Engineering advanced dynamic biomaterials to optimize adoptive *T*-cell immunotherapy

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ABSTRACT

Adoptive *T*-cell therapy (ACT) is a promising therapeutic approach based on the concept of potent *T*-cell mediated immunity against the tumor. The outcome of antigen-specific *T*-cells responses relies on the interaction between *T*-cells and antigen-presenting cells, which provides signals for generating different *T*-cell phenotypes with different roles in tumor removal. However, such interaction is often not optimal *in vivo* and results in low therapeutic efficacy. To reach the full potential of the *T*-cell response, current research put effort into developing dynamic biomaterials as artificial antigen-presenting cells to study and regulate the *T*-cell activity for controlling *T*-cell fate. In this perspective, we provide (1) an overview of ACT and general *T*-cells behaviors, (2) explore the insight on how biomaterials can be used for studying and regulating *T*-cell behaviors, (3) and discuss conceptual gaps in knowledge for biomaterials-based immunotherapy.

1. Introduction

1.1 Overview of adoptive T-cell immunotherapy

Despite the advance in cancer therapeutics (e.g., surgery, chemotherapy, and radiotherapy) for improving the survival rate of cancer patients, the adverse effects of these therapeutics and tumor recurrence remain significant challenges in clinical practice [1–4]. Adoptive *T*-cell immunotherapy (ACT) is an emerging and promising strategy by leveraging tumor-specific immune cells, with a special emphasis on autologous cytotoxic *T*-cells derived from patients with cancers since the 1990s that shows theoretically low side-effect. Typically, tumor-infiltrating *T*-cells can be isolated from the surgery removed tumor for expansion (on 10^{11} scale) *in vitro* (Fig. 1a). Upon infusion of the autologous *T*-cells, they can recognize and attack the specific tumor cells [5–8]. To personalize the course of treatments, the gene encoding mutation-derived tumor antigens can be identified *via* genomic analysis, and the antigens are introduced into antigen-presenting cells (APCs), such as dendritic cells (DCs, Fig. 1b) [8]. The APCs can also activate the bystander *T*-cells into tumor-specific *T*-cells with additional expansive capacity during co-culture *in vitro*. Subsequently, these activated *T*-cells can be an additional source for the ACT. To meet the practical need of ACT, a short, simplified, and efficient strategy for activating and expanding *T*-cells *in vitro* is critical to maximizing the anti-tumor *T*-cell responses

In principle, ACT relies on the autologous or allogeneic isolation, activation and expansion *ex vivo*, and transfer of antigen-specific *T*-cells *in vivo*. Each of these steps requires an optimized condition for the highest outcome of the therapy [7]. In particular, *T*-cell activation involves three primary signals: (1) *T*-cell stimulation through the engagement between its *T*-cell receptor (TCR) and a tumor epitope presented by major histocompatibility complex (pMHC) or leukocyte antigen (HLA) in human on APC surface, or unspecifically through activating anti-CD3 Fab' (*α*CD3 or UCHT1); (2) co-stimulatory signal *via* the binding between CD28 and CD80/86 expressed APCs or anti-CD28 Fab' (*α*CD28) that crucial for triggering *T*-cell proliferation; (3) the presence of essential cytokines (e.g., interleukin-7 (IL-7) and IL-15 for memory *T*-cells survival and proliferation, respectively) for directing *T*-cell differentiation and sustaining proliferation (Fig. 2a) [5,6,10–12]. Taken together, these signals can decide the outcome of a targeted *T*-cell response.

Conventional *T*-cell expansion strategies involving cytokine cocktail relying on signal (3) can be an approach to expand the extracted *T*-cells but are costly and challenging [14]. Thus, recent studies have been optimizing signals (1,2), which significantly depend on the antigen affinity and sensitivity of *T*-cells against tumor cells. For instance, immobilized pMHC monomers on APCs or immobilized *α*CD3 and *α*CD28 on artificial APCs (aAPCs) surface (two-dimensional or three-dimensional) rather than the soluble forms can significantly activate *T*-cells (Fig. 2b) [15]. Chimeric antigen receptor *T*-cells (CAR *T*-cells) have been engi-
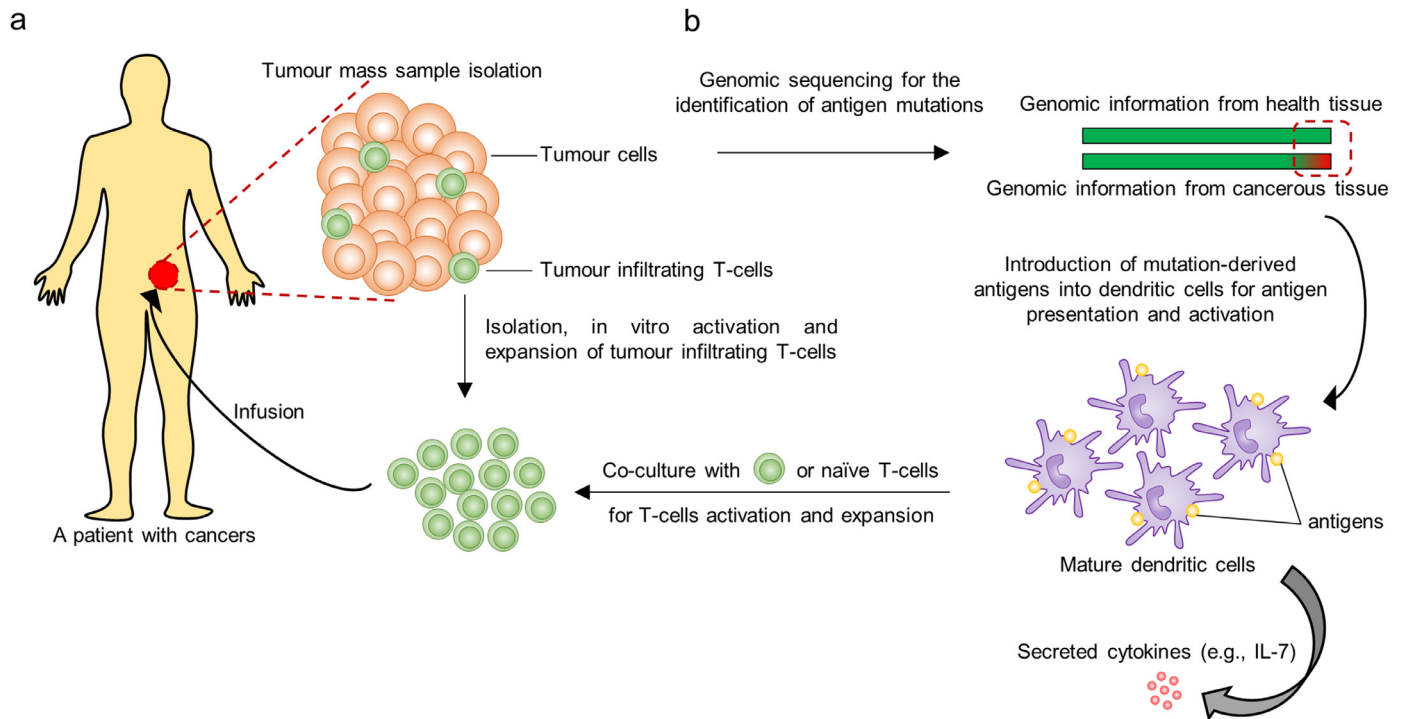
Fig. 1. *General approach of adoptive T-cell immunotherapy (ACT).* It involves (a) isolation, activation, and expansion of tumor infiltrating *T*-cells from the surgery removed tumor mass specimens. (b) The extracted tumor mass can be utilized for tumor-derived antigen mutations identification through genomic sequencing. Such antigens can be introduced into dendritic cells, which can be co-cultured with and activate tumor infiltrating *T*-cells/naïve *T*-cells for expansion. The activated DCs can secret cytokine signals to further stimulate *T*-cell, such as IL-7 [9], despite the presented antigens and co-stimulatory ligands. These expanded *T*-cells can ultimately be infused into the patients to eradicate the remaining tumor cells to prevent relapse.
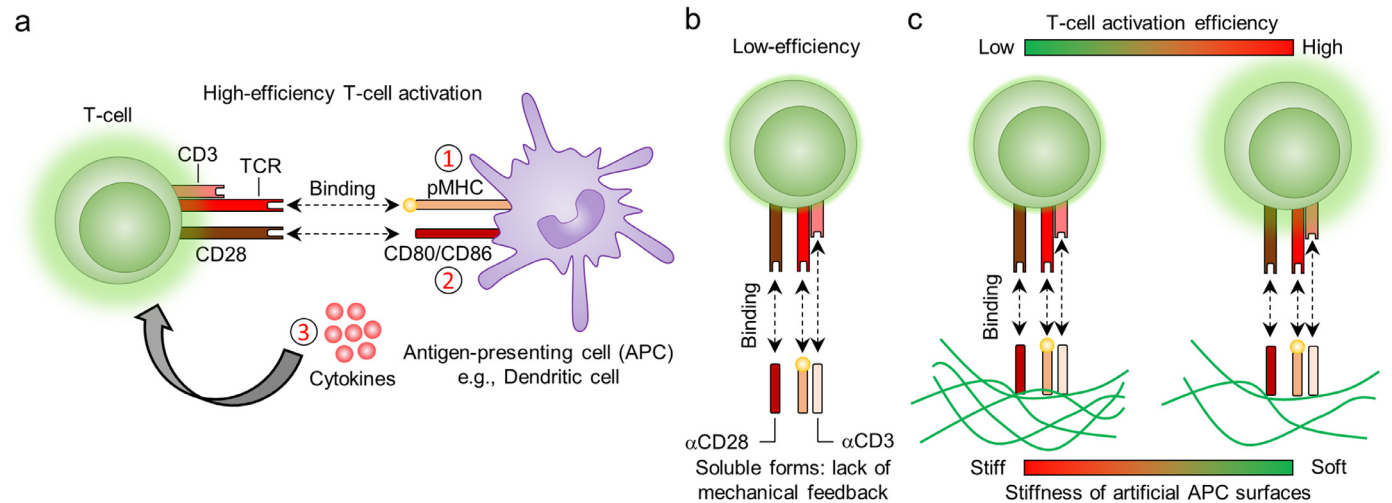

Fig. 2. *Essential conditions of T-cell activation.* (a) three primary signals involve (1) engagement between TCR and stimulatory signal pMHC for specific activation (or *α*CD3 for non-specific activation), (2) binding between CD28 and co-stimulatory signal CD80/86 (or *α*CD28) for proliferation, and (3) presence of cytokines for differentiation. (b) soluble stimulatory signals are lack of mechanical feedback, which causes low activation efficiency. (c) Immobilization of stimulatory signals on soft aAPCs leads to higher *T*-cell activation efficiency than those on stiff aAPCs [13].

neered by genetic modification to produce an artificial TCR, which can concentrate tumor-specific CD8+*T*-cells *ex vivo* and have achieved significant clinical success [5]. However, the activation and expansion of CAR *T*-cells still require high dynamic contacts between the antigen-recognizing receptor and the specific antigen. To ascertain the degree of this optimization, engineering the mechanical properties of various aAPC materials has provided new insight into the *T*-cell activation process to reach the full potential of *T*-cell immunotherapy. In this perspective, we recapitulate the current advanced biomaterials designs that

optimize the biophysical and physiological conditions to study and regulate *T*-cell behaviors for ACT against cancers. Moreover, we provide insights from unexplored parameters as determining attributes of a *T*-cell response, based on well-established biomaterials.

1.2 T-*cell differentiation models*

T-cell phenotype profile is an active area of research since *T*-cell functions vary among subsets. For instance, effector memory *T*-cells (T$_{EM}$-
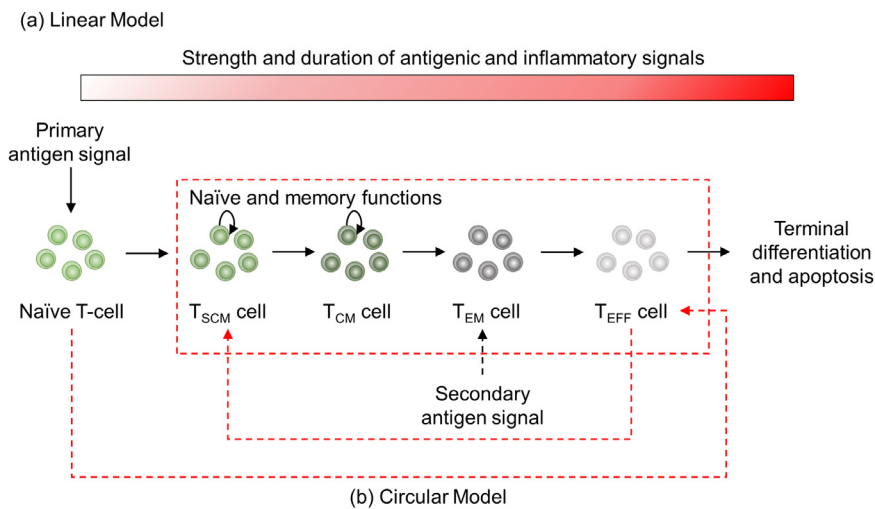
(a) Linear Model

Fig. 3. *Proposed models of CD8+ T-cell differentiation pathway model* [17]. (a) Developmental or linear differentiation model (solid black line) proposes a progressive acquisition of effector function by naïve CD8+ T-cell: Naïve→T_{SCM}→T_{CM}→T_{EM}→T_{EFF}, which will eventually terminate differentiation and undergo apoptosis. The strength and duration of antigenic and inflammatory signals are key determinants of T-cell differentiation. (b) The circular model (red dotted line) proposes a recurring cycle of T-cell differentiation by naive CD8+ T-cell: Naïve→T_{EFF}→T_{SCM}→T_{CM}→T_{EM}→T_{EFF}, which either undergoes apoptosis or differentiates into memory T-cell subsets following antigen withdrawal.

cells) are relatively cytotoxic, and central memory T-cells (T_{CM}-cells) are relatively more proliferative and producing more IL-2 [16]. According to Waddington's model on T-cell stemness, as discussed by Gattinoni et al., T_{EM}-cells are thought to be more differentiated and closer to senescence or death than T_{CM}-cells. Thus, the low proliferative feature of T_{EM}-cells should be taken into consideration for a long-term therapeutic application [16]. According to the linear differentiation model, T-cell differentiates following the order: naïve T (T_N)-cells, stem cell memory T (T_{SCM})-cells, central memory T (T_{CM})-cells, effector memory T (T_{EM})-cells, and effector T (T_{EFF})-cells (Fig. 3a) [17]. In this model, differentiation of cell is strength and duration-dependent, i.e., repetitive and robust signal can drive the differentiation toward effector phenotype, whereas weak signal only allows T-cell differentiation to memory phenotype [16,17]. Therefore, the activation strength and duration of antigen exposure in biomaterials design are the key parameters to modulate T-cell phenotype profiles. Potentially, biodegradable aAPCs may influence the material rigidity or mechanical feedback of immobilized TCR ligands sensed by T-cells. This may slow down T-cell differentiation into effector phenotype along with the degradation of aAPCs by weakening the TCR activation signals.

Beside, a circular differentiation model has been proposed to reveal the dedifferentiation and redifferentiation features of T-cells in multiple antigen exposure events (Fig. 3b) [17]. T_N cells differentiate to T_{EFF} cells after primary antigen exposure. These T_{EFF} cells may undergo apoptosis or have been proposed to enter a cycle of differentiation order: T_{SCM} cells, T_{CM} cells, T_{EM} cells, and T_{EFF} cells upon the same antigen exposure [17]. In terms of adoptive T-cell immunotherapy, the tumor-specific T-cell population isolated from the patient may also enter such a differentiation cycle. Therefore, alteration of the T-cell differentiation dynamics by advanced biomaterials is highly desirable to change relative populations of T-cell subsets.

It has been proposed that the features of high plasticity and self-renewal potential of T_{SCM}-cells are attractive for immunotherapy [16]. Together with their tumor-specificity and multipotency, T_{SCM}-cells can maintain a high population of different T-cell subsets for prolonged immunotherapy [16]. Therefore, we suggest that biomaterials presenting prolonged and robust exposure to stimulatory signals may not be the right choice for the ACT as they may drive a large population of T-cells toward terminally differentiated T-cells before implantation. In contrast, a biomaterial allowing well-controlled activation strength and stimulatory signal exposure duration may help preserve the stemness of T-cells and achieve long-term immunotherapy. Taken together, we believe that both the quantity and phenotype profile of T-cells are crucial in ACT applications.

1.3 T-cell exhaustion

In the tumor microenvironment, chronic exposure of tumor antigen causes T-cell tolerance, which is progressive developed into functional exhaustion by upregulating the expressions of co-inhibitory receptors, such as PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). This is featured by low proliferative capacity and cytokine production, which served as the significant challenges in the ACT [18–20]. These co-inhibitory receptor ligation reduced T-cell activity [21]. A stronger activating signal is required to overcome the inhibitory signal to activate exhausted T-cells [22]. In other words, either a strong inhibitory signal or weak stimulatory signal limits the T-cell activation in exhausted T-cells that highly hampers ACT.

Beside the inhibitory signals to T-cells *via* PD-1/PD-L1 interaction for anti-apoptotic signals, the expression of PD-L1 on cancer cells was associated with proliferative signalings, e.g., PI3K-Akt pathway and mTOR, which leads to enhanced glycolytic metabolism [23]. Therefore, T-cell dysfunction may positively correlate to enhanced tumorigenic activity. Nevertheless, how regulation of cancer cell behavior by different inhibitory pairs unexplored (Fig. 4).

As highlighted in the 2018 Nobel Prize in Physiology or Medicine, the addition of antibodies that target the inhibitory receptor can block the inhibitory receptor ligation, hence rescuing T-cell exhaustion [24,25]. Thus, targeting T-cell exhaustion would be a potential strategy to achieve more effective immunotherapy, such as the selective expansion of intermediately exhausted T-cells (PD-1^{int}) from highly exhausted T-cells (PD-1^{high}) pool 20,26]. Moreover, it has been shown that PD-1 surface expression can be downregulated through FBXO38-mediated ubiquitination-induced degradation, which is a potential therapeutic target of preventing T-cell exhaustion [27]. Despite PD-1 and CLTA-4, other inhibitory receptors such as lymphocyte activation gene-3 (LAG-3) and mucin domain-3 (TIM-3) may also be expressed on the T-cell surface [28]. Therefore, a cocktail of antibodies targeting multiple inhibitory receptors is shown to promote T-cell expansion [20]. However, this strategy is expensive. Also, the regulations of surface expression of other inhibitory receptors remain unclear.

As aforementioned, IL-2 is important for T-cell proliferation. However, one recent report demonstrated that IL-2 contributes to CD8+ T-cell exhaustion through STAT5 signaling, dependent on both IL-2 dose and exposure time. This T-cell exhaustion is featured by upregulations of LAG-3 and TIM-3 [29]. This finding implies that a high dosage of IL-2 can drive T-cells in exhausted phenotype and hence is unfavorable to sustain the anti-tumor therapeutic application. Therefore, the parameters of exposure duration of T-cell stimulatory factors and cytokines should be considered for the biomaterials design for immunotherapy.
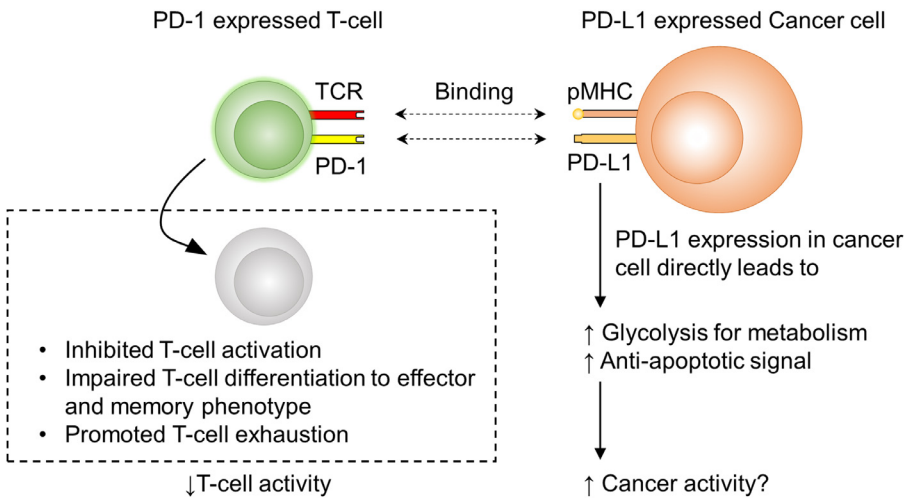
PD-1 expressed T-cell   PD-L1 expressed Cancer cell

Fig. 4. *Schematic illustration for the summary of PD-1/PD-L1 interaction for inducing T-cell exhaustion* [23]. Upon ligation between PD-1 of *T*-cells and PD-L1 of cancer cells, *T*-cell activation and differentiation into effector and memory phenotypes are inhibited. These consequences promote T-cell exhaustion and downregulation of T-cell activity. On the other hand, the expression of PD-L1 in tumor cells can elevate anti-apoptotic signal and glycolytic activity for metabolism, improving cell survival and proliferation. Therefore, T-cell exhaustion in the tumor microenvironment may indirectly enhance cancer activity.

2. Mechanotransduction for enhanced *T*-cell activity

2.1.1 Mechanotransduction is crucial for T-cell activation through T-cell receptors

It is known that the mechanical properties of aAPC materials, such as stiffness, can govern the binding dynamics between TCR and its ligands. Multiple reports showed that soft rather than stiff two-dimensional materials improved *T*-cell activation, including both CD4$^+$ and CD8$^+$ T-cells, through this engagement (Fig. 2c). In particular, CD4$^+$CD25$^-$FoxP$_3^-$ (Forkhead-family transcription factor forkhead box P3, a master regulator of Treg development, function, and homeostasis) *T* regulatory precursors cells can be driven to be CD4$^+$CD25$^+$FoxP$_3^+$ *T* regulatory cells by seeding on soft (100 kPa) but not on stiff (3 MPa) aAPC PDMS (polydimethylsiloxane) substrates [13,30,31]. These findings suggest that TCR is mechanosensitive to biophysical cues. Specifically, Schneck et al. revealed that CD8$^+$ *T*-cells cultured on soft (0.5 kPa) hyaluronic acid hydrogel with immobilized αCD3, αCD28 and integrin-ligand (RGD) exhibited a much larger cluster size of CD3 than the cells cultured on stiff (3 kPa) gel [13]. Since CD3 is a protein complex with TCR, a large cluster of CD3 is indirect evidence of TCR clustering critical to amplify the signal transduction for *T*-cell activation [32]. However, this behavior of TCR is unlike the response of other well-known mechanosensitive receptors such as integrin, of which the lateral clustering for focal adhesion maturation is positively proportional to the strength of mechanical force transmission from the ligands tethered to a substrate/matrix [33,34]. Potentially, soft aAPCs have higher deformability than stiff aAPCs, and hence the TCR-ligand complex can aggregate to amplify the activation signal on soft aAPC by forming a large cluster. The exact mechanism of how TCR aggregates to a large cluster in response to the ligands conjugated on soft 2D substrate remained further studied.

2.1.2 Antigen-specific T-cell response requires mechanical force at piconewton scale between TCR and ligand

Salaita et al. have reported a nanoplatform consisted of TCR-binding ligand conjugated to a hairpin DNA-based nanoparticle as a fluorescent tension probe for studying the mechanical force mediated by individual TCR complexes on pMHC binding [35]. This fluorescent probe is initially dual-quenched by both the molecular quencher through fluorescence resonance energy transfer (FRET) and the plasmon of the AuNP *via* nanometal surface energy transfer (NSET). DNA hairpins generate fluorescence when the applied force exceeds the unfolding force (designed by the known number of base-pairs) and provide unprecedented sensitivity, generating a ~100-fold fluorescence increase in response

to pN forces. With this advanced design, they reported that the TCR complexes tension, pY (pan-phosphotyrosine), and Zap70 (zeta-chain-associated protein kinase 70) signals, which represent *T*-cell activation quantitatively, decreased with ligand potency (binding strength of the agonist). These results imply a strong correlation between TCR tension and *T*-cell activation.

In the same study, Salaita et al. further reported a DNA tension gauge tether (TGT) tailored to dissociate at force levels (12 pN and 56 pN) that exceed its mechanical tolerance. In general, *T*-cells binding to a strong agonist (high ligand potency) exerted a strong mechanical tension through TCR to the bonded ligand and amplified *T* cell signal. In contrast, there was only a weak tension when the *T*-cells were binding with a weak agonist (low ligand potency). Also, the results revealed that more potent ligands immobilized on TGT contributed to more significant *T*-cell activation signals under 56-pN TGT than 12-pN TGT, while *T*-cell activation remained similar when less potent ligands were tethered in TGT at both force levels. This observation provides important evidence of specificity discrimination by mechanical force as a feature of *T*-cell. Furthermore, they demonstrated an improved DNA probe that can store cell-generated mechanical information and unravel the role of mechanical force in the immune checkpoint [36]. Intriguingly, this study revealed that *T*-cells exerted force on the antibody, anti-programmed cell death protein 1 (PD1) through PD1 receptor. Taken all these together, there is an intrinsic feedback system to gauge TCR-agonist tension, which regulates the *T*-cell signal. This finding implies a possible correlation between the activation of immune checkpoints and mechanics, which can modulate *T*-cell exhaustion (reduced *T*-cell functionality). Nevertheless, more research is required to support this speculation.

2.2 Integrins subtypes support TCR-mediated activation via mechanotransduction

In addition to TCR-ligand engagement, membrane integrins also play a critical role in *T*-cell response. Schneck and his colleagues further demonstrated that the presence of immobilized RGD (typical β_1- and β_3-class ligand) on soft hydrogel promoted CD8$^+$ *T*-cell proliferation ~1-fold greater than that of the hydrogel without RGD [13]. In contrast, stiff hydrogel suppressed cell proliferation and abrogated this proliferation difference in the presence or absence of RGD. These results imply that the activation of integrin additionally supports *T*-cell activation.

It is known that RGD immobilized on stiff matrices can enhance integrin-mediated signalings and cytoskeleton *F*-actin assembly, leading to the development of actomyosin contractility and cytoskeletal tension, which result in nuclear localization of a mechanosensor, Yes-associated protein (YAP) [37]. In contrast, soft matrices suppress this cellular response and lead to the major distribution of YAP in the cytoplasm. A
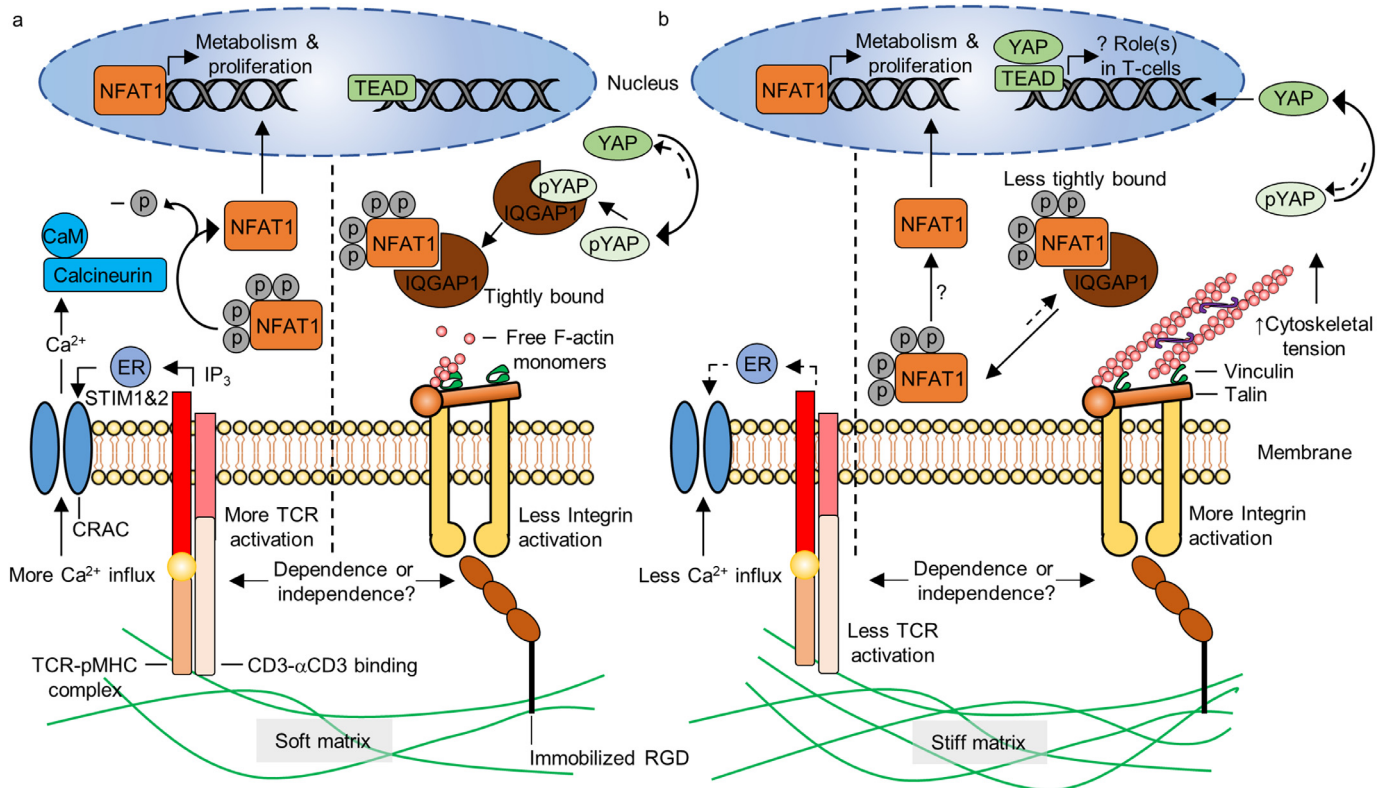
Fig. 5. *Mechanoregulation of T-cell metabolism and proliferation via NFAT1 signalings* [38,39]. (a) In the canonical pathway, stimulatory signals-bearing soft-substrate enhanced TCR activation leads to the production of IP_3, which opens IP_3 receptor channels in ER. This opening causes an efflux of Ca^{2+} from ER that activates STIM1&2, which binds to and opens CRAC channels, causing an influx of Ca^{2+} outside the membrane. Subsequently, this increase of intracellular Ca^{2+} activates CaM and the serine/threonine phosphatase calcineurin to dephosphorylate cytosolic NFAT1, resulting in nuclear localization of NFAT1. However, stiff matrices do not favor this TCR-mediated signaling. (b) In contrast, stiff matrices promote integrin-mediated mechanosensing signalings in the presence of immobilized RGD peptides. This causes more nuclear localization of YAP and less cytosolic pYAP interacting with IQFAP1. Subsequently, the NFAT1 can easily unbind from the NFAT1-IQFAP1 complex. While the dephosphorylation of the NFAT1 mechanism in this signaling is unknown, more nuclear localization of NFAT1 occurs. The presence of more cytosolic pYAP can lead to a tightly bound state between IQFAP1 and NFAT1.

recent report by Butte et al. studied the role of integrin in the cell proliferation and metabolism of CD4$^+$ T-cells when co-cultured with APCs on RGD-conjugated alginate hydrogels with varying stiffnesses [38]. Their work showed that soft gel (4 kPa) retained a majority of YAP in the cytoplasm, which promoted the interaction between Ras GTPase-activating-like protein (IQGAP1) and nuclear factor of activated T-cells 1 (NFAT-1). This interaction suppressed the nuclear transport of NFAT-1 and downregulated the NFAT-1 dependent metabolism and proliferation program. In contrast, stiff gel (40 kPa) translocated most cytoplasmic YAP into the nucleus, and hence the NFAT-1 dependent signaling can be "switched on". This trend of integrin-mediated mechanosensing behavior in response to the matrix stiffness is opposite to the softness-enhanced TCR activation in NFAT1-related signalings (Fig. 5b). In the canonical pathway, pMHC of APCs stimulates TCR to produce more inositol-1,4,5-trisphosphate (IP_3) [39]. The expressed IP_3 can open IP_3 receptor channels in the endoplasmic reticulum (ER). Subsequently, this ER opening decreases ER Ca^{2+} concentration that activates stromal interaction molecule 1 (STIM1&2). STIM1&2 can bind to and open Ca^{2+} release-activated Ca^{2+} (CRAC) channels formed by ORAl1 and ORAl2 proteins in the membrane. Ultimately, these events trigger influx of Ca^{2+} through CRAC channel and activated calmodulin (CaM) and the serine/threonine phosphatase calcineurin. The activated calcineurin will dephosphorylate serine/threonine residues in the regulatory domain of NFAT1 (and also NFAT2), resulting in a conformational change, exposing nuclear localization signals, and hence nuclear import of NFAT1 (Fig. 5a). However, little evidence show whether these two receptors (integrin and TCR) can interfere with the NFAT1 signaling events of each other. It is

unknown whether TCR-mediated and integrin-mediated NFAT1 signalings directly participate in each other pathway. This "mechanical checkpoint" provides a novel insight into the regulation of T-cell behavior by the physical microenvironment.

3. The requirement of ligand nano-spacing in three dimensions for T-cell activation

Kinetic-Segregation (KS) model describes a restriction of TCR ligand at the nanoscale as a requirement for initiating T-cell activation (Fig. 6) [40]. The distribution of TCR, tyrosine phosphatases with large ectodomain (e.g., CD45), Src kinases (e.g., Lck: lymphocyte-specific protein tyrosine kinase) can diffuse freely in resting T-cells [41]. LCK plays a role in phosphorylation zeta-chain-associated protein kinase of 70 kDa (ZAP70), which is recruited by tyrosine-based activation motifs (ITAMs) of TCR-CD3 complexed for T-cell activation. In parallel, CD45 maintains the activity of LCK, but continuously dephosphorylates LCK and ZAP70. In this case, phosphorylation and dephosphorylation of T-cell receptors are balanced due to evenly distributed CD45 and Lck (Fig. 6a). Upon contacting an APC, the TCR-ligand complex dimension defines the intermembrane distance (generally ~13 nm) between the T-cell and APC, which can physically exclude CD45 (~20 nm domain outside the membrane) outsite this immuno-synapse in a size-dependent manner (Fig. 6b) [40,42–44]. Therefore, the TCR phosphorylation and dephosphorylation dynamics become imbalance, resulting in exceeding in basal level of TCR phosphorylation for T-cell activation initiation.
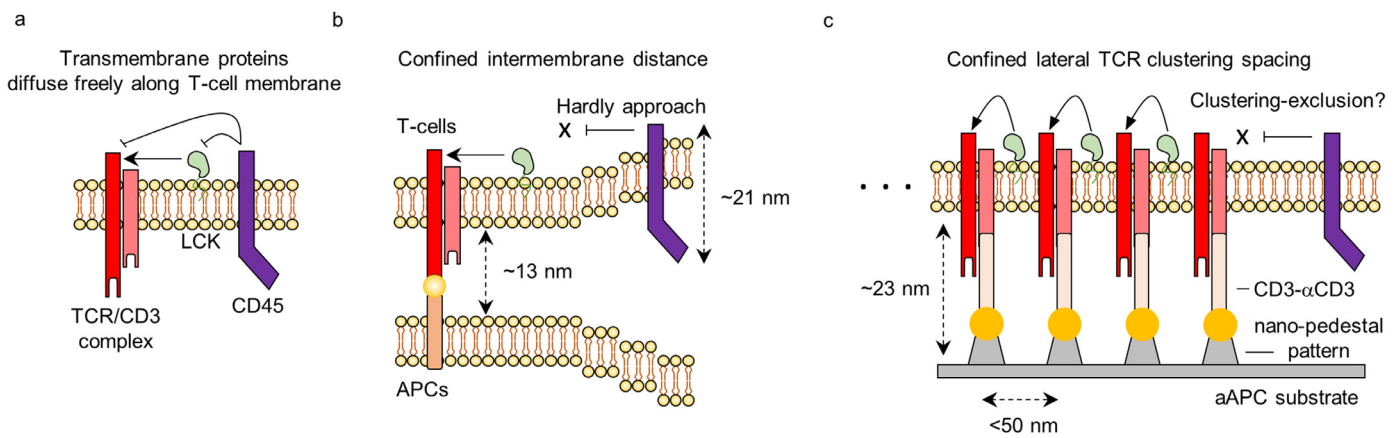
Fig. 6. *Kinetic segregation (KS) model associated dynamics in T-cells* [44]. (a) At resting state, key transmembrane proteins diffuse freely along the T-cell membrane in the KS model. The phosphorylation and dephosphorylation of TCR are balanced by LCK and CD45, respectively. (b) Upon TCRs binding with pMHCs of APCs, the intermembrane distance is confined ~13 nm that can physically exclude the approach of rigid CD45 that causes more phosphorylation of TCR for initiating T-cell activation. (c) This is hypothesized that successive TCR/CD3-αCD3 binding complex lateral clustering spacing <50 nm can also effectively exclude the lateral approach of CD45 to TCR, regardless of the intermembrane distance.

Choudhuri et al. transfected APCs to present varying dimensions of pMHC domains for studying the critical intermembrane spacing between APCs and T-cell for TCR triggering [40]. APCs (CHO cells) expressing single-chain trimer (SCT) maintained a 13.1 ± 3.1 nm intermembrane distance with pOVA/H-2K^b-specific murine T-cell hybridoma B3Z. In contrast, APCs expressing SCT-CD4 (linear conjugation of the two proteins) confined 17.8 ± 4.6 nm intermembrane distance (CD4 has the length 10–12 nm) with the B3Z cells. Strikingly, a more robust T-cell response (such as IL-2 release) is associated with a shorter ectodomain length of pMHC presented on APC. Furthermore, they demonstrated that the B3Z exhibited early depletion of CD45 (2 min of culture with the APCs), followed by late enrichment of CD45 (5 min of culture with the APCs) *via* immunofluorescence staining. However, this biphasic effect was observed in neither B3Z cultured with APCs expressing long dimension length (SCT-CD4) nor the B3Z pre-incubated with Src kinase inhibitor PP2. These findings highlighted that elongation of SCT is associated with less effective depletion of CD45 from the T-cell–APC interface.

Wind and colleagues recently developed a two-dimensional aAPC substrate with precisely controlled vertical (ligand dimension) and lateral (inter-ligand distance) nano-spacings within a nanoarray cluster and ligand density (density of nanoarray cluster) to study the biophysical parameters that govern T-cell activation at the nanoscale [44]. Their results showed that a lower inter-ligand distance (ranged from 120 nm to 40 nm) of UCHT1 contributed to a more robust T-cell activation (evidenced by stronger immunofluorescent staining of phosphotyrosine) at ~13 nm vertical nano-spacing (T-cell–substrate contact distance). More importantly, their result highlighted that increasing the vertical nano-spacing by introducing a nano-pedestal pattern (~10 nm) allowed the diffusion of CD45 toward the TCR-CD3 complexes (Fig. 6c). The result agreed with KS model that the involvement of CD45 inhibited the T-cell activation due to failure of the kinetic segregation of the key membrane proteins. Interestingly, this inhibition of T-cell activation was rescued when the lateral spacing was below 50 nm (lateral nanospacing at 40 nm), even with the nano-pedestal pattern. This T-cell behavior indicates a potential model for TCR activation: CD45 exclusion from the TCR-ligand complexes requires the lateral clustering of TCR with <50 nm spacing for triggering T-cell responses. This model provides a critical parameter for designing aAPC to potentiate the phenotype and proliferation of T-cells. From this model, we postulate that soft matrices potentially provide a more flexible network, where T-cells exert contractile force to aggregate the immobilized TCR ligand-TCR complexes with a closer inter-receptor spacing (<50 nm) to exclude CD45 proteins

than that of the stiff matrices and hence promote the T-cell activation. However, this hypothesis needs further validation.

4. Dynamic control of tcr clustering boost T-cell activation by micro/nanomaterials

In general, the commercially available product Dynabeads® CD3/CD28 (micro-size spherical polystyrene beads) as aAPCs is the most widely used and recognized platform for separation and *in vitro* expansion of T-cells [45]. Recent research also comprehensively developed novel advanced materials that resemble the features of Dynabeads® with improved physical properties for a more effective and efficient tumor-specific T-cell expansion. Consistent with the result of preferential activation on soft two-dimensional matrices, soft polydimethylsiloxane (PDMS)-based version of Dynabeads® (7.3 MPa, Sylgard 184 formulation) stimulated CD4$^+$−CD8$^+$ mixed T-cells with an extended proliferative phase compared to the conventional Dynabeads® [46]. This finding demonstrates the importance of integrating specific nanoscale structures and rigidity into aAPC for improving T-cell expansion.

Despite the contact softness, the geometry of aAPCs can also modulate the effective contact area between ligands and the T-cell membrane. In particular, Green et al. showed that micro-size ellipsoidal biomimetic aAPCs with a high aspect ratio (AR) significantly induced higher T-cell responses against a specific tumor *in vitro* and *in vivo* than the spherical aAPCs with a similar particle volume and stimulatory factor content [47]. Apart from using micro-size ellipsoidal aAPC, Meyer reported that nano-size ellipsoidal aAPC could improve T-cells proliferation and aAPC distribution in blood circulation [48]. Similarly, Fahmy and colleagues demonstrated that bundled carbon nanotubes with a high surface area provided a high density of immobilized T-cell stimuli on the cell surface, reducing the thousand-fold soluble cytokine IL-2 in T-cell proliferation which was required using Dynabeads® for CD8$^+$ T-cell expansion [49]. These results indicate that aAPC geometry is a critical design criterion to govern the surface area of contact in aAPC/T-cell for enhanced cancer immunotherapy. From these work, we speculate that the combination of softness and high aspect ratio into aAPCs allows a high degree of body deformation that maximizes the accessibility between ligands and TCR to optimize the outcome of T-cell responses.

Micro-size aAPC can mimic the cell-cell interaction, while nano-size aAPCs are a valuable tool for the fundamental study of T-cell behavior at the nanoscale. Notably, Hickey and colleagues showed that large aAPCs with a diameter of ~300 nm activated CD8$^+$ T-cell more efficiently than small aAPCs with a diameter of ~50 nm [50]. Increasing the ligand

density on the small aAPCs did not further stimulate the response of CD8$^+$ T-cells. This reveals the size of aAPC is critical for effective T-cell activation. Thus far, their findings confirm that the ability of aAPCs to activate T-cells is size-dependent for consecutive receptor-ligand clustering and are consistent with a previous report which indicated that the lateral organization of MHC class I enhances the sensitivity of T-cell recognition of agonist peptide [51]. Moreover, Perica et al. have reported iron-Dextran nanoparticle (50–100 nm) bearing MHC-Ig dimer (signal 1) and αCD28 (signal 2), forming nano-aAPC for magnetic enrichment and expansion of antigen-specific T-cells (Thy1.1þ pmel TCR transgenic T cells specific for Db-GP100 melanoma antigen) from a polyclonal CD8$^+$ T-cell population (Thy1.2+ CD8 T-cells from wild type B6 mice) [52]. Specifically, the T-cell expansion positively depended on the strength of the magnetic field and duration, which were optimized at 3 h and 0.2T, respectively [53]. The reason behind this expansion is potentially because of magnetically aggregated nano-aAPCs enhancing the clustering of TCR. To study effective TCR clustering, Kosmides et al. using MNP for the optimization of ACT protocols. Rather than decorating both signal 1 (αCD3 or MHC-Ig) and 2 (αCD28) on the MNPs (~30 nm) as traditional aAPC, they immobilized a single signal onto either one particle to simplify the fabrication and characterization procedures. Thus, the composition of the signals per particle can be easily tuned. These separate signal-bearing MNPs can be simultaneously clustered under magnetic field alignment, and hence the co-stimuli can activate CD8+ murine T-cells. Also, the authors claimed that the particles presenting separate signals approach create homogeneous single signal "islands", which may interact with the T-cells differently than endogenous APCs. However, further research is required to unravel the mechanisms of these two approaches [54]. These platforms not only provide powerful tools for optimizing T-cell activation but also validates the requirement of signal clustering within the "close-contact" in both two- and three-dimensions. We have further summarized current advanced aAPC platforms with selected design feature(s), types of T-cell studied, the interaction of aAPCs and T-cells, and their key finding(s) for the ACT in Table 1.

5. Future perspective

The mechanism of how cell-adhesive (e.g., RGD-presenting) extracellular matrices regulate T-cell function remains poorly understood. More importantly, the as-mentioned physical parameter, the influence of matrix stiffness (or elasticity) of biomaterials on T-cell activation, has been extensively studied. T-cells can sense substrate elasticity by gauging resistance through developing traction forces on the substrate. Nevertheless, those aAPCs may be composed of covalently cross-linked polymers, such as hyaluronic acid hydrogel, exhibiting purely elastic substrates with time-independent storage or elastic modulus. Hence, the presentation of co-stimulatory signals by these aAPCs is rather like a "static" interaction between TCR and the ligands since the mechanical property of the aAPCs is predefined and constant. On the other hand, reconstituted matrices, such as collagen, may demonstrate a stress relaxation or a decrease in elastic modulus over time when a constant strain is applied. With this property, the matrices resisting the exerted cellular traction forces relaxed over time due to the flow and remodeling of the matrix. This substrate stress-relaxation has been shown to enhanced stem cell integrin-mediated adhesion, spreading, and mechanosensing signaling (YAP). This property not only can simulate the microenvironmental ECM remodeling process but also allow dynamic interaction between membrane receptors and ligands. Nevertheless, very little research pays attention to the effect of matrix stress-relaxation on the regulation of T-cell behaviors. We have mentioned the role of YAP in controlling T-cell metabolism and proliferation, as proposed by Butte et al. [38]. Since soft matrices enhance activation of TCR while stiff matrices promote integrin activation, an aAPC with stress-relaxation property can be adopted to harness and establish the integrin-mediated T-cell adhesion (stiff matrice stage) and stable mechanosensing for the "close contact" between

T-cell and aAPC at the initial stage. The YAP-mediated NFAT1 signalings may drive T-cells to a readily proliferated state even before their activation. Subsequently, the relaxation of the aAPC (soft matrice stage) may facilitate aggregation/clustering of TCR for T-cell activation over time. We believe that this approach presents a dynamic change of biophysical cues that upregulate two potential independent signaling pathways as a function of time for optimizing T-cell activation.

Conventional aAPC materials with persistent activation signal 1 & 2 exposure can lead to T-cell exhaustion. Recent literature demonstrated the importance of a transient rest to CAR T-cells for restoring functionality epigenetically from exhaustion [61]. Thus, a remote-controllable platform to switch T-cell stimulatory signal "ON" and "OFF" duration may prevent T-cell exhaustion for improved ACT with persistent and non-exhausted T-cells in the long term. Recently developed smart-culture platforms demonstrated the manipulations of ligand-tether mobility, oscillation, exposure/hidden, etc., via external stimuli to govern RGD presentation dynamics [33,62–65]. For instance, YAP nuclear localization in mesenchymal stem cells was promoted by strengthening the integrin-ligand binding magnetically and hence regulates stem cell differentiation. Thus far, developing these dynamic platforms is highly desirable for regulating metabolism and proliferation and assisting fundamental studies of T-cells for the ACT. In general, T-cell behavior is hardly adjusted after recognizing TCR-ligand, co-stimulatory ligand for TCR without cytokines. Therefore, it is possible that those dynamic platforms can control YAP mechanical checkpoint by altering mechanical signals in the microenvironment for boosting T-cells of desired phenotypes.

Dynamic control of cytokines presentation is another crucial issue for regulating T-cell phenotype, especially preventing exhaustion. We had mentioned that a high dosage and long-term exposure of IL-2 to CD8+ T-cells could drive them to exhaustion in Section 1.3 of our original manuscript. To address this issue, Sun et al. reported a human red blood cell (RBC) membrane-based aAPC system (5–8 μm) that tethered with pMHC I, αCD28 and IL2 (presenting signals 1–3) [57]. Such aAPC has the advantages of (1) excellent biocompatibility, (2) high surface-to-volume ratio and excellent membrane flexibility for presenting the signals, (3) appropriate size of regular human cells, and (4) long-circulating life in blood for increasing interaction chances with T-cells if administrated intravenously. Most importantly, they demonstrated that RBC-based aAPC-mediated T-cell activation achieved a more robust T-cell proliferation and IFN-γ and TNF-α secretion compared to those stimulated by soluble forms of the stimulatory signals in vitro. Furthermore, a recent study developed poly(lactic-co-glycolic acid) (PLGA)-based microparticles that bear oligonucleotides scaffold for capturing corresponding stimulatory ligands and cytokine, IL-2 with high density on the particle surface [66]. These particles were intratumorally injected to prime the tumor-infiltrated AND-gate (SynNotch) CAR T-cells that were intravenously administered. The results were striking and consistent with the work done by Sun et al. that the primed CAR T-cells exhibited much less exhaustion after expansion compared to the cells activated by Dynabeads over 14 days of stimulation, thereby suppressing tumor size more effectively. The idea of immobilizing T-cell stimulatory factors/cytokine on aAPC seems to be the crux to extend T-cell expansion half-life and lower systemic cytotoxicity (lower dosage of cytokines). Since T-cells require different cytokines at different stages [12], potentially, a regularly presenting/withdrawal of different signal 3 (cytokines) may further alleviate T-cell exhaustion, similar to that of "ON" and "OFF" switching of signal 1 and 2. Thus far, low dosage (immobilization) and dynamic control ("ON" and "OFF") of T-cell stimulatory signals are the key parameters for improving the quality and quantity of desired T-cell phenotypes for the ACT. We expect these strategies will be taken into consideration in future biomaterial design to achieve effective, safe, and low-cost T-cell expansion.

Apart from fundamental studies, biomaterials designs for the ACT should also focus on and aims for clinical translation. Stephan et al. reported using a macroporous alginate-based scaffold (modified by a

Table 1

Recent aAPC platforms for enhanced *T*-cell activation and immunotherapy.

Reference	Design features	*T*-cell type(s) involved	*T* cell-APC mimietic interation	Key findings
Perica et al. [53]	Dextran-coated paramagnetic iron nano aAPC of 50–100 nm coated with signal 1 + 2	CD44⁻naive CD8⁺ *T* cells and CD8⁺ *T* cells from pmel TCR and 2C TCR transgenic mice	*T*-cells were mixed with nano aAPC	• Nano aAPC binded with more TCR on activated T-cells compared to naive T cells • Magnetic field-induced TCR clustering results in CD3 aggregation and 3 h with 0.2 T could promote 10-fold T cell expansion after 1 week
Fadel et al. [49]	Carbon nanotube-polymer composite (CNP): Carbon nanotube presenting signal 1 + 2 and conjugating with PLGA nanoparticle loaded with signal 3 (IL-2) and magnetite.	CD8⁺ *T*-cells from human and OT-1 mice	Functionalized carbon nanotube – PLGA composite are in suspension form, T-cells were added directly to suspension.	• CNP promoted T-cell expansion, activated phenotype (higher CD27 expression) T-cell cytotoxicity better than that of using dynabead or soluble antigen-presenting tetramer with exogenous IL-2. • CNP expands cytotoxic *T* cells *in vitro* to a level comparable to clinical standards using 1000-fold less soluble IL-2.
Sunshine et al. [47]	PLGA microspherical particle and microellipsoidal particle presenting signal 1 and signal 2	CD8⁺ *T* cells from pMEL TCR/Thy1ᵃ Rag-/- transgenic mice and C57BL/6J	T-cells are mixed with micro aAPC	• Ellipsoidal micro aAPC enhanced T-cell proliferation compared to spherical micro aAPC • Ellipsoidal micro aAPC provided better T cell–aAPC conjugates and contact length compared to spherical micro APC
Stephan et al. [55]	GFOGER* conjugated alginate scaffold, embedded with micro aAPC (surface: anti-CD3/28/137, soluble factor loaded: IL-15/IL-15Rα) GFOGER is an $\alpha_2\beta_1$ collagen receptor (on lymphocyte) targeting peptide[55]	CD8⁺ *T* cells from BALB/c mice and chimaeric natural-killer receptor-modified T-cells from C57BL/6 J mice CD8⁺ *T* cells	The cell suspension was added on a lyophilized scaffold, cells then infused into the scaffold	• Implantation of T-cells encapsulated scaffold treated inoperable or incompletely removed tumor more effectively than T-cells injection • Scaffold encapsulated T-cells proliferated more robust and retained non-exhausted phenotype compared to the injected *T*-cells *in vivo*
Meyer et al. [48]	PLGA nano-ellipsoidal aAPC, presenting signal 1 + 2	CD8⁺ *T*-cells from PMEL mouse	*T*-cells were mixed with nano aAPC	• Nanoellipsoidal aAPC promoted *T*-cell with higher proliferation compared to nano-spherical aAPC • Nanoellipsoidal aAPC maintained a higher concentration in blood circulation compared to nano-spherical aAPC after intravenous injection *in vivo*
Perica et al. [52]	Iron-Dextran Nanoparticle presenting MHC-Ig Dimer and anti-CD28	CD8⁺ *T*-cells from Pmel TCR/Thy1a Rag-/- transgenic mice, C57BL/6 J mice, and CD8⁺ *T* cells from healthy donors	*T*-cells were mixed with nano-aAPC	• Enrichment: antigen-specific *T*-cells can be isolated by mixing antigen-specific nano-aAPC and polyclonal *T*-cells population through the magnetic column. • Enrichment with expansion: selected antigen-specific *T*-cells expanded in the presence of nano aAPC
Kosmides et al. [56]	PLGA microparticle presenting signal 1 + 2 and anti-PD-1 was added to the culture for immune checkpoint blockade	CD8⁺ *T*-cells from PMEL, 2C mouse, and C57BL/6 mice	*T*-cells were mixed with micro aAPC, soluble anti-PD-1 was added	• aAPC and anti-PD-1 could provide a synergistic effect on promoting IFN-γ production during PD-1ʰⁱ *T* cell and PD-L1ʰⁱtumor cell culture • An increasing amount of aAPC or anti-PD-1 loading could improve IFN-γ production
Lambert et al. [46]	PDMS microbead presenting anti-CD3 and anti-CD28, the unconjugated surface was passivated with albumin. Three formulations were used: Sylgard 184, MED-6215, MED-4086.	Mixed CD4⁺ and CD8⁺ *T* cells from healthy adult donors	T cells are mixed with micro aAPC	• *T*-cells expansion could be improved by softer microsize aAPC (~7.3 MPa) compared to Dynabead (Polystyrene, GPa scale)

Table 1 (*continued*)

Hickey et al. [50]	Nano aAPC of 50, 300, 600, 4500 nm, superparamagnetic iron oxide nanoparticles coated with signal 1 + 2.	CD8$^+$ *T*-cells from 2C transgenic mouse	*T*-cells are mixed with nano aAPC	• The ability of cognate *T*-cells stimulation by 50 nm aAPC is lower than that by aAPC above 300 nm, even though the concentration of bound signal 1 remains constant. • Large aAPC required less pMHC (per *T*-cell) for *T*-cell activation than small aAPC • Clustering of small, but not large, aAPC by magnetic field improved *T*-cell expansion
Sun et al. [57]	Engineered red blood cell (RBC) presenting signal 1 + 2, and surface tethered IL-2	Splenocytes from OT1 mice and C57 mice	*T*-cells were mixed with RBC based aAPC	• RBC based aAPC with surface tethered IL-2 achieved more robust T cell proliferation, IFN-γ, and TNF-α production compared to that without surface IL-2 decoration but with free IL-2 administration
Kosmides et al. [54]	Paramagnetic or non-paramagnetic nanoparticles coated with signal 1 or signal 2 (separating signal 1 and signal 2 in different nanoparticles)	Naive CD8$^+$ and CD8$^+$ *T* cells from C57BL/6 mice, PMEL TCR/Thy1^a Rag-/- transgenic mice	T cells are mixed with nanoparticles (separated signals)	• Nanoscale nanoparticle (separated signal 1 and signal 2) can better promote *T*-cell expansion than the dynabeads (microscale) • Aggregation of signal 1 and signal 2 together could better promote *T*-cell expansion compared to aggregation of particle bearing signal 1 + 2
Dang et al. [30]	Aligned or unaligned electrospun fiber composed of mixed PDMS/PCL or PCL alone. Meshes were coated with anti-CD3 and anti-CD28, providing signal 1 and signal 2 to *T*-cells, respectively.	Mixed CD4 and CD8 human *T* cells from either healthy donors or chronic lymphocytic leukemia (CLL) patients.	*T*-cells were seeded on a mesh substrate	• Fibers with lower mechanical rigidity promoted *T* cell expansion. • The *T*-cell activating mesh platform successfully rescued *T*-cells expansion from CLL patients
Nataraj et al.[31]	PDMS substrate coated with anti-CD3 and anti-CD28. Rigidity was controlled by varying curing agent to elastomer base ratio.	CD4$^+$/CD25$^-$ conventional *T*-cells and CD4$^+$/CD25$^-$Tregs (from GFP-linked FoxP3 B6 mice).	CD25$^-$ cells were seeded on PDMS substrate (antibodies coated). CD25$^+$ cells act as positive control for FoxP3$^+$ Tregs.	• Treg is induced when *T*-cells are cultured on a low rigidity substrate (~100 kPa) compared to high rigidity substrate (~3 MPa).
Cheung et al. [11]	Antigen-presenting cell-mimetic scaffolds (APC-ms): Mesoporous silica micro-rods (MSRs) adsorbed with IL-2. *T*-cell cues (signal 1 and 2) were attached to the surface of the rod through biotin-streptavidin conjugation.	Primary human *T*-cells CD19 CAR-T cells, primary mouse T-cells (C57BL/6 J and Balb/c mice)	*T*-cells infiltrated to matrix pores, resulting in a 3D scaffold cell culture model.	• Anti-CD3 and anti-CD28 on rod surface more effectively improved polyclonal T-cell expansion compared with Dynabeads under IL-2 supplement. • Substitution of pMHC over anti-CD3 enables specific *T*-cell expansion. • For CAR *T*-cells polyclonal expansion, CD4-to-CD8 ratio preserved after restimulation with APC-ms, while Dynabead skews CD4 population.
Mi et al. [58]	PLGA nanoparticle presenting anti-PD-1 and anti-OX40	OT1 CD8$^+$ *T*-cells	*T*-cells were mixed with nanoparticle	• Nanoparticle decorated with anti-PD-1 and anti-OX40 can block PD-1 (negative signal) and stimulate OX40 (positive signal) simultaneously, achieving more IFN-γ producing cells and higher IFN-γ activity, higher effector- to central-memory ratio compared to administration of mixed, free antibodies

synthetic collagen-mimetic peptide, GFOGER), encapsulating stimulatory signals-bearing microparticles for the ACT [55]. The biopolymer implant was demonstrated to treat (1) incompletely resected tumors, and (2) inoperable tumors with high therapeutic effectiveness compared to injection of tumor-specific *T*-cells alone. They further showed that intravenous injection of tumor-specific *T*-cells led to an accumulation of the *T*-cells in the spleen and liver, and local injection of the *T*-cells into resected tumor sites led to *T*-cell exhaustion. These findings revealed the challenges of tumor-specific *T*-cell delivery for clinical practice. In contrast, they showed that scaffold-delivered *T*-cells were more robustly proliferated and less subjective to exhaustion. Inspired by this work, we suggest other advanced bioadhesive hydrogel designs could also be utilized for *T*-cell delivery. Injectable hydrogels are attractive biomaterials for tissue engineering and regenerative medicine [67,68]. Recently, a promising outcome has been achieved to treat retinoblastoma by using injectable hydrogel that controlled-release of GD2-specific CAR *T*-cells and IL-15 [69]. Beside preventing tumor relapse, we believe that bioadhesive of the injectable hydrogel are critical for promoting *T*-cell adhesion in tumor resection sites, especially with irregular shapes. Moreover, pH-independent gelation is attractive for regenera-

Table 1 (*continued*)

Matic et al. [59]	anti-CD3 conjugated gold nanoparticle nanoarray with defined spacing and anti-CD28 was added as a soluble form	Human $CD4^+$ T cells	T-cells were seeded on nanoarray	• Lower spacing (~35 nm) promoted CD69 expression (activation marker), IL-2 secretion, and T cell proliferation compared to ~150 nm spacing
Hickey et al. [13]	Hyaluronic acid based hydrogel 2D surface conjugated with anti-CD3 or pMHC dimer signal 1 and anti-CD28 (signal 2) cues.	$CD8^+$ T-cells from B6, 2C, and PMEL transgenic mice, healthy human donors	T-cells are seeded on top of a 2D matrix surface	• Soft 2D matrix presenting signal 1 + 2 enhanced T cell expansion • Soft 2D matrix presenting signal 1 + 2 promote TCR clustering size • 2D matrix of 0.5 kPa presenting signal 1 + 2 skewed effector memory phenotype over central memory phenotype compared to 1 kPa matrix • Hyaluronic acid hydrogel promotes proliferation and memory phenotype compared to tissue culture plate
Huang et al. [60]	PLGA microparticle functionalized with anti-PD-L1, or ligands targeting CAR T-cells, or signal 1 + 2, or IL-2	AND-gate CAR- T cell (requires 2 specific antigens to be activated), human primary CD4+ and CD8+ T cells, and murine immune cell lines: 32D, BA/F3 and EL4	T-cells were mixed with micro aAPC	• PLGA microparticle could be functionalized with various immune modulators (through DNA hybridization linker), achieving respective immunomodulation • Surface tethered IL-2 promoted primary CD4+ and CD8+ T cells expansion much better than dyanbead with IL-2 supplement

tive medicine in acidic wounds, such as gastric ulcers [70]. For human tumor microenvironments, the extracellular matrix is generally slightly acidic (pH 6.4–7) in humans and can be as low as 5.6 [71–73]. Acidic resistant, bioadhesive hydrogels are desired for maintaining polymer structure for T-cell attachment and stimulatory signals presentation in the tumor resection site.

Taken altogether, we call for next-generation advanced biomaterials that combine unprecedented biomechanical properties, biological functions, and translational capacity for adoptive T-cell therapy.

6. Conclusion

Adoptive T-cell therapy focuses on immune system defense against a tumor, but requires many tumor-specific T-cells. Fundamental concepts of T-cells activation, differentiation, and exhaustion are introduced in this perspective, providing primary guidance for engineering design in ACT application. Recent advanced T-cell culture platforms have explored the requirements and mechanisms of T-cell activation, aiming for low-cost and efficient cell expansion. aAPCs biomaterials have merits of stable production and consistent characterization compared to viable APCs. These aAPCs are expected to optimize T-cell activity and expansion against tumors for improving the survival rate of cancer patients in clinical trials.

Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Wai Ki Wong: Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Bohan Yin:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Anel Rakhmatullina:** Writing - original draft. **Jingying Zhou:** Writing - original draft, Writing - review & editing. **Siu Hong Dexter Wong:** Conceptualization, Visualization, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

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