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Augmentation of T-Cell Activation by Oscillatory Forces and Engineered Antigen-Presenting Cells

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Abstract

Activation of T cells by antigen presenting cells (APCs) initiates their proliferation, cytokine production, and killing of infected or cancerous cells. We and others have shown that T-cell receptors require mechanical forces for triggering, and these forces arise during the interaction of T cells with APCs. Efficient activation of T cells in vitro is necessary for clinical applications. In this paper, we studied the impact of combining mechanical, oscillatory movements provided by an orbital shaker with soft, biocompatible, artificial APCs (aAPCs) of various sizes and amounts of antigen. We showed that these aAPCs allow for testing the strength of signal delivered to T cells, and enabled us to confirm that that absolute amounts of antigen engaged by the T cell are more important for activation than the density of antigen. We also found that when our aAPCs interact with T cells in the context of an oscillatory mechanoenvironment, they roughly double antigenic signal strength, compared to conventional, static culture. Combining these effects, our aAPCs significantly outperformed the commonly used Dynabeads. We finally demonstrated that tuning the signal strength down to a submaximal "sweet spot" allows for robust expansion of induced

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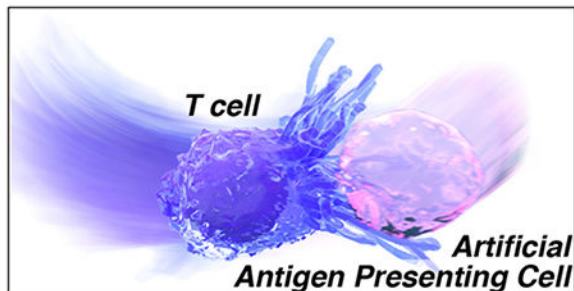[▽] These authors contributed equally.

regulatory T cells. In conclusion, augmenting engineered aAPCs with mechanical forces offers a novel approach for tuning of T-cell activation and differentiation.

Graphical Abstract

Keywords

T cell; alginate; antigen presenting cell; mechanobiology; force; regulatory T cell

INTRODUCTION

T lymphocytes circulate throughout the body and coordinate the immune response against pathogens by recognizing their proteome as foreign. Activation of T cells begins by T-cell receptors (TCRs) engaging with antigenic peptides proffered by the major histocompatibility complex (p-MHC) of antigen presenting cells (APCs). Triggering of the TCR by pMHC requires a mechanical force that pulls on the TCR.[1,2] Those pulling forces are at least partially generated by the T cells themselves through a series of oscillatory pulling movements.[3] We have previously shown that these forces can be provided by an exogenous source, for example, ligands tethered to the cantilever of an atomic force microscope.[3] Those experiments entailed contact with a single T cell at a time and, thus, did not allow for scaling the application of forces toward the large numbers of T cells needed for industrial, clinical purposes. In this paper, we investigated if the provision of exogenous forces in vitro could augment the force-based triggering of TCRs in a large number of T cells. A positive outcome could open the door to biomedical applications of mechanically activated T cells.

Ex vivo cultivation of T cells is important for manufacturing cellular therapies, such as CAR-T cells. Therefore, the optimization of approaches for polyclonal T-cell cultivation is clinically important.[4] In the activation of T cells for biomedical engineering applications, such T cells are commonly cultured with beads coated with stimulatory antibodies, or artificial antigen presenting cells (aAPCs). Many groups have developed aAPCs using microtechnology and nanotechnology approaches, developing particles that can be co-cultured with T cells or engineered surfaces that offer stimulatory signals.[5] In this work, we compared our particles with Dynabeads, which are the most popular, commercial aAPC, comprising a rigid, polystyrene shell roughly the size of resting T cells (4–5 μm diameter) around a superparamagnetic particle, and coated with stimulatory antibodies.

The key to T-cell activation is offering simulation to the TCR, either in the form of pMHC or by using antibodies that trigger the CD3 chains of the TCR complex. Naïve T cells also require costimulation of the CD28 receptor for complete activation, and so antibodies that cross-link and activate CD28 are almost always included in the formulation of aAPCs. The amount of antibodies provided by the aAPC is proportionate to their cost, and so many approaches have attempted to identify and minimize the amount of signal needed. T cells stimulated on a nanopatterned antigen array comprising a planar lipid bilayer showed that the number of antigens on a surface is more important than their surface density, with a density of 90–140 pMHC/μm^2, yielding maximal stimulation.[6] Nanoarrays formed by block copolymer micellar nanolithography (BCML) functionalized with gold nanoparticles found a plateau of maximal response when the distance between antibodies was 60 nm or less.[7] Because surface curvature may also contribute to T-cell activation,[8,9] it is difficult to extrapolate antigen amounts from experiments done on planar surfaces.

To measure the effect of strength of stimulatory signals on T-cell activation, we fabricated aAPC particles and conjugated anti-CD3 and anti-CD28 antibodies at different densities. To test the effect of various degrees of curvature and different surface areas of contact with T cells, our aAPCs comprised spheres of different sizes. To test whether an external mechanical stimulus upon the TCR could promote activation, we also engaged an oscillatory movement to the aAPCs. Overall, we found conditions that dramatically improved activation beyond conventional, Dynabead-based stimulation. In another example, we chose aAPC conditions that offered a "sweet spot" of signaling to maximize the production of induced regulatory T cells (iTreg), the development of which is actually hindered by high levels of stimulation. The particles were also endowed with the ability to secrete cytokines to further promote iTreg development. These examples show that the provision of an oscillatory mechanoenvironment coupled with aAPCs of tunable size/curvature, signal density, and cytokine section offer the possibility to fruitfully engineer T cells for a variety of clinical and experimental needs.

DESIGN CONSIDERATIONS

An important design consideration was to endow the particles with the ability to be easily separated from cells during modification, washing steps, and especially after co-culture. Our solution to this problem was to make the beads magnetic by embedding superparamagnetic iron oxide nanoparticles (SPIONs) during their microfluidic synthesis. The processing conditions were tuned to encapsulate ~2.8 ± 0.3 vol % SPIONs per particle (see Table S1 in the Supporting Information), which was identified in pilot work to be sufficient for magnetic separation.

Our polymeric, microparticle aAPCs are composed of alginate, which is ionically cross-linked by the addition of divalent cations such as calcium. Ionic cross-linking of alginate provides sufficient working stability; however, we saw irreversible changes (hysteresis) in particle morphology during pilot magnetic separation and traction experiments. To overcome this issue, we used our recently developed combinatorial cross-linking technique which provided both chemical and physical cross-linking.[10]

During our experiments, we adjusted the number of aAPCs to provide a constant activation surface area of 9.5×10^7 μm^2 per 1.5×10^6 T cells (see Table S2 in the Supporting Information). This calculation was equivalent to the surface area of Dynabeads in co-culturing one Dynabead per one primary T cell, the manufacturer's recommendation. This adjustment allowed us to then appropriately consider the impacts of particle size and antigen density on T-cell activation and also compare our aAPCs to the conventional approach.

RESULTS AND DISCUSSION

To develop our artificial APCs, we utilized a microfluidic droplet generator that encapsulates alginate polymer and magnetic nanoparticles (Figure 1A). A constant fraction of cross-linker (4-arm PEG hydrazide) was mixed with the alginate polymer in the main channel. During droplet formation, we encapsulated within our beads magnetic nanoparticles that were 100-nm-diameter, carboxylated SPIONs. The microfluidic approach produced a homogeneous collection of particles, as verified by dynamic light scattering (Figure 1C). To tune the size of particles ranging from 150 nm to 10 μm, we varied the ratio of central to sheath flow (Figure 1B). To employ a broad range of particle sizes in our experiments (over one order of magnitude), we selected three sizes of particles with average radii of 307 nm ("0.3"), 824 nm ("0.8"), and 4540 nm ("4.5 μm") (Figure 1C). In pilot work, we found that nanoparticles of diameters in the range 50–250 nm could not be fully separated from each other or from T cells due to partial internalization or trapping on the rough cell surface, whereas particles larger than 300 nm could be separated from cells with >95% efficiency (not shown). The resulting alginate particles were then collected in a bath of 200 mM $CaCl_2$, followed by a ~40 min incubation to reach complete gelation. Eventually particles were subjected to overnight chemical cross-linking through the hydrazine linker. Excess calcium and cross-linker were removed by serial washing with phosphate buffered saline. The mechanical stiffness of these aAPC microparticles was measured by nanoindentation and found to be 14.6 kPa (Figure 1D), a substrate stiffness that allowed for maximal spreading of T cells.[11]

We next conjugated stimulatory antibodies to the surface of the microparticles. The carboxylic groups of alginate provide a versatile platform for antibody conjugation. Using NHS/EDC chemistry, we conjugated anti-CD3 and anti-CD28 antibodies (see the Materials and Methods section in the Supporting Information) and washed away excess antibodies and quenching unreacted groups through repeated washing with phosphate buffered saline solution containing 0.5% w/v BSA. To characterize the conjugation of antibodies, we imaged them by confocal microscopy and found that over 80% of antibodies were conjugated to the outside the particles (see Figures S1A–S1E in the Supporting Information). Based on pilot experiments, we chose three different densities of antibodies, representing 10-fold dilutions, to coat beads representing high ("1"), medium ("0.1"), and low ("0.01") amounts of antigenic signaling. An average of 2692 ± 420, 266 ± 41, and 33 ± 7 antibody molecules per square micrometer were immobilized as high, medium, and low conjugation densities, respectively (Figure 1F). For comparison, a theoretical limit of ~12 732 antibodies could be packed into a square micrometer, assuming that an antibody has a radius of ~5 nm.[12] Thus, what we call a "high" labeling density in this Letter corresponds to ~21% of the theoretical limit. The size of the interface between T cells and antigen presenting cells (Figure 1E) varies based on cytoskeletal state of the T cell,[13] with most

contacts falling in the range of 5–25 μm^2. If one assumes an area of 10 μm^2 for a typical immune synapse, the large particles (2.25 μm radius) would offer a hemispheric area of ~32 μm^2, so that an immune synapse-sized 10 μm^2 would engage ~$^1/_3$ of the hemisphere and would engage ~22 000 antibodies (high density conjugation), 2192 antibodies (medium), or 251 antibodies (low). For the medium-sized particles (0.3 μm radius), a hemispheric immune synapse offers an area of ~1 μm^2 and 2785, 291, and 36 molecules, at these respective conjugation densities (high, medium, low). For the smallest particles (0.15 μm radius), a hemispheric immune synapse would engage 433, 41, and 5 molecules, respectively. The experimentally observed minimum amount of signaling needed to activate a T cell ranges from 1 to 4 engaged TCRs.[14,15] Thus, in all cases, the number of T-cell receptors our particles can engage in the immune synapse should be exceed that minimum.

To assess the impact of external, gentle mechanical stimulation on co-cultures of T cells with aAPCs, we used an orbital shaker to deliver a continuous oscillatory movement of either ~250 rpm rotational speed ("dynamic") or switched off ("static") (Figure 2A). The impact of the aAPCs on T cells were compared with that of the popular, commercially available CD3/CD28 T-cell expansion beads (Dynabeads from Life Technologies). Primary T cells were obtained from spleens of wild-type mice, enriched by negative magnetic-bead selection, and cultured with aAPCs under either static or dynamic conditions.

By day 2 or 3 of culture, polyclonal, primary mouse T cells formed large clusters with the beads. The clusters were obviously larger in the dynamic culture than in static culture (Figure 2B). The T cells were separated from the beads and imaged by 3D confocal microscopy to assess their growth. Cell volume was quantified since the volume changes as a cell grows, proliferates, or differentiates. Cell volume can also change in response to external physical cues.[16] We found that cell growth was larger in dynamic culture versus static culture across all particle sizes and conjugation densities (Figure 2C; statistical comparisons are given in Figure S2A in the Supporting Information). The average volume of T cells co-cultured with Dynabeads under static conditions ($n = 22$) was 321 ± 26 μm^3 (mean, ±95% CI), which represents an average diameter of 8.5 ± 0.34 μm. The largest cells were those resulting from co-culture with 4.5 μm particles at the highest density of ligands, which had average volume of 580 ± 74 μm^3, corresponding to a diameter of 10.3 ± 0.82 μm ($n = 25$).

To assess their proliferative response, we counted T cells after 3 days of co-culture with the various particles. Under all conditions, dynamic culture resulted in significantly higher expansion of T cells than static culture (Figure 2D; statistical comparisons are given in Figure S2B in the Supporting Information). The average fold expansion of T cells co-cultured with Dynabeads under static conditions ($n = 3$) was (5 ± 1.8)-fold (mean, ±95% CI). T cells proliferated much more in culture with our mechanically soft particles of the same size and antibody loading as Dynabeads than with Dynabeads, suggesting that the softer mechanics of our aAPC offers an additional stimulus for activation and proliferation ((8.6 ± 1.8)-fold expansion, $p = 0.006$, compared to static Dynabeads). The largest expansion of T-cell count was observed under conditions where T cells were cultured in oscillating conditions with our 4.5 μm particles at the high density of stimulatory antibodies, resulting in an increase of 12.5 ± 1.2 fold ($p = 0.004$, compared to static Dynabeads).

Averaging across all particle sizes and antigen doses, mechanical oscillation increased the proliferation of the cells by 2.0-fold, compared to static culture (ANOVA considering movement, size, and dose; movement $p = 1.5 \times 10^{-12}$).

Generally, cytotoxic CD8+ T cells have a higher proliferative capacity than CD4+ T cells. Cytotoxic T cells have important applications in engineered cancer immunotherapies. We assessed the ability of these particles to promote cytotoxic T-cell expansion by monitoring the CD8-to-CD4 T-cell ratio during proliferation. We separately purified CD4+ T cells and CD8+ T cells from mice, then mixed them to achieve the physiological ratio of one CD8+ T cell to two CD4+ T cells. We co-cultured T cells with particles as above, and, after 5 days, we measured the ratio of CD8 to CD4 T cells by flow cytometry (Figure 2E; statistical comparisons are given in Figure S2C in the Supporting Information). The average CD8-to-CD4 ratio of T cells co-cultured with Dynabeads under static conditions ($n = 3$) was 2.75 ± 1.5 (mean, ±95% CI). The largest increase in the cellular ratio was observed in the condition where T cells were cultured with 4.5 μm particles with the highest density of ligands, resulting in a CD8-to-CD4 ratio of 30.1 ± 9.8 ($p = 0.005$, compared to static Dynabeads). Averaging across all particle sizes and antigen doses, mechanical oscillation increased the CD8-to-CD4 ratio of the cells 2.1-fold, compared to static culture (ANOVA considering movement, size, and dose; movement $p < 10^{-16}$).

We noted that the larger particles resulted in more expansion, and especially CD8 expansion, of the T cells than the smaller particles, even though the density of antibodies across the beads of different sizes was almost identical (Figure 1E) (ANOVA considering size $p < 2 \times 10^{-16}$). This result suggests that the immune synapse integrates the aggregate number of molecular signals across the interface, rather than the density of antigenic ligands.

We further examined the proliferative responses of T cells upon stimulation with our aAPCs by using a dye-dilution approach to follow the proliferation pattern. Sequential generations of daughter cells result in roughly 2-fold dilution of the fluorescent signal (Figure 3A). The percentage of T cells that underwent proliferation when co-cultured with Dynabeads under static conditions ($n = 3$) was 91.0 ± 5.8% (mean, ±95% CI) (Figure 3B; statistical comparisons are given in Figure S3A in the Supporting Information). The maximum proliferation was observed under the condition where T cells were cultured with 4.5 μm particles with dynamic oscillations at the highest density of antibodies, resulting in proliferation of 98.8 ± 1.9% ($p = 0.005$ compared with static Dynabeads). Averaging across all particle sizes and antigen doses, mechanical oscillation increased the percentage of T cells that underwent proliferation by 1.72-fold, compared to static culture (ANOVA considering movement, size, and dose; movement $p = 0.011$). To measure not just whether the cells divided but also how many times they divided, we also calculated the division index, which is defined as the average number of cell divisions that a T cell in the original population underwent (the average includes cells that never divided at all) (see Figure S3B in the Supporting Information). Because not all cells proliferated, we also compared the proliferation index, which is defined as the average number of divisions for just the responding population (Figure S3C in the Supporting Information). These show that the maximum number of divisions was observed under the condition where T cells were dynamically cultured with 4.5 μm particles at the highest density of antibodies.

We also examined expression of T-cell activation markers CD25 and CD44 by flow cytometry after activation and found that expression of these markers trended similarly to proliferation (Figures 3D–G; statistical comparisons are given in Figures S4A and S4B in the Supporting Information). As with absolute expansion, activation and proliferation were greater for larger beads than smaller beads, even when antibody density is held constant. Together, these results show that activation and proliferation are proportional to the amount of antigen rather than its density.

We showed in prior work that the size of the immune synapse in naive versus effector (recently activated) T cells controls the amount of signal they accumulate in their interactions with APCs.[13] We sought to test the coupling of signal accumulation and synapse size in a reductionist manner. After OT-II T cells were activated with aAPC particles, as above, for 24 h, we purified away the stimulatory particles and co-cultured them with the B-cell lymphoma line LB27.4 that was loaded with ovalbumin peptide antigen. We measured the average volume of immune synapses formed between T cells activated under different culture conditions and B cells based on the accumulation of the integrin leukocyte function-associated antigen 1 (LFA-1) measured by the volume of positive pixels (Figure 4A). The average synapse size for T cells co-cultured with the 4.5 μm particles at high levels of stimulatory antibodies under static conditions ($n = 3$) was 32.8 ± 4.1 μm^2 (Figure 4B). The maximum synapse size was observed under the conditions where T cells were cultured with 4.5 μm particles with the high level of antibodies under mechanical oscillation conditions ($n = 3$) 36.7 ± 4.1 μm^2 ($p = 0.04$, compared to static). Averaging across all particle sizes and antigen doses, mechanical oscillation increased the size of synapses by 1.3-fold, compared to static culture (ANOVA considering movement, size, and dose; movement $p = 2 \times 10^{-7}$). These results show that the size of the immune synapse is larger when the cells are more activated. Our previously published result compared the size of the synapses for naïve T cells versus effector T cells (lymphoblasts after 3–5 days of culture),[13] and showed that activation of cofilin in effector T cells enabled changes to the cytoskeleton that allowed for larger synapses and a lower threshold of activation than naïve T cells. In other words, the threshold of activation was determined by the size of the synapse and thus the dynamic ability of the cytoskeleton to rearrange when in contact with an APC. The results here go further: we demonstrate here that the dynamic size of the synapse is not just binary, but rather gradated based on signal strength.

Activating T cells with high signal strength allows for massive expansion, which is needed for transducing chimeric antigen receptors (CARs) and having sufficient transduced cells for a therapeutic dose. The opposite problem arises when expanding T cells in culture for the purposes of generating engineered regulatory T cells. In vivo, regulatory T cells can be elicited to foreign antigens when they are provided at low levels, rather than at high signal strength.[17] Induction of regulatory T cells is improved by provision of TGF-β and IL-2.[18] We recently demonstrated that alginate microparticles could be loaded with cytokines to skew T cells to iTregs,[19] which served as a motivation to combine that capability with the tunable signal strength of the system presented here. To evaluate the effect of orbital shaking and antigen strength on iTreg formation, we loaded Alg-Hep particles with TGF-β and IL-2. We demonstrated the release of these factors from the particles over time (Figure S5 in the Supporting Information). We sorted rigorously naïve CD4+ T cells to eliminate natural

regulatory T cells, and then co-cultured these cells with our microparticles. Dynabeads were used for comparison, providing an equivalent amount of soluble TGF-β in the media.

We assessed the development of Tregs by intracellular staining for the key transcription factor Foxp3, followed by flow cytometry. The mean fluorescence intensity of the Foxp3 transcript correlates to their regulatory ability,[20] and so Foxp3 expression level was measured as well.

The percentage of iTregs induced in culture with Dynabeads plus TGF-β under static conditions ($n = 3$) was 25.7 ± 10.1% (mean, ±95% CI) (see Figure 5A). The maximum iTreg induction was observed under the condition where T cells were statically cultured with 4.5 μm particles at the lowest density of antibodies (0.01), resulting in iTreg induction of 71.6 ± 9.6%, almost 3-fold higher ($p = 0.005$, compared to static Dynabeads). Generally, averaging across all particle sizes and antigen doses, mechanical oscillation did not alter the rate of iTreg induction at all (1.02-fold difference). Curiously, the lowest amounts of signal strength, as seen in proliferation and activation assays above, were not able to elicit high yields of iTreg induction. In fact, a "sweet spot" of signal was needed, either in the form of larger beads with lower antibody coating or smaller beads with higher antibody coating amounts (Figure S6A in the Supporting Information). The "sweet spot" fell where all three particle sizes could be compared, that is, had comparable numbers of antibodies interacting with T cells (i.e., the largest particles with the lowest antigen density, the medium particles with medium antigen density, and the smallest particles with the highest antigen density). In other words, even as multiple small (300 nm diameter) particles interacted with a single T cell, and offered a comparable antigen amount as a larger particle, the generation of Tregs still favored the situation with the larger particle, i.e., the lower curvature. Thus, in situations where antigen amounts are comparable, larger aAPCs/lower curvature are favored over smaller ones/higher curvature.

The expression of Foxp3 on a per-cell basis was highest under the conditions that elicited the highest induction of iTregs (Figure 5B), and again showed a "sweet spot" of signal strength (Figure S6B in the Supporting Information). These results showed that larger immune synapses with low antigen amounts resulted in the highest induction of Tregs.

We examined the stability of the Foxp3 protein expression, because transient expressions of Foxp3 do not yield highly suppressive regulatory T cells.[17] The T cells generated through culture with the 4.5 μm particles were separated from the particles and then were maintained in culture with IL-2. We assessed for the expression of Foxp3 by flow cytometry at day 4 and day 8 of culture (Figure 5C). We found that the expression of Foxp3 was dramatically reduced under the conditions where the induction was highest, by the 4.5 μm particles that offered the lowest antigen signal (0.01). Dynamic culture mitigated the loss of Foxp3 expression modestly (29.9% ± 13.1% decrease in dynamic culture versus 37.6% ± 17.7% in static culture). In contrast, the antigenic strengths that were moderate (0.1) and highest (1) had the most stability in culture (4%–5% diminishment). Overall, at day 8, the highest expression of Foxp3 was still seen in the co-culture conditions with 4.5 μm particles that offered the lowest antigen signal (0.01), 34 ± 8.8% ($n = 3$).

The ultimate in vitro test of regulatory T-cell function is assessed by their ability to suppress the effector responses of conventional, activated T cells. We co-cultured iTregs induced under a variety of conditions above with conventional T cells at a cellular ratio of 0, 1, 10, and 30 CFSE-labeled conventional, naïve T cells to one iTreg and stimulated with anti-CD3 and anti-CD28. Proliferation of the naïve T cells was assessed without Tregs, and the percentage inhibition was measured by subtracting the proliferation as seen when Tregs were co-cultured. We found that maximal inhibition—and, thus, maximal regulatory function—was enacted by the iTregs that were generated in the "sweet spot" condition of culture with 4.5 μm particles at medium levels of activating antibodies (0.1) (Figure 5D). Together, these results show that the activation and generation of regulatory T cells can be optimized by culturing with particles of large size but low to medium antigenic strength, resulting in the highest stability of regulatory T cells and the most inhibition of effector T-cell proliferation.

CONCLUSION

T cells form an interface with antigen presenting cells (APCs) called the "immune synapse" that allows for triggering of TCRs by pMHCs. Artificial antigen presenting cells (aAPCs) can be fabricated to emulate this response and allow for productive expansion of T cells in vitro. By manipulating the size and density of stimulatory signals on aAPCs, we can learn about what is required in the TCR-pMHC interface to reach the threshold for T-cell activation and thus improve cultivation of engineered T-cell therapies. No other work, to our knowledge, has harnessed advanced materials to provide mechanically soft artificial antigen presenting cells combined with mechanical stimulation as we did here.

Orbital shaking provides a mixture of mechanical cues, including oscillatory forces on the immune synapse, stirring of the media to promote T-aAPC engagement, and potentially even shear forces upon the T cells. We showed, in prior work, that the oscillatory mechanical forces needed to trigger the TCR are in the piconewton range.[1,2] In their engagement with APCs, T cells generate these forces themselves through actindependent pushing and myosin-dependent pulling.[3] The cellular pushing forces occur first, last for ~1 min, and through cofilin-mediated cleavage of actin filaments promote the rapid expansion of the lamellipodium to engage the APC.[11,13,21,22] T cells then pull against the engaged TCR-pMHC bonds, generating the mechanically induced conformational changes of the TCR and initiation of the biochemical cascade that represents triggering of the TCR.[23,24] By providing exogenous mechanical forces here by an orbital shaker, we showed that T-cell triggering can be amplified above and beyond their own capabilities. Specifically, we showed overall that gentle mechanical stimulation of the TCR offers an ~2-fold increase in signal strength to T cells, compared to conventional static cultures.

Other work regarding the use of aAPCs has demonstrated an ability to optimize T-cell signaling and proliferation, akin to the work presented in the first part of this paper. AAPCs fabricated from biomimetic scaffolds—namely, mesoporous silica nano-rods coated with a lipid bilayers—allowed for maximizing signal strength and an allowed for impressive expansion of T cells in vitro,[25] but did not allow for obviously tuning down the signal as we did here. Another aAPC system was demonstrated recently with the conjugation of pMHC

onto the surface of yeast cells, and this work revealed that density of pMHC was correlated to activation of T cells.[26] Conversely, our work here and other reductionist approaches[15] demonstrated that absolute amount of antigenic signal, rather than density, is more important. Nano-aAPCs 100 nm in diameter, coated with anti-CD3 and CD28, were able to elicit modest proliferation in vitro but in the in vivo context could traffic down lymphatics to draining lymph nodes more readily than micrometer-scale particles.[27] Recent work with aAPCs comprising polyisocyanopeptide polymers 100–1000 nm long, functionalized with anti-CD3 and IL-2, showed that closer spacing of the anti-CD3 and IL-2 contributed to T-cell activation.[28] These aAPCs together reveal that tuning the amount of antigenic signals and their spatial separations has utility and offers an advantage over biological antigen presenting cells when developing engineering solutions to scaling the production of T cells for therapeutic purposes.

Others have shown that the mechanical stiffness of the surface where antigens or antibodies are tethered has a significant impact on the proliferation and effector responses of T cells. In response to polyacrylamide (PA) surfaces of different stiffness coated with anti-CD3 antibodies, T cells appear to activate strongly in the context of stiff PA surfaces, rather than very soft ones,[29] and demonstrate peak spreading on soft surfaces of ~20 kPa.[11] Our work utilized mechanically soft particles (alginate) as well as extremely stiff, commercially available ones (Dynabeads) and found that culture with softer particles could actually outperform stiffer ones, although the stiffness axes was not directly probed in our work and could be explored in future work, because alginate is quite tunable for mechanical stiffness. [19] The ability of conventional Dynabeads or stiff microparticles to serve as a source of cytokines is inherently limited, as shown by recent work demonstrating that IL-2 activity is abrogated when immobilized on very rigid aAPCs.[28] Our aAPCs, on the other hand, demonstrated the ability to enhance regulatory T-cell development through gradual secretion of IL-2 and TGF-β. In summary, we demonstrate many advantages of utilizing the advanced material properties of tunable, cytokine-secreting microparticle aAPCs to both control the activation and engineer the differentiation of T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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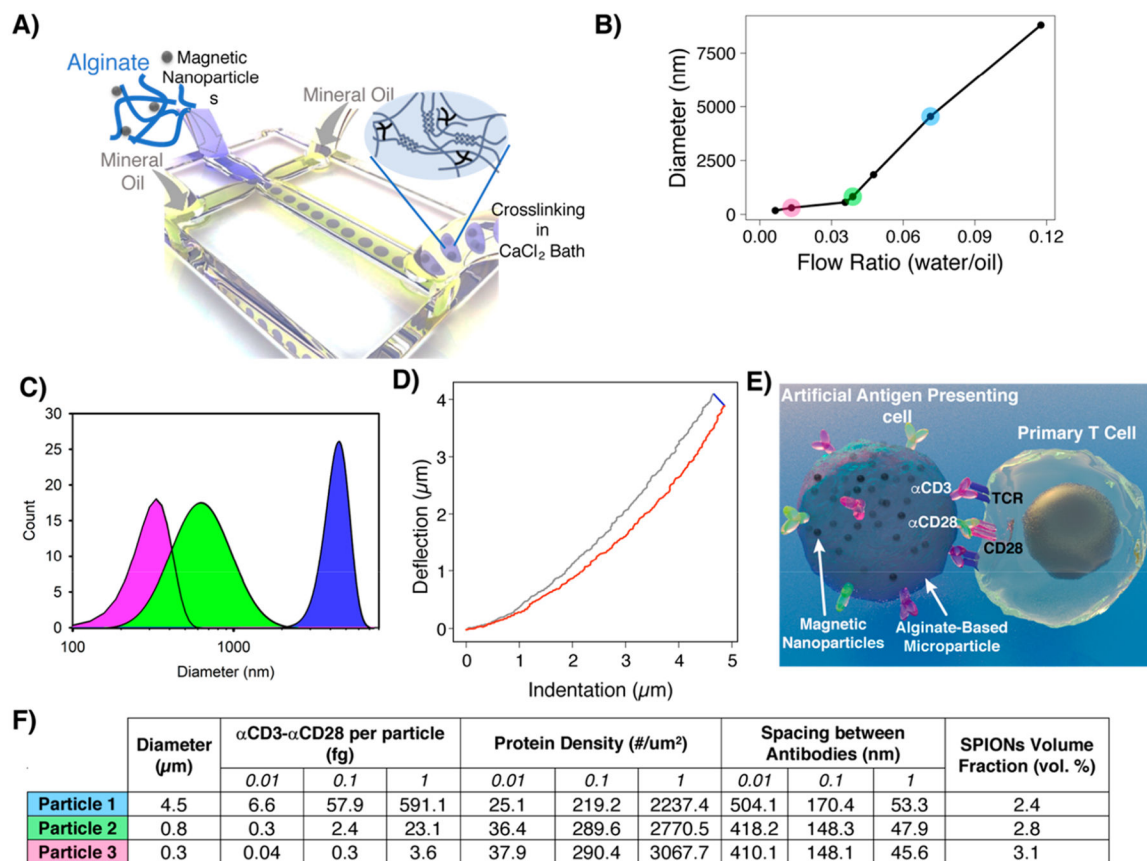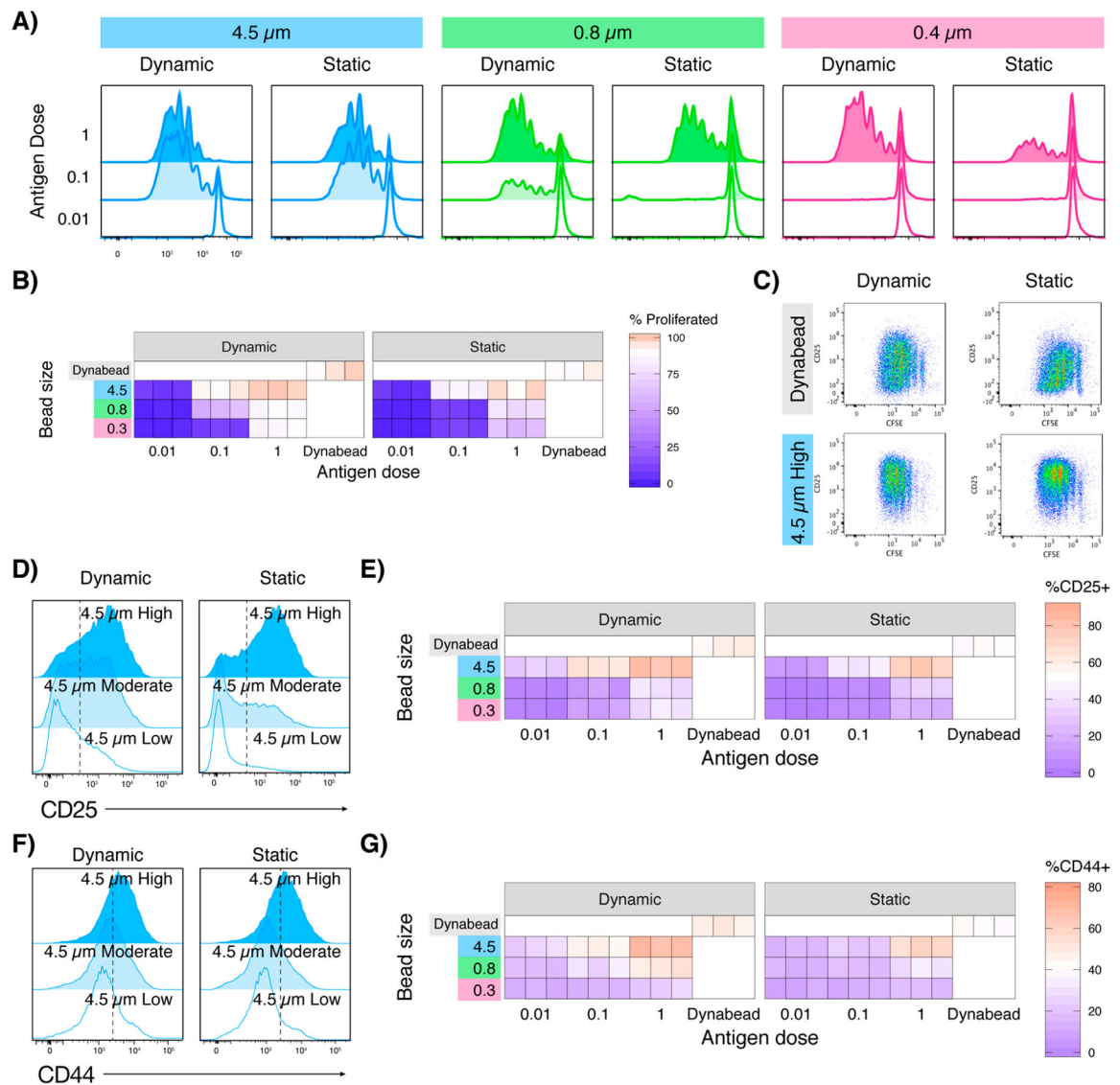
Figure 1.

(A) Schematic representation of microfluidic generation of alginate nano/microparticles encapsulating magnetic nanoparticles. (B) Size distribution analysis of prepared microparticles at different flow rates. (C) Size distribution analysis of selected particles (0.3, 0.8, 4.5 μm). (D) Nanoindentation to assess the elastic modulus (red, indentation; gray, retraction). (E) Schematic representation of proposed interactions between artificial antigen presenting cells and primary T cells. (F) Summary of physical characteristics of prepared library of particles to present broad range of antigens on their surfaces.
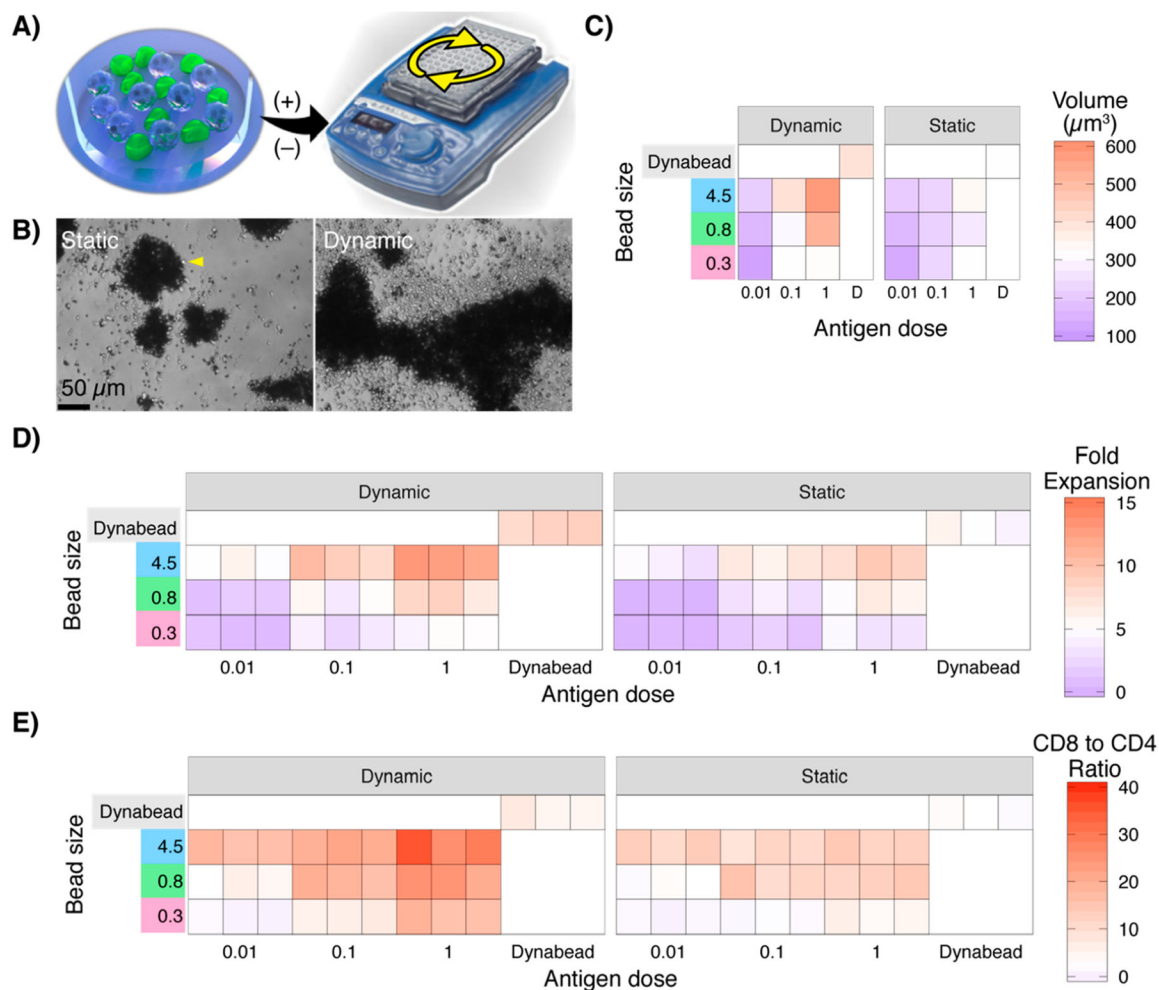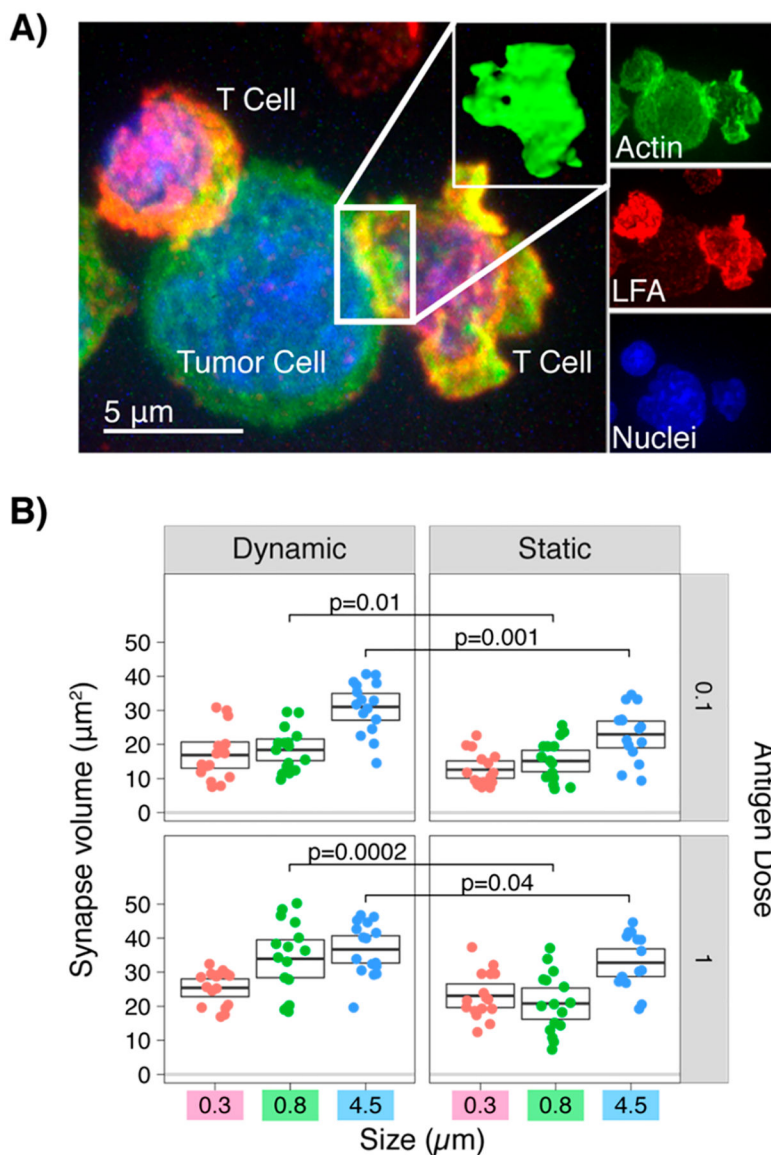
Figure 2.
(A) Particles and T cells were co-cultured under static and dynamic conditions. (B) Representative bright-field microscopy images of formed clusters by primary mouse T cells cultured with 4.5 μm aAPCs at a constant dose (1:1 particle/T-cell ratio) under static and dynamic (mechanical oscillation) cultures. Scale bars = 50 μm. (C) The mean volumes of T cells activated and expanded using various formulations of particles, as indicated. (D) Expansion of primary mouse CD4+ T cells by varying the antigen dose, particle size or the culture conditions after 4 days. (E) FACS quantification of CD8-to-CD4 ratio of T cells cultured with varying formulations of particles, compared to Dynabeads. The starting ratio for all conditions was 0.5.
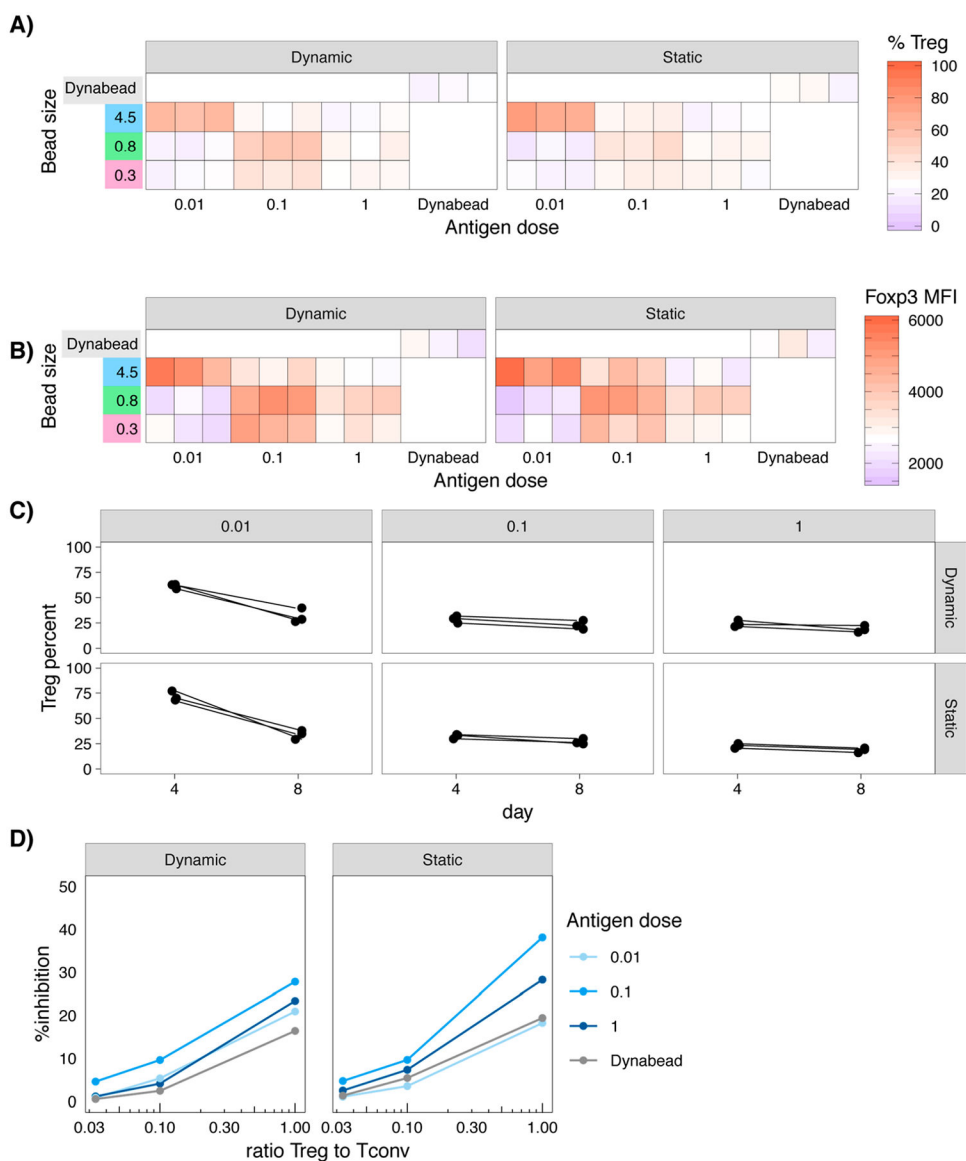
Figure 3.
Proliferation and activation analyses of CD4+ T cells cultured under static or dynamic conditions in the presence of varying formulation of particles. (A) Flow cytometry histograms CFSE dilution and (B) percentage of proliferated T cells 3 days after co-culturing with different formulation of engineered particles. (C, D) CD25 expression histograms after 24 h of co-culturing of primary naïve CD4+ T cells with 5 μm microparticles, presenting various surface densities of antibodies under static or dynamic culture. (E) Percentage of CD25+ T cells 24 h after activation with various formulation of particles or Dynabeads. (F) CD44 expression histograms after 24 h of co-culturing of primary naïve CD4+ T cells with 5 um microparticles presenting various surface densities of antibodies under static or dynamic culture. (G) Percentage of CD44+ T cells 24 h after activation with various formulation of particles or Dynabeads.

Figure 4.
(A) Immune synapses formed by OT-II T cells activated with 4.5 μm (1) microparticles interacting with (antigen-pulsed) antigen presenting cells (B lymphoma cells) were imaged by confocal microscopy. Images show overlap of confocal slices. Representative cells that had the median immune synapse volume were chosen. (B) Analysis of immune synapse volumes (in μm^3) formed by primary naïve T cells activated with various particle sizes with high or low antibody conjugation level cultured under static or dynamic conditions. Each dot represents an immune synapse between a T cell and an antigen presenting cell ($n = 16$ per condition). Boxes show means and 95% CI values. Results are representative of three independent experiments.

Figure 5.
Flow cytometric analysis of iTreg development was assessed by flow cytometry for Foxp3 and CD25 coexpression after co-culture of naïve CD4+ T-cells with particles at various formulations either under dynamic or static conditions for 4 d. (A) Percentage of induced Tregs and (B) mean fluorescence intensity (MFI) of Foxp3 expression in T cells 4 d after activation with various formulation of particles or Dynabeads. (C) Stability of formed T-regs as assessed by measuring the change in the population of iTregs (T cells expressing CD4, CD25, and Foxp3+) after 4 and 8 days. (D) T-cell suppression assay. Flow sorted Tregs cocultured with naïve primary CD4+ T-cells (Tconv) at three different ratios of cell counts (1:1, 1:10, and 1:30 of Treg to Tconv) in the presence of surface-coated anti-CD3 and soluble anti-CD28 for 3 d.