BL/6 and enriched as described above) to assist in presentation of OVA antigen. The resulting co-culture system was incubated for 5 days. Following incubation, proliferation of responders was monitored by dilution of CellTrace Violet and induced FoxP3 expression was tracked by conversion of RFP⁻ to RFP⁺ cells, both via flow cytometry.

T_{reg} suppression assay

In vitro T_{reg} function was measured by examining the capacity of T_{reg} to suppress the proliferation of a responder T cell population, as described previously [49]. T_{reg} generated *in vitro* using APC-TGF-β1, termed *in vitro*-induced T_{reg} (iiT_{reg}), were generated as described above. Briefly, 10⁵ APC-TGF-β1 were co-cultured with α-CD3/28 dynabeads and 10⁵ CD4⁺CD62L^{hi} naïve T cells for 5 d. Subsequently, cultures were harvested, from which iiT_{reg}, classified as CD4⁺FoxP3^{RFP+}, were sorted using BD FACSAria cell sorter. For direct

comparison with iiT_{reg} , $CD4^+ FoxP3^{GFP+}$ natural T_{reg} (nT_{reg}), freshly sorted from the spleens of $FoxP3^{GFP}$ -B6 mice, were used. The harvested T_{reg} , either iiT_{reg} or nT_{reg} , were cultured with 5×10^4 $CD4^+ FoxP3^{GFP-}$ T cells (termed “responder T cells” and labeled with CellTrace Violet) and 5×10^4 APCs and α -CD3 (stimulating agents for the responder T cells). The ratio of T_{reg} to responder T cell was increased from 1:16 to 1:1. Cell proliferation of the $CD4^+GFP^-$ responder T cells was monitored by dilution of CellTrace Violet signaling after 3 d of co-culture.

Flow cytometry and cell purification

For cell-surface phenotype analysis, murine splenocytes were stained with APC-Cy7 live/dead staining (Life Technologies), Alexa Fluor 700 anti-CD4 (BD Biosciences), PE-Cy7 anti-CD25 (BD Biosciences), PE-Texas Red anti-CD44 (BD Biosciences), PerCP Cy5.5 CD62L (Biolegend). Fluorescence-activated cell sorting analysis was performed using Flowjo 7.6.1 software (Tree Star). FoxP3 expression was tracked by GFP or RFP fluorescence after gating for viable $CD4^+$ cells. Cell proliferation was tracked by dilution of CellTrace Violet (Life Technologies). Cells were washed with HBSS with 0.5% BSA. Total number of events collected for analysis was 25,000–50,000 per sample.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation, with $n = 3$ for each experimental group. Three independent experiments were made for each study. Statistical analysis was performed on all samples using *t*-test and one-way ANOVA and a Tukey's multiple comparison test to evaluate significant differences between groups with $p < 0.05$.

Results

PEGylated TGF- β 1 induces FoxP3 expression in $CD4^+ CD62L^{hi}$ GFP-naïve precursors

Staudinger ligation, the chemoselective conjugation of a phosphine to an azide, was selected as the scheme of choice for the covalent linkage of TGF- β 1 to surfaces. To achieve this, TGF- β 1 was first functionalized with PEG-MDT (1-methyl-2-diphenylphosphino-terephthalate) via NHS-PEG-MDT (Figure 1A). The resulting conjugate, TGF- β 1-PEG-MDT, was termed MDT TGF- β 1. As PEGylation of proteins can lead to diminished function, MDT TGF- β 1 was screened for its capacity to generate T_{reg} and compared to unmodified TGF- β 1. For this test, T cells were isolated from reporter FoxP3 mice, which permit ease in tracking conversion of naïve T cells to FoxP3-expressing cells as the fluorescent protein is only co-expressed with FoxP3. Two mouse models were used in these studies, $FoxP3^{GFP}$, which co-expresses GFP with FoxP3, and $FoxP3^{RFP}$, which co-expresses RFP with FoxP3. Naïve $CD4^+ CD62L^{hi} GFP^-$ (or RFP^-) T cells were sorted from isolated splenocytes with high purity ($> 98\%$), labeled to permit tracking of proliferation, and stimulated using α CD3/CD28 activator beads. Soluble TGF- β 1, either unmodified (ζ TGF- β 1) or PEGylated (MDT TGF- β 1), was also added to the culture media and the responder cells were assessed after 3 d. To quantify the generation of T_{regs} , cells were gated for viable $CD4^+$ T cells prior to analysis of FoxP3 GFP expression and proliferation (Figure 2A, representative dot plot). As showing in Figure 2B, significant T_{reg} conversion was not observed for naïve T cells cultured without TGF- β 1; however, T_{reg} generation was

significantly increased when $\text{MDT-TGF-}\beta 1$ was added to the culture. The dose response of $\text{MDT-TGF-}\beta 1$ was characteristic of that observed in previous publications [50] and comparable to unmodified soluble $\text{TGF-}\beta 1$ ($\text{sTGF-}\beta 1$) at the same concentration ($p = 0.83$, Figure 2C). These results confirm that functionalization of the TGF- $\beta 1$ via PEG-MDT did not result in functional impairment.

Coating of TGF- $\beta 1$ on idealized surfaces

The capacity of MDT functionalized TGF- $\beta 1$ to form a stable, covalent bond with a biomaterial surface in a chemoselective manner via Staudinger ligation was evaluated using two-dimensional silicon wafers. The silicon planar substrate was functionalized with azide groups, followed by incubation with $\text{MDT-TGF-}\beta 1$. The binding of the $\text{MDT-TGF-}\beta 1$ to the planar surface was assessed via ellipsometry. Surfaces exposed to $\text{MDT-TGF-}\beta 1$ demonstrated a marked increase in film thickness with modest disparity (Figure 3A). Further, the chemoselectivity of this coating was substantiated via exposure of azide surfaces to PEGylated TGF- $\beta 1$ terminated with CH_3 ($\text{CH}_3\text{TGF-}\beta 1$). The lack of the MDT group led to no binding, as evidenced by no significant increase in film thickness. Exposure of the resulting tethered TGF- $\beta 1$ coating to repeated rinsing with PBS did not result in a statistically significant change in film thickness, demonstrating the stability of the TGF- $\beta 1$ conjugation.

Visualization of the surface tethered MDT-PEG-TGF- $\beta 1$ was performed using glass beads functionalized with N_3 . To visualize the resulting coating via fluorescent confocal microscopy, a carboxyfluorescein was embedded within the NHS-PEG-MDT linker on the functionalized TGF- $\beta 1$ protein, termed $\text{MDT-CF-TGF-}\beta 1$. Incubation of this labeled $\text{MDT-CF-TGF-}\beta 1$ with the azido-glass beads resulted in conjugation via Staudinger ligation, as shown in Figure 3B where high fluorescence and uniform conjugation can be visualized. Resulting coatings were also stable following rigorous PBS rinsing. Of note, while unreacted fluorescent label linker was purified from the $\text{MDT-TGF-}\beta 1$ solution prior to exposure to the azide beads, it cannot be fully discounted that residual unreacted linker may contribute to the overall resulting signal. Comparison of these images to that collected when the control $\text{AC-CF-TGF-}\beta 1$ protein was used further demonstrates the chemoselectivity of the reaction. Overall, these results demonstrate the capacity to form uniform, covalently linked TGF- $\beta 1$ coatings on three-dimensional spherical surfaces.

Tethered TGF- $\beta 1$ induces FoxP3 expression in $\text{CD4}^+ \text{CD62L}^{\text{hi}}$ GFP-T cells

Following conjugation to three-dimensional glass beads, the functional capacity of these surfaces to direct immunoregulatory pathways, specifically to induce T_{reg} generation, was evaluated. As with soluble TGF- $\beta 1$, glass beads presenting tethered TGF- $\beta 1$ were co-cultured with naïve $\text{CD4}^+ \text{CD62L}^{\text{hi}}$ GFP^- T responder cells, polyclonally stimulated with $\alpha\text{CD3/CD28}$ activator beads (see schematic Figure 4A). After 3 days, T_{reg} conversion was monitored via GFP^+ expression. The effect of TGF- $\beta 1$ surface density was evaluated by screening glass beads treated with 3 different concentrations of MDT-PEG-TGF- $\beta 1$ during the surface modification procedure, termed $\text{G-TGF-}\beta 1^{\text{LOW}}$, $\text{G-TGF-}\beta 1^{\text{MED}}$, and $\text{G-TGF-}\beta 1^{\text{HIGH}}$. For controls, MDT-PEG-BSA was used, termed: $\text{G-BSA}^{\text{LOW}}$, $\text{G-BSA}^{\text{MED}}$, and $\text{G-BSA}^{\text{HIGH}}$. The presence of medium or high TGF- $\beta 1$ conjugated beads resulted in

significantly enhanced T_{reg} generation when compared to untreated controls (Figure 4B, $p = 0.002$ and < 0.0001 for $G_{TGF-\beta 1}^{MED}$ and $G_{TGF-\beta 1}^{HIGH}$, respectively) or BSA coated beads ($p < 0.0001$ for both $G_{TGF-\beta 1}^{MED}$, and $G_{TGF-\beta 1}^{HIGH}$, respectively). The lower conjugation of TGF- $\beta 1$ ($G_{TGF-\beta 1}^{LOW}$) did not impart a significant increase in T_{reg} when compared to untreated controls ($p = 0.94$) or BSA coated beads ($p = 0.17$). Further, a dose-dependent effect was observed, with the degree of T_{reg} generation for $G_{TGF-\beta 1}^{HIGH}$ beads demonstrating comparability to the soluble TGF- $\beta 1$ ($S_{TGF-\beta 1}$) control ($p = 0.13$). This data demonstrate the functional ability of TGF- $\beta 1$, tethered to a material surface, to direct activated T cells towards a regulatory phenotype.

Cell surface functionalization with TGF- $\beta 1$ generates T_{reg} cells

While surface conjugation of TGF- $\beta 1$ to biomaterial surfaces exhibits a unique platform for directing host immune T cells towards a regulatory phenotype, of additional interest is exploring this approach for cell surface engineering. To evaluate this approach, splenocytes enriched for antigen presenting cells (APC) were used. The cell surface of the APCs was first grafted with linear NHS-PEG- N_3 , followed by surface conjugation of $MDT_{TGF-\beta 1}$ to the PEG brush via Staudinger ligation (Figure 1).

The resulting polymer grafted APCs, presenting either surface bound TGF- $\beta 1$ or BSA (termed $APC_{TGF-\beta 1}$ and APC_{BSA} , respectively), were then co-cultured with naïve $CD4^+CD62L^{hi}RFP^-$ T responder cells, which were polyclonally stimulated using $\alpha CD3/CD28$ activator beads (Figure 5A). When $APC_{TGF-\beta 1}$ were incubated with $CD4^+$ T cell responders at ratios of 1:2, 1:1, 2:1, a significant enhancement of T_{reg} conversion over untreated controls was observed (Figure 5B, $p = 0.0004$, $p < 0.0001$, and $p < 0.0001$ for 1:2, 1:1, and 2:1 ratios of $APC_{TGF-\beta 1}:CD4^+CD62L^{hi}RFP^-$ responder cells, respectively). This trend was in stark contrast to control surfaces presenting BSA ($p < 0.0001$ for all ratios). Thus, surface engineering TGF- $\beta 1$ to the APC was uniquely capable of promoting significant T_{reg} conversion.

APC tethered with TGF- $\beta 1$ converts naïve $CD4^+CD62L^{hi}RFP^-$ T cells to antigen-specific T_{reg}

Recent reports suggest that alloantigen-specific T_{reg} may be more efficient than polyclonal populations [51, 52]. To determine whether our TGF- $\beta 1$ biomaterial platforms can convert naïve T cells into FoxP3-expressing cells in an antigen-specific manner, we modified the culture system. First, OVA-specific naïve $CD4^+CD62L^{hi}RFP^-$ T responder cells ($CD4^+CD62L^{hi}RFP^-$ isolated from OTII-FoxP3 RFP mice) were used to ensure antigen-specific clonal expansion (i.e. the effector cells are specific to OVA antigen only). Additionally, T responder cells were activated using OVA peptide, in lieu of general $\alpha CD3/CD28$ activator beads. Further, unmodified APCs were boosted into the system as a third cell population to efficiently present the OVA peptide to the naïve T responder cells. Using cells clonally specific for OVA and only OVA peptide for stimulation results in highly antigen specific responses, ensuring that any and all T_{reg} generated will be antigen-specific. $APC_{TGF-\beta 1}$ were added to the culture system at ratios of 1:2, 1:1, 2:1 $APC_{TGF-\beta 1}:CD4^+CD62L^{hi}RFP^-$ T responder cells. Following incubation, significant antigen-specific T_{reg} conversion was observed when $APC_{TGF-\beta 1}$ were present when compared to both the control group (Figure 6B, $p < 0.0034$, $p < 0.0003$, $p = 0.1252$, for 1:2, 1:1, 2:1 ratios of $APC_{TGF-\beta 1}:CD4^+CD62L^{hi}RFP^-$

responder cells) and respective BSA tethered APCs ($p = 0.0003$, $p < 0.0001$, $p = 0.012$, for 1:2, 1:1, 2:1 ratios of $_{APC}TGF-\beta 1:CD4^+ CD62L^{hi} RFP^-$ responder cells). Overall, this data demonstrates that functionalized $_{APC}TGF-\beta 1$ can adequately provide the necessary TGF- $\beta 1$ to not only convert naïve T cells into FoxP3-expressing T cells, but also to generate antigen-specific T_{reg} when co-stimulated with the complementary antigen.

T_{reg} induced by $_{APC}TGF-\beta 1$ suppress $CD4^+$ T cell activation

While the capacity of TGF- $\beta 1$ grafted surfaces to generate FoxP3 $^+$ $CD4^+$ T cells was firmly established, the functional nature of these cells was unproven. Given that transient Foxp3 expression in T cells without suppressive activity has been observed in human cells [53], the suppressive function of T_{reg} generated *in vitro* by TGF- $\beta 1$ grafted surfaces, termed herein as *in vitro*-induced T_{regs} or iiT_{reg} , should be validated. The suppression assay involves the addition of T_{regs} to polyclonally stimulated $CD4^+FoxP3^-$ T responder cells. If the T_{regs} are functional, significant suppression of responder T cell proliferation should be observed. To further compare the functional activity of the iiT_{reg} , natural T_{reg} (nT_{reg} ; endogenous $CD4^+FoxP3^+$ T cells isolated from spleens) were screened in parallel (Figure 7A). As shown in Figure 7A–B, potent suppression of responder T cell proliferation was observed when iiT_{reg} were added. This suppression was concentration dependent, as an increase in the number of T_{reg} to responder T cells resulted in enhanced suppression of proliferation, with a 1:1 ratio resulting in the most substantial suppression. While statistically distinct at 1:4, 1:2, and 1:1 ratios, the trends of iiT_{reg} to nT_{reg} suppression were remarkably similar, demonstrating analogous functional capacity. These results support that TGF- $\beta 1$ grafted cell surfaces were capable of generating functionally competent T regulatory cells.

Discussion

The modification of biomaterial surfaces with bioactive agents is a rapidly growing approach to direct host responses following transplantation. While most approaches seek to engineer materials to link bioactive motifs capable of directing cellular adhesion and migration, differentiation, or vascularization [54–56], the concept of engineering immunomodulatory biomaterials capable of instructing immune responses has gained legitimacy in recent years [15, 57–60]. A salient advantage of this approach is that it serves as a means to bypass side effects associated with soluble delivery of these agents. Moreover, active proteins can be expressed on the material surface without the need to genetically modify cells, bypassing the complexities associated with gene therapy. Finally, by providing early and local instruction to the microenvironment, the potential for tolerance is raised.

Covalent stabilization of the biologic to the biomaterial is of critical importance, particularly within the immunologically hostile and physiologically dynamic transplant environment. Herein, we outline the covalent tethering of TGF- $\beta 1$ using Staudinger ligation, a biorthogonal reaction that chemoselectively crosslinks azide to a MDT-functionalized TGF- $\beta 1$, resulting in a stable amide bond. Following Staudinger ligation, we demonstrated the capacity of TGF- $\beta 1$ modified surfaces to direct T cells towards a regulatory phenotype. After general co-stimulation of TCR via activator beads and TGF- $\beta 1$ signaling with our engineered biomaterial, naïve T cells were effectively converted to T_{reg} . Thus, by tethering

TGF- β 1 to the surface of a material or cell, this approach has efficiently created a mimetic of T_{reg} , with the surface capable of conferring a regulatory phenotype onto responding T cells.

Comparing T cells responses towards our biomimetic platform to that of the native cell surface-bound presentation of TGF- β 1 can provide some context. Cell surface-bound TGF- β 1 consists of latency-associated peptide (LAP), the N-terminal part of pro-TGF- β 1, non-covalently associated with mature TGF- β 1 (termed LAP/TGF- β 1), which can be bound to the outer cell membrane through the receptor glycoprotein-A repetitions predominant (GARP) [28, 61]. Cell surface-bound TGF- β 1 expressed on T_{reg} has been found to be involved in cell contact-dependent immunosuppression [33, 62]. Further, these studies hypothesize that the major role of surface-bound TGF- β 1 is to convert responder T cells into T_{reg} , when both populations are activated in concert via their TCRs. Our study strengthens this very view that T_{reg} may be induced from CD4⁺ CD62L^{hi} FoxP3⁻ precursors through co-stimulation of TCR and tethered TGF- β 1 signaling. Further, the mechanism by which native TGF- β 1 exerts function from the latent GARP/LAP complex is still a subject of debate. While it is known that T_{reg} express surface membrane-bound TGF- β 1 following TCR stimulation [30, 33], whether TGF- β 1 directly mediates T cell suppression remains controversial. Recently, Edwards et al. suggested latent TGF- β 1/GARP complex found on active T_{reg} bestows it with potency to convert conventional T cells into T_{reg} [63]. The high stability resulting from our covalent strategy further supports that T_{reg} conversion is likely executed through the tethered TGF- β 1, as opposed to cleavage products. Tethered TGF- β 1 is likely not involved in T_{reg} mediated suppression, evidenced by recent studies, which revealed that the suppressive abilities of both GARP-deficient T_{reg} and TGF- β 1-deficient T_{reg} were unimpaired [64]. The absence of direct suppression by TGF- β 1 in our studies is consistent with this view.

While the creation of general T_{reg} may be a desirable approach, the generation of antigen-specific T_{reg} would be more efficacious and less likely to induce broad immunosuppression. Natively, suppression by T_{reg} is antigen-dependent, which ensures self versus nonself discrimination. In *ex vivo* T_{reg} expansion, antigen-specific T_{reg} have been shown to be more efficient at preventing diabetes onset in the NOD autoimmune model than polyclonal T_{reg} [35]. In our studies exploring T_{reg} generation in the context of OVA antigen, naïve T cells enriched from OTII mice efficiently converted to T_{reg} in the presence of TGF- β 1 modified APCs. As such, these platforms promote T_{reg} conversion in a monoclonal context. These studies lay the foundation for the generation of monoclonal T_{reg} tailored to the offending antigen, which would not only enhance the strength of the immune suppression, but ensure competency of the immune system towards other antigens or pathogens.

Further, T_{reg} generated using TGF- β 1 grafted surfaces were functionally competent, demonstrating significant suppressive function when co-cultured with activated T cells. This effect was dependent on the number of T_{reg} present, with a positive correlation between T_{reg} number and the suppression of responder T cell proliferation. Notably, the suppressive influence of T_{reg} generated using TGF- β 1 grafted surfaces was only slightly less than endogenous or natural T_{reg} . This observation is notable when compared to the published depressed functional potency of *in vitro*-induced T_{reg} generated via soluble TGF- β 1 when compared to natural T_{reg} [65,66].

Comprehensively, TGF- β 1 in its soluble form inhibits immune responses (i.e. inhibition of production of IL-2, contribution to CTLA-4 mediated suppression) and confers infectious tolerance [67, 68]. Our study demonstrates the mechanism of action of tethered TGF- β 1 is sufficient and specific – converting a naïve population into a tolerogenic one. Through the suppression assay, our characterization of *in vitro*-induced T_{reg} generated by tethered TGF- β 1 demonstrate their efficacy. To best of our knowledge, this is the first gain-of-function study that investigates surface-bound TGF- β 1 alone, parsing out the “infectious” property of the T_{reg}.

Of interest, the general PEGylation of APCs was found to result in suppression of antigen presentation pathways. In the OTII study where OVA peptide was used to stimulate responder T cells, a boosting with an unmodified APC population was necessary to sufficiently activate the OTII CD4⁺ T cells. This result suggests that the PEGylation of the APCs to generate APC-TGF- β 1 may have resulted in the lessened efficiency of the APCs to present OVA peptide to the CD4⁺ T cells, resulting in decreased T cell stimulation. This supports literature reports, which found cell surface PEGylation capable of masking MHC and blocking direct cell-cell interactions [69–71]. Of practical relevance, these results suggest that the combination of an immunoprotective cell coated with TGF- β 1, in absence of an additional stimulating factor, will not result in T_{reg} expansion. It has been postulated, however, that antigen shedding from the coating biomaterial will inevitably lead to indirect antigen presentation and ensuing T cell expansion, activation, and recruitment to the graft site [72, 73]. In this context, the TGF- β 1 surface would direct these peripheral effector T cells to T_{reg}. Ergo, the combination of cellular encapsulation strategies capable of masking surface antigens and MHC with immunomodulatory agents capable of directing immune cells towards regulatory phenotypes may be highly complementary.

Of consideration for *in vivo* translation of this immunomodulatory platform is the capacity to generate T_{reg} cells at the site of the implant. Several studies have highlighted the challenge of both ensuring robust engraftment of *in vitro* generating T_{reg} and retaining their phenotypic stability [74–76], rendering *ex vivo* expansion of T_{reg} and subsequent infusion not ideal for long term tolerance. Surface modification of biomaterials at the transplant site presents a unique approach for T_{reg} generation, as it serves to convert T_{eff} cells to T_{reg} cells at the implant interface, therefore bypassing engraftment issues and ensuring close context for instruction. Further, by linking TGF- β 1 to the surface, the impact of TGF- β 1, which is known to induce fibrosis in soluble form, on host responses should be mitigated [77, 78]. A final concern for *in vivo* translation is the potential of the biomaterial itself to instigate a foreign body response (FBR). While no material is completely stealth from innate immune responses, grafting of PEG to materials has been shown to diminish FBR. Thus, it is suspected that the PEG grafting approach used in our study would favor a minimal host response [58, 79]. Overall, we are intrigued by the underexplored potential of this TGF- β 1-conjugated biomaterial platform to generate a local tolerogenic microenvironment at the transplant site and plan to explore this more deeply.

Conclusion

In summary, we have shown that tethered TGF- β 1 functions as an “infectious” T_{reg} when tethered to inert and cell surfaces. Future efforts are required to assess their ability to generate tolerance, as well as their mechanism of action *in vivo*. From a therapeutic outlook, T_{reg}-mediated suppression is complex three-partner interaction (i.e. the T_{reg} cell, the T helper cell to be suppressed, and the APC) challenged further by the low frequency of antigen-specific T cells at the graft site. Our approach tenders a unique method to generate antigen-specific and localized T_{reg} for potential therapy. The flexibility of the ligation chemistry, with its basis in a biorthogonal conjugation scheme, provides ease in translating this approach to innumerable biomaterial platforms to modulate transplant environments.

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Figure 1. Schematic illustrating TGF-β1 functionalization (A) and subsequent surface tethering to inert or viable surfaces (B). A) TGF-β1 was modified to express the functional handle 1-methyl-2-diphenylphosphino-terephthalate (MDT) through reaction of free amines with NHS-PEG-MDT; generating $_{MDT}TGF-\beta 1$. B) Inert spheres or cells were first grafted with NHS-PEG- N_3 prior to incubation with $_{MDT}TGF-\beta 1$, whereby the complementary N_3 and MDT groups reacted to form an amide bond (inset) via chemoselective Staudinger Ligation.

Figure 2. TGF- β 1 conjugated with MDT-PEG efficiently converts naïve T cells to T_{reg}
 Generation of FoxP3⁺ T_{regs} was assessed by FACS analysis of FoxP3^{GFP+} expression after co-culture of naïve CD4⁺CD62L^{hi}GFP⁻ T cells with α CD3/CD28 activator beads and MDT-PEG-TGF- β 1 (MDTTGF- β 1) or unmodified TGF- β 1 (γ TGF- β 1) for 3 d. Control groups received only activator beads. Three concentrations of MDTTGF- β 1 were evaluated (0.3, 3, or 6 ng/mL). A) Flow cytometry data from one representative experiment (TGF- β 1 concentration of 3 ng/mL), where responder cells were gated for viable CD4⁺ T cells prior to analysis of FoxP3^{GFP} expression and proliferation (via CellTrace). Percentage in upper left corner of each dot plot indicates the percent of proliferating CD4⁺FoxP3^{GFP+} cells B) Summary of generation of viable proliferating T_{reg}, as characterized by FoxP3^{GFP+} cells, when MDTTGF- β 1 was added at the indicated concentrations. C) Comparison of modified MDTTGF- β 1 and unmodified γ TGF- β 1 at 3 ng/mL. Data (B and C) are representative of 3 independent experiments. Error bar = standard deviation. * $p < 0.05$; *** $p < 0.0001$ when compared to control group (0 ng/mL TGF- β 1)

Figure 3. MDT-PEG-TGF-β1 chemoselectively conjugates to N₃ surfaces

A) Ellipsometry film thickness measurement of TGF-β1 conjugates: MDT-PEG-TGF-β1 and CH₃-PEG-TGF-β1 (control), demonstrating chemoselective linkage of MDT-PEG-TGF-β1 via Staudinger ligation. * $p < 0.05$. B) Confocal microscopy single slice (a) and multislice projection (b–c) images of azide-coated glass beads following incubation with MDT-PEG-carboxyfluorescein-TGF-β1, demonstrating uniform coating of idealized surfaces. (d) Confocal microscopy multislice projection image of control azide-coated glass beads following incubation with Ac-PEG-carboxyfluorescein-TGF-β1, demonstrating chemoselectivity of reaction. Scale bar = 100 μm for (a) and 50 μm for (b–d)

Figure 4. TGF-β1, tethered to glass beads, efficiently converts naïve T cells to T_{reg}

A) Schematic of experiment where generation of FoxP3⁺ T_{reg}s was assessed by FACS analysis of FoxP3^{GFP+} expression after co-culture of naïve CD4⁺CD62L^{hi}GFP⁻ T cells with αCD3/CD28 activator beads and TGF-β1 or BSA functionalized glass beads for 3 d. Control groups received only activator beads. sTGF-β1 group received activator beads and soluble TGF-β1. Three degrees of protein conjugation on the glass beads (1500 beads/mL) were evaluated, labeled GTGF-β1^{LOW}, GTGF-β1^{MED}, and GTGF-β1^{HIGH} for TGF-β1 and GBSA^{LOW}, GBSA^{MED}, and GBSA^{HIGH} for BSA. **B)** Summary of generation of viable proliferating T_{reg}, as characterized by FoxP3^{GFP+} cells. Data are representative of 3 independent experiments. Error bar = standard deviation. *** *p* < 0.0001. **C)** Flow cytometry data from one representative experiment, where responder cells were gated for viable CD4⁺ T cells prior to analysis of FoxP3^{GFP+} expression and proliferation (via CellTrace). Percentage in upper left corner of each dot plot indicates the percent of proliferating CD4⁺Foxp3^{GFP+} cells.

Figure 5. TGF-β1, tethered to cell coating, efficiently converts naïve T cells to T_{reg}

A) Schematic of experiment where generation of FoxP3⁺ T_{regs} was assessed by FACS analysis of FoxP3^{RFP+} expression after co-culture of naïve CD4⁺ CD62L^{hi} RFP⁺ T cells with αCD3/CD28 activator beads and mitomycin C-treated APC functionalized with TGF-β1 or BSA for 3 d. Control groups received only activator beads. Three ratios of APC^{TGF-β1} to CD4⁺FoxP3^{RFP} responder cells were evaluated: 1:2, 1:1, and 2:1, respectively. **B)** Summary of generation of viable proliferating T_{reg}, as characterized by FoxP3^{RFP+} cells. Data are representative of 3 independent experiments. Error bar = standard deviation. ****p* < 0.0001. **C)** Flow cytometry data from one representative experiment, where responder cells were gated for viable CD4⁺ T cells prior to analysis of FoxP3^{RFP} expression and proliferation (via CellTrace). Percentage in upper left corner of each dot plot indicates the percent of proliferating CD4⁺FoxP3^{RFP+} cells.

Figure 6. TGF-β1, tethered to cell surface, efficiently generates monoclonal T_{regs} from naïve T cells

A) Schematic of experiment for monoclonal FoxP3⁺ T_{reg} generation. Naïve OTII CD4⁺CD62L^{hi}RFP⁻ T responder cells were stimulated using OVA peptide and unmodified APC. Mitomycin C treated APCs, surface modified with either TGF-β1 or BSA, were added to the culture and FoxP3^{RFP+} conversion was assessed by FACS analysis after 5 d. Control groups received only APC and peptide. Three ratios of APC^{TGF-β1} to CD4⁺CD62L^{hi} RFP⁻ responder cells were evaluated: 1:2, 1:1, and 2:1. **B)** Summary of generation of viable proliferating T_{reg}, as characterized by FoxP3^{RFP+} cells. Data are representative of 3 independent experiments. Error bar = standard deviation. ***p* < 0.01 ****p* < 0.0001. **C)** Flow cytometry data from one representative experiment, where responder cells were gated for viable CD4⁺ T cells prior to analysis of FoxP3^{RFP} expression and proliferation (via CellTrace). Percentage in upper left corner of each dot plot indicates the percent of proliferating CD4⁺Foxp3^{RFP+} cells.

Figure 7. T_{reg} induced by APC TGF-β1 suppress CD4+ T cell activation

A) Schematic of experiment, where nT_{reg}, freshly isolated from spleens, or iT_{reg}, generated by co-culture with APC TGF-β1, were added to CD4⁺ FoxP3⁻ T responder cells stimulated with α-CD3 in the presence of APC. Proliferation of the responding CD4⁺ FoxP3⁻ T cells was assessed by FACS analysis by tracking proliferation via Cell Trace. Five ratios of CD4⁺ FoxP3⁻ T cells to T_{reg} were used for each T_{reg} source at: 1:16, 1:8, 1:4, 1:2, and 1:1, responder T cell:T_{reg}. **B)** Flow cytometry data from one representative experiment (1:1 ratio), where responder cells were gated for viable CD4⁺ T cells prior to analysis of proliferation via CellTrace. **C)** Summary of suppression of viable proliferating CD4⁺ T cells, as characterized by percentage of divided population. Data are representative of 3 independent experiments.