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Ex vivo Induction of Regulatory T cells from Conventional CD4⁺ T cells is Sensitive to Substrate Rigidity

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Abstract

The immune system maintains a balance between protection and tolerance. Regulatory T cells (Tregs) function as a vital tolerance mechanism in the immune system to suppress effector immune cells. Additionally, Tregs can be utilized as a form of immunotherapy for autoimmune disorders. As T cells have previously been shown to exhibit sensitivity to the rigidity of an activating substrate upon activation via IL-2 secretion, we herein explore the previously unknown effect of substrate rigidity on the induction of Tregs from conventional naïve mouse CD4⁺ T cells. Substrates with modulatable rigidities ranging from a hundred kilopascals to a few megapascals were fabricated via poly(dimethylsiloxane). We found that there was a significant increase in Treg induction at lower substrate rigidities (i.e., E ~ 100 kPa) compared to higher rigidity levels (i.e., E ~ 3 MPa). To confirm that this significant difference in induction rate was truly related to T cell mechanosensing, we administered compound Y-27632 to inhibit myosin contractility. In the presence of Y-27632, the myosin-based contractility was disrupted and, as a result, the difference in Treg induction caused by the substrate rigidity was abrogated. This study demonstrates that mechanosensing is involved in Treg induction and raises questions about the underlying molecular mechanisms involved in this process.

Keywords

Regulatory T cells; Substrate rigidity; Poly(dimethylsiloxane); T cell immunotherapy; Mechanosensing

Introduction

The immune system maintains a balance between protection and self-tolerance. Two major players of the immune system, CD4⁺ helper T cells and CD8⁺ cytotoxic T cells, work together towards protective immunity by identifying target antigens, initiating activation of other immune cells, and killing infected cells. On the contrary, regulatory T cells (Tregs) act as a crucial brake mechanism by suppressing the function of these effector T cells, thus promoting immunologic tolerance. Differentiated Tregs release cytokines such as TGF- β

and IL-10, which work to suppress immune response by limiting T cell proliferation, inflammation, and MHC expression.¹

There are two primary subsets of Tregs: nTregs, native to the thymus, and iTregs, induced in the periphery. Both subsets of Tregs are committed to and maintained in their lineage by the Forkhead-family transcription factor forkhead box P3 (FoxP3). FoxP3 is a master regulator of Treg development, function, and homeostasis, and therefore, is the most reliable marker for identifying both Tregs.² Tregs also express certain distinctive markers including CD4, CD25, and CTLA-4 and require downstream signaling of IL-2 and TGF- β to maintain a Treg phenotype. nTregs arise in the thymus during the final stage of T cell development, whereas iTregs are induced in the periphery in secondary or non-lymphoid tissues from naïve conventional T cells (Tconvs) and play a critical role in preventing inflammatory responses to benign non-self-antigens.³

The importance of Tregs for immune system function is evidenced by several diseases. For example, a mutation in the human gene *FOXP3* can lead to IPEX (immune dysregulation polyendocrinopathy, X-linked) syndrome, a fatal autoimmune disorder,² which causes autoimmune disease, inflammatory bowel disease, and allergies. Additionally, impaired Treg function is associated with a range of diseases including multiple sclerosis⁴ and rheumatoid arthritis (RA).^{5,6} Type 1 diabetes, which can be found in one out of 500 juveniles, is a chronic condition in which pancreatic β cells become incapable of producing insulin due to autoimmune destruction.⁴ Currently, kidney, pancreas, or islet cell transplants are considered the only clinically available cures for type 1 diabetes.

An emerging treatment for autoimmune disorders is adoptive immunotherapy,^{7,8} in which a patient's own Treg cells are leveraged as the therapeutic agent. Central to this process is the *ex vivo* stimulation and proliferation (expansion) of nTregs and the subsequent transfusion of these cells back into the patient. This form of immunotherapy has indeed gained traction over the past few years, starting in 2009, where Trzonkowski and colleagues successfully demonstrated that *ex vivo*-expanded Tregs can be used in humans to alleviate symptoms and reduce pharmacologic immunosuppression against graft-versus-host disease.⁹ Many other trials since then have achieved moderate success in treating autoimmune disorders, but many parameters have yet to be optimized for safety and efficacy to avoid compromising general immunity or blunting responses to infectious agents.^{10,11} An alternative strategy to augment Tregs for adoptive immunotherapy is the *ex vivo* induction of iTregs from conventional T cells (Tconvs, i.e., effector, naïve, and memory T cells).^{12,13} As peripheral blood presents itself as a readily accessible and viable source for conventional T cells, leveraging this population may unlock numerous avenues for adoptive immunotherapy; notably, this method may produce more potent and long-lasting Tregs than via the expansion of nTregs.^{14,15}

The current clinical gold standard for *ex vivo* Treg expansion is the Dynabeads® platform, which is composed of polystyrene microbeads coated with activating antibodies, anti-CD3 and anti-CD28, along with significant amounts of IL-2.¹⁶ However, immune cells *in vivo* experience many different microenvironments with variable rigidities and hydrodynamic forces, and thus modulate their structure and function to account for these different biomechanical cues.¹⁷ Poly(dimethylsiloxane), PDMS, has been used widely to study

mechanosensing characteristics of various cell types^{18–20} due to its versatility and convenience of controlling its rigidity ranging from a few kilopascals to tens of megapascals. Modulation of PDMS rigidity towards a more physiologically relevant level can be achieved by altering the mixing ratios of its base polymer and curing agent, as well as designing various aspect ratios of micro-pillar arrays.^{21,22} To this end, a series of endeavors has been made to improve the efficiency of T cell expansion by mimicking the physiological mechanical microenvironment of the system. Softer materials composed of PDMS or polyacrylamide gels, in which the Young's modulus falls in the 10–500 kPa range, have demonstrated significantly higher T cell activation and cytokine secretion compared to conventional culture substrates such as glass or polystyrene.^{23,24} In addition to the planar substrate format, the microscale beads format of PDMS materials from various manufacturers was tested. It was demonstrated that the number of T cell population doublings increase when activated by softer beads compared to that by rigid Dynabeads® after 19 days.²⁵ A similar result was reported recently with a mesh format.²⁶ These works were followed by many studies on lymphocyte responses to varying substrate rigidities which examine B cell activation, actin dynamics, and microtubule organizations of T cells.^{27–29}

Here, we explore the effect of substrate rigidity on the induction of Tregs from naïve CD4⁺ Tconvs (Fig. 1). We hypothesized that softer planar PDMS substrates would enhance the induction rate of iTregs from Tconvs compared to more rigid PDMS slabs. The information gained from this study will contribute to a better understanding of how Tregs develop in the periphery to maintain tolerance and the mechanosensing properties of T cells, and it may eventually aid in the development of better treatments for autoimmune disorders.

Materials and Methods

Synthesis of PDMS substrates

Poly(dimethylsiloxane) (PDMS) slabs were fabricated via the conventional Sylgard 184 (Dow Corning) formulation, prepared by varying weight ratios of curing agent to elastomer base. Substrate rigidity was modified by altering this ratio, in decreasing rigidity (1:5, 1:10, 1:25, and 1:50). PDMS preparations were poured into tissue culture plates, degassed, and cured for three hours at 60 °C. Cured PDMS slabs in multiwell plates were stored at room temperature for 24 hours and subsequently coated with 3:1 mol/mol mix of anti-mouse CD28ⁱ/CD3ⁱⁱ antibodies for 2 h. Coated PDMS slabs were washed with and then stored in PBS at 4 °C overnight until used the next day.

Young's modulus estimation

The Young's modulus (E) for each corresponding PDMS surface was estimated using an indentation apparatus. Slabs of PDMS made in a 6-well plate with 6 mm thickness were deformed using a flat, 5 mm-diameter cylindrical head, to which a calibrated mass was applied. The deflection formed allowed for an estimation of the Young's Modulus. Young's

ⁱeBioscience, anti-CD28 IgG, clone 37.51

ⁱⁱeBioscience, anti-CD3 IgG, clone 145-2C11

modulus was estimated via the following equation given a measured head diameter d , vertical substrate deflection h , mass m , and Poisson's ratio ν :

$$E = \frac{mg(1 - \nu^2)}{dh}$$

Here, the gravitational acceleration g was assumed to be 9.81 m/s^2 and $\nu = 0.5$. Varying weights were used to cause deflection based on the PDMS sample. For the 1:5, 1:10, and 1:25 samples, a 0.170 kg weight was used. For the 1:50 sample, a 0.020 kg weight was used.

ELISA assay for antibody concentration

In order to determine antibody adsorption levels to ensure equal levels of activating antibody for each sample, an ELISA colorimetric assay with a microplate reader was performed per manufacturer's directions. Briefly, antibodies were coated at varying concentrations at a 3:1 mol/mol ratio of anti-CD28 to anti-CD3, starting at $28 \mu\text{g/mL}$ (100%), and diluted to $21 \mu\text{g/mL}$ (75%), $14 \mu\text{g/mL}$ (50%), and $7 \mu\text{g/mL}$ (25%) for two hours at 37°C . Substrates were then washed and blocked overnight with 4% w/v bovine serum albumin (BSA) in 1x PBS. Horseradish peroxidase (HRP)-conjugated goat anti-hamster IgG was subsequently coated onto the surface as a secondary capture antibody for 30 minutes at the same concentrations. Following this, 3,3',5,5'-tetramethylbenzidine (TMB), the substrate to HRP enzyme, was added. After five minutes, a stop solution was added to end the reaction. The resulting color change at 450 nm was measured to quantify the HRP concentration.

Mouse T Cell Isolation

FoxP3-GFP linked B6 miceⁱ were used for induction of Tregs from $\text{CD4}^+ \text{CD25}^-$ mouse T cells. Naïve mouse CD4^+ T cells were first isolated from spleen and further isolated to CD25^+ and CD25^- cell populations using a mouse Treg isolation kitⁱ. The fluorescence-activated cell sorting (FACS) analysis showed that 98 % of the isolated $\text{CD4}^+ \text{CD25}^-$ cells are turned out to be CD25^{low} and $\text{FoxP3}^{\text{low}}$ (Supplemental Figure 1).

Cell Activation and Induction

For cell activation, the surfaces of a 24-well cell culture plate were coated with a 3:1 mix of anti-CD28/CD3 activating antibodies. The ELISA titration assay showed an equal adsorption of antibodies among samples when the tissue culture plate (TCP) sample was coated at $28 \mu\text{g/mL}$ and the other PDMS samples (1:5, 1:25, 1:50) were coated at $14 \mu\text{g/mL}$. Based on these results, the samples were coated accordingly in order to equalize final antibody adsorption.

Isolated CD25^- cells were then seeded onto the various surfaces and cultured in RPMI 1640 media, supplemented with $20 \mu\text{M}$ L-glutamine, 10 % fetal bovine serum (FBS) and 1 mM HEPES. The complete RPMI media was further supplemented for iTreg induction with 1 mM sodium pyruvate, MEM non-essential amino acids solution, $\text{TGF-}\beta^{\text{ii}}$, and IL-2^{iii} . After

ⁱJackson Farm, six-week-old, stock no: 006772 B6.Cg-Foxp3tm2Tch/J

ⁱMiltenyi Biotec

3 days of induction (37 °C, 5% CO₂), cells were fixed (4% paraformaldehyde, 15 min, room temperature) and subjected to flow cytometry. CD25⁺ cells were fixed immediately after isolation from mice and used as a positive control for FoxP3⁺ Tregs.

Fluorescence-Activated Cell Sorting (FACS) Analysis

FACS^{iv} analysis was performed to determine the percentage of cells expressing FoxP3 by measuring the FITC channel (GFP expression). Cells were capped at 50,000 events and gated based on positive controls (CD25⁺ isolated cells) and negative controls (no antibody or cytokine stimulation). Induction percentages were compared between samples to determine if rigidity had an effect on induction.

Compound Y-27632 as an inhibitor for actomyosin-based contractility

Y-27632, (+)-(R)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamideⁱ, was used to determine if the observed differences in Treg induction were truly related to mechanosensing. At the time of seeding, water-soluble Y-27632 was mixed into supplemented RPMI media as previously described at concentrations of 0, 0.5, and 5 μM. The rest of the procedure was the same as the previous induction procedure; Treg induction proceeded for 3 days and cells were fixed and analyzed by flow cytometry for numerating FoxP3^{high} cells.

Results

Rigidity of PDMS substrates

In order to control PDMS elastomer rigidity, the ratio of curing agent-to-base polymer in the Sylgard 184 formulation was modulated between 1:5, 1:10, 1:25, and 1:50 formulations, resulting in an average bulk Young's modulus of the activating substrates of 3.8 MPa, 3.4 MPa, 1.3 MPa, and 100 kPa, respectively, from five independent experiments, as shown in Fig. 2. Because the 1:10 substrates were found to not significantly differ in rigidity from the 1:5 substrates ($P = 0.07$ in Fig. 2), induction experiments focused on the 1:5, 1:25, and 1:50 substrates.

ELISA assay to equalize antibody adsorption

Initially, the TCP and PDMS substrates of various rigidities were coated with the same concentration of mouse anti-CD3 and anti-CD28 (28 μg/mL of the 3:1 mix of anti-CD28/CD3 activating antibodies). However, the resulting adsorbed antibody concentration on TCP was found to be different from those on PDMS substrates (Supplemental Fig. 2). To ensure the same amount of activating antibody on each substrate, we titrated the concentration of antibody solution with 25, 50, 75 and 100% of full concentration and tested the adsorbed concentration on each substrate again by ELISA. As a result, we chose to coat the TCP sample with 28 μg/mL of the 3:1 mix of anti-CD28/CD3 activating antibodies and the remaining PDMS samples with 14 μg/mL. Fig. 3 shows the results from these adjusted

ⁱⁱPeprotech, recombinant human TGF-β1, 10 ng/mL

ⁱⁱⁱPeprotech, recombinant human IL-2, 10 ng/mL

^{iv}FlowJo v10.0.7

ⁱEMD Milipore, InSolution

coating concentrations and confirms that the adsorbed activating antibodies are not significantly different ($p > 0.05$) among samples based on a one-way ANOVA test.

Treg induction is sensitive to PDMS substrate rigidity

CD4⁺CD25⁻ Tconvs and CD4⁺CD25⁺ Tregs isolated from green-fluorescent protein (GFP)-linked FoxP3 B6 mice were compared in Fig. 4 (a-b). FoxP3 expression is the hallmark of Treg development and function.^{1,2} Isolated CD4⁺CD25⁻ Tconvs did not produce significant signal on the green channel, thus showing that they did not express FoxP3, while approximately 70% of CD4⁺CD25⁺ isolated Tregs were observed to be FoxP3-positive. Note that a purity level of approximately 70% in FoxP3⁺ (i.e., GFP-positive) cells after isolation of CD25⁺ cells (Fig. 4c and Supplemental Fig.1) is not unreasonable due to the nature of the magnetic bead-based positive isolation method relying on CD25 ligands on nTreg cell surfaces, not by FoxP3 expression.

Flow cytometry was subsequently used to quantify the degree of induction in number of cells and FoxP3-GFP expression level. FoxP3-positive cells were gated according to the FITC-channel detection in negative controls (antibody and cytokine-free on 1:25 PDMS) (Fig. 5). Notably, Treg induction levels were found to depend on the PDMS substrate formulation (Fig. 6): a significant increase in induction level was observed between the 1:5 and the 1:25 substrates, average of 29% and 45%, respectively. Also, there was a further increase in induction rate between 1:25 and 1:50 substrates, average of 45% and 56%, respectively ($p = 0.09$, $n = 14$). TCP samples were included in every experiment as a control surface to reconcile any changes in mice, reagents, or media. These samples typically revealed an average of 43% ($\pm 10\%$) Treg induction rate with a standard deviation of approximately 10% across all surfaces. While the trends in induction rate on the varying rigidities were maintained in the seven repeated experiments with duplicated samples, the values of induction rate were observed to fluctuate. Notably, the surface characteristics of TCP are very different from PDMS and thus did not follow the trend shown in PDMS surfaces. Overall, results suggest that induction of Tregs from Tconvs is sensitive to PDMS substrate rigidity ranging from 100 kPa to 5 MPa.

Sensitivity of Treg induction to rigidity was abrogated by Y-27632

Y-27632 is a widely used inhibitor for Rho-kinase family, which acts on its downstream effectors including Rho-associated protein kinases (ROCK-I/II) and myosin light chain kinases (MLCK) and therefore, is known to be directly and indirectly involved in actomyosin-based contractility. We tested Treg induction in the presence of Y-27632 in order to confirm if the Treg induction is truly mechanosensitive. As shown in Figure 7, the induced Treg population on the 1:50 PDMS surfaces after 3-day induction was significantly higher than on the 1:5 surfaces, as indicated in % FoxP3^{high} cells. Upon treatment with Y-27632, the Treg induction rate was found to increase significantly on both 1:5 and 1:50 substrates. Notably, this increase resulted in a similar Treg population on 1:5 and 1:50 surfaces with the 5 μ M Y-27632 treatment. These results indicated that disrupting myosin-based contractility, via treatment with Y-27632, abrogated the sensitivity of T cells to rigidity and, therefore, the difference in Treg induction owing to the substrate rigidity.

Discussion

As a part of the effort to better understand the physiological mechanical microenvironment of the immune system, studies have indicated that stimulating and culturing naïve T cells on softer materials result in significantly higher T cell activation and cytokine secretion compared to on conventional stiff materials such as glass or polystyrene.^{23–26} More importantly, cell proliferation composes a critical aspect of Adoptive Cell Therapy (ACT). It was shown that polyclonal expansion of peripheral blood T cells (naïve CD4⁺ and CD8⁺ T cells) is greater on softer flat PDMS substrates compared to stiff ones²³. Similar results with fiber formats were reported, as well, that higher proliferation was shown with softer fibers (i.e., various mixtures of PDMS and PCL) than stiff fibers (i.e., PCL only)²⁶. Our motivation in this study was to determine if this phenomenon applies to the induction of Tregs from Tconvs, a cell population which is abundant in human blood.

Ex vivo Treg induction is one of the existing strategies to either augment the number of Tregs or to replace impaired Treg cells in patients. Crucial signals required for Treg induction include TCR stimulation,^{30,31} CD28 binding,³² and the presence of soluble cytokines³³ such as TGF- β and IL-2. This is the first study to our knowledge that explores the mechanosensitivity of Treg induction; our results suggest that the induction of Tregs from naïve CD4⁺ Tconvs is indeed sensitive to the rigidity of the activating substrate, as demonstrated via modulating the stiffness of planar PDMS slabs for culture. Specifically, a decrease in the rigidity of PDMS substrates was seen to improve Treg induction. These findings further imply that Tconvs may actively sense mechanical microenvironments and determine whether they would maintain the phenotype and proliferate as Tconvs or differentiate into Tregs. The inhibition of the ROCK activity by Y-27632 promotes phosphorylation of myosin light chain (MLC),^{34,35} and disables myosin contractility. The introduction of Y-27632 effectively abrogated the difference in Treg induction and showed that choosing to proliferate or differentiate is a mechanosensitive process. In this study, Y-27632 was chosen over blebbistatin to inhibit myosin contractility. Although blebbistatin inhibits myosin contraction more directly than Y-27632, it is often used in place of blebbistatin³⁶ when the platform is a PDMS substrate or a PDMS-based microfluidic chip, because hydrophobic small molecules, such as blebbistatin, are very well known to be readily absorbed in PDMS.^{37–39} Further, autofluorescence of blebbistatin overlaps with GFP³⁶, and thus, in our case, with FoxP3 fluorescence. Our results readily open new avenues towards the investigation of potential T cell signaling pathways and how different factors can ultimately determine T cell fate.

However, the results of this report raise several questions. First, we have only analyzed the effect of rigidity on induction rate for PDMS slabs. Our control in this experiment was a standard tissue culture plate, which has Young's modulus of approximately 2–3 GPa.⁴⁰ Interestingly, the Treg induction rate on TCP was similar to that on the 1:25 PDMS formulation, which had a measured Young's modulus of approximately 1.3 MPa. This similarity in induction rate on substrates of different rigidities may be due to completely different physicochemical properties between the two materials such as surface energy, oxygen profiles, and hydrophilicity/hydrophobicity, which will all undoubtedly affect cell adhesion. It is important to note that antibody adsorption levels did not cause the

inconsistency because the final concentration of coated activating antibody was equalized. Observation of cell morphology indicated that cells on PDMS tended to form clusters that grew over time, thus indicating that robust cell stimulation was ongoing. On the other hand, cells cultured on TCP were observed to grow individually and not form clumps (data not shown). It should further be noted that the 100 kPa rigidity of our 1:50 PDMS slabs is much stiffer than physiologically relevant values, and we have used mouse T cells from a FoxP3-GFP transfected mouse strain instead of human T cells for ease of CD25⁺ T cell isolation and quantification of Treg induction level via expression of FoxP3-GFP. In order to better address human need, future work will examine materials that reach single digit kPa and the response of human T cells to these substrates.

Lastly, biological characterization remains. Although we have confirmed that a higher population of Tregs was induced on softer PDMS slabs by measuring the size of the FoxP3^{high} population compared to the entire population, their suppressive capabilities⁴¹ and their stability³⁰ as functional Tregs were not tested. In fact, the maintenance of stability of induced Tregs phenotype *in vivo* under relevant physiological conditions is currently one of the biggest challenges in induced Treg for ACT.^{3,7,30} Higher proliferation levels of Tregs with stronger FoxP3 expression may be induced with longer induction time and with assistance of other reagents such as retinoic acid.¹³ However, we kept the induction conditions as simple as possible in this preliminary study in order to properly identify the role of substrate rigidity in Treg induction.

We have targeted the potential utility of this soft platform to be T cell-based ACT partly because the expanded T cells can be easily detached from the surfaces and infused to patients, leaving the PDMS material behind. Knowing the effect of elastic moduli on improved adhesion and proliferation of various cell types such as fibroblasts, smooth muscle cells and epithelial cells,^{42–44} perhaps, the effect of rigidity demonstrated here might have direct applications in tissue engineering of both immune and non-immune systems. Future studies will focus on optimizing this approach to maximize Treg production using even softer materials that can reach physiological values, as well as application of this concept to cells of human origin, a key step in emerging immunotherapy approaches to autoimmune-based diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Treg induction from Tconvs is sensitive to substrate rigidity. Flat PDMS slabs of various rigidities, ranging from 100 kPa to 3 MPa, were obtained by varying ratios of PDMS curing agent to base polymer. $\text{CD4}^+ \text{CD25}^-$ Tconvs were seeded on the surfaces coated with activating anti-CD3 and CD28 in the presence of IL-2 and TGF- β for 3 days. FoxP3^+ population was significantly higher at 100 kPa surface than 3 MPa substrate.

Figure 2.

The Young's modulus (kPa) of Sylgard activating substrates of varying ratios of curing agent and base polymer two days post-curing. Data was analyzed by two-way ANOVA based on 5 repeated experiments with at least triplicated samples. $P < 0.05$.

Figure 3. Relative antibody concentration absorbed on surfaces quantified by ELISA and represented in absorbance. Antibody coating concentration was modulated to ensure a normalized adsorption to the cell substrates. Data was analyzed by one-way ANOVA based on 2 repeated experiments with at least 4 repeated samples.

Figure 4.

FoxP3-EGFP transfected mouse T cells are shown in FITC channels. FoxP3⁺ cells fluoresce in green. (a) Tconvs: CD4⁺CD25⁻ T cells isolated (b) Tregs: CD4⁺CD25⁺ T cells isolated (c) FoxP3-EGFP intensity comparison per cell type. Dotted line represents the cut-off intensity of FoxP3 high cells.

Figure 5.

FoxP3 expression (FITC) levels three days after Treg induction as measured via flow cytometry. (a) Gating procedure for T cells from PDMS and TCP samples and negative control (+)(-), (b) Treg induction levels as a function of PDMS formulation, TCP, associated negative controls; (+)(-) indicates control with activating antibody coating on 1:25 PDMS but no IL-2 or TGF-b cytokines, (-)(-) indicates control with no antibody coating on 1:25 PDMS and no cytokines, TCP denotes tissue culture plates, and ratios denote Sylgard PDMS substrates with various mixing ratios of curing agent and base polymer by weight.

Figure 6.

Percentage of FoxP3⁺ cells from naïve CD4⁺CD25⁻ T cells after 3 days of induction *ex vivo*. (+)(-) indicates control with activating antibody coating but no IL-2 or TGF- β cytokines on 1:25 PDMS, (-)(-) indicates control with no antibody coating and no cytokines on 1:25 PDMS, TCP denotes tissue culture plates, and ratios denote Sylgard PDMS substrates with various mixing ratios of curing agent and base polymer by weight. Data were analyzed by two-way ANOVA based on 7 repeated experiments with duplicates. $P < 0.05$.

Figure 7.

Effect of compound Y-27632 on Treg induction on different substrates. Y-27632 was administered at concentrations marked above at the time of seeding in RPMI with 1mM sodium pyruvate, 1 $\times$ MEM- NEAA, IL-2 10 ng/mL, and TGF-b 10ng/mL. Data were analyzed by two-way ANOVA based on three repeated experiments with duplicates. $P < 0.05$.