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Review

Engineering of synthetic cellular microenvironments: Implications for immunity

Shimrit Adutler-Lieber [a], Irina Zaretsky [b], Ilia Platzman [c], Janosch Deeg [c], Nir Friedman [b], Joachim P. Spatz [c], Benjamin Geiger [a, *]

[a] Department of Molecular Cell Biology, Weizmann Institute of Science, 234 Herzl St., Rehovot 7610001, Israel
[b] Department of Immunology, Weizmann Institute of Science, 234 Herzl St., Rehovot 7610001, Israel
[c] Max Planck Institute for Intelligent Systems & University of Heidelberg, Heisenbergstr. 3, 70569 Stuttgart, Germany

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ABSTRACT

In this article, we discuss novel synthetic approaches for studying the interactions of cells with their microenvironment. Notably, critical cellular processes such as growth, differentiation, migration, and fate determination, are tightly regulated by interactions with neighboring cells, and the surrounding extracellular matrix. Given the huge complexity of natural cellular environments, and their rich molecular and physical diversity, the mission of understanding "environmental signaling" at a molecular-mechanistic level appears to be extremely challenging. To meet these challenges, attempts have been made in recent years to design synthetic matrices with defined chemical and physical properties, which, artificial though they may be, could reveal basic "design principles" underlying the physiological processes. Here, we summarize recent developments in the characterization of the chemical and physical properties of cell sensing and adhesion, as well as the design and use of engineered, micro- to nanoscale patterned and confined environments, for systematic, comprehensive modulation of the cells' environment. The power of these biomimetic surfaces to highlight environmental signaling events in cells, and in immune cells in particular, will be discussed.

Very different systems, yet a very similar strategy: a personal prelude

I began my Ph.D. research in Ruth Arnon's laboratory towards the end of 1972, over 40 years ago. At the time, my own research project centered around the immunochemistry of enzymes; yet the Arnon lab, as a whole, encompassing long-term projects carried out in close collaboration with Michael Sela, dealt with synthetic antigens and their use as models for natural immunogens, potential synthetic vaccines, or specific modulators of immune responses. Since then, I have drifted away from immunology to the study of cell adhesion and migration, mostly in non-immune cells. Yet while preparing this review article concerning synthetic cellular environments, I have come to realize how close in essence our current research strategy is to the original Sela-Arnon approach, already undertaken in the 1960's and 70's [1–4]. Their strategic synthetic approach was driven by the rationale that natural antigens such as proteins or viruses are quite large and complex, containing multiple epitopes, the responses to which can be rather heterogeneous, and may interfere with our ability to understand the basic mechanisms underlying immunogenicity. To address this challenge, they chose to design and utilize synthetic polypeptides, whose immunogenic complexity may be narrower than that of classical immunogens, and may thus be more suitable for conducting mechanistic studies. As discussed below, our current work, as well as the overall focus of this article, clearly parallels the original Sela-Arnon strategy. We, and others in the field, realize that the complexity, diversity and multi-faceted nature of the extracellular matrix renders it difficult to study the molecular basis for cell adhesion-mediated sensing and signaling; thus, the use of well-defined, synthetic matrices offers an attractive solution.

(B. Geiger)

1. Cross-talk between cells and their microenvironment: challenges and prospects for the engineering of synthetic cellular niches

The evolution of metazoan life, some 600 million years ago, introduced new challenges to evolving multicellular organisms; namely, the need for precise spatial and temporal coordination

* Corresponding author. Tel.: +972 52 348 8848.
E-mail addresses: Shimrit.Lieber@weizmann.ac.il (S. Adutler-Lieber), ira.zaretsky@weizmann.ac.il (I. Zaretsky), plazman@is.mpg.de (I. Platzman), janosch.deeg@is.mpg.de (J. Deeg), nir.friedman@weizmann.ac.il (N. Friedman), spatz@is.mpg.de (J.P. Spatz), benny.geiger@weizmann.ac.il (B. Geiger).

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between the constituent cells, essential for maintaining the body's structural and functional integrity. This formidable challenge required the development of new mechanisms whereby cells continuously collect specific positional cues from the surrounding cellular and non-cellular environments, integrate this information, and specifically respond to it. Development of a robust response to the signaling environment is an extremely demanding task: the molecular complexity and diversity of the pericellular space, the need to simultaneously respond to multiple chemical, physical, positional, and temporal cues and, above all, the capacity to integrate all these signals and respond in a specific and coherent manner, must all be taken into account.

To illustrate the magnitude of this challenge, consider the following: a central component of the environment is the extracellular matrix (ECM), consisting of over 400 proteins, proteoglycans and glycosaminoglycans [5]. The ECM is recognized by the cells via multiple transmembrane adhesion receptors (e.g., integrins), which display varying degrees of specificity to the matrix components. In addition, cells also interact with their neighbors via diverse cell–cell adhesion receptors (e.g., cadherins, IgG family receptors, and different lectins [6,7]), which add yet another level of complexity to the environmental sensing mechanism. Apart from the transmembrane receptors, adhesion sites also contain cytoplasmic proteins, which include many cytoskeleton-associated "scaffolding molecules," with over 200 for integrin adhesions [6,8], and over 170 for cadherin adhesions [7]. These molecules contribute to the stability and mechanical robustness of the adhesion sites, alongside multiple signaling molecules that can activate the cellular signaling machinery, and, at the same time, modify the adhesion site and modulate its stability [9]. Beyond the incredible compositional complexity of adhesion sites, it has recently been shown that cells also react to different physical properties of the ECM, including internally generated or externally applied forces [10–12], microtopography [13–16], anisotropy [17,18], rigidity [19,20], and dimensionality [21–23].

Moreover, cells respond not only to different surface adhesive ligands [24], but also to their precise spatial nano-/micrometric distribution [25,26], through differential signaling of integrin-based complexes [27–30]. In addition, diverse matrix proteins and their degree of folding [15], coupled with differing structures of the adhesive epitope [24], have a huge impact on the selection of specific integrin receptors, causing differences in the initiation and progression of the adhesion process [31,32].

Finally, the cell's adhesive responses to different matrices are quite diverse, and may include changes in proliferation rate, differentiation, cell viability, strength of adhesion, and migratory activity (illustrated schematically in Fig. 1).

Given the complex nature and poor multi-parametric definition of native matrices (an example of the immune niche is discussed in Section 2), as well as limited functional information on the cell's sensory machinery operating at adhesion sites, it is difficult to determine the molecular mechanisms underlying physiological, adhesion-mediated signaling.

To tackle the challenges imposed by the diversity of the ECM, complexity of the adhesion machinery, and varying physiological cellular responses to adhesion-mediated cues, three major experimental approaches have been utilized: development of synthetic matrices; systematic modulation of gene expression; and systems-level analysis of multiple cellular responses to the adhesion-dependent cues. Indeed, advanced approaches for synthesis of a wide range of adhesive surfaces with distinct chemical and mechanical properties have been established (discussed in Section 3). As shown schematically in Fig. 1, artificial adhesive surfaces can entail different chemical properties (indicated by ECM1……ECMn), as well as different physical features.

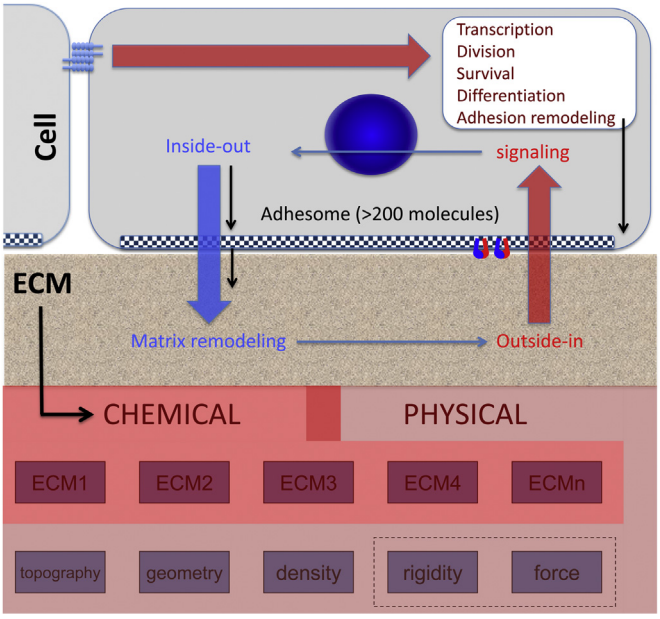

Fig. 1. A schematic description of the cross-talk between cells, their neighbors, and the underlying ECM. Diverse "outside-in" signals from the ECM can be induced by different matrix components (ECM1, ECM2……ECMn), as well as by specific physical characteristics. Cues from the ECM, as well as from cell–cell adhesions, activate the cellular signaling machinery (red arrows), which regulate key cellular processes such as gene expression, proliferation, viability and differentiation. In turn, matrix-induced signaling events can also drive "inside-out" processes, such as the remodeling of the ECM itself (blue arrow), and potentially affect its signaling activity.

The molecular complexity of the cellular adhesive system is commonly addressed using advanced molecular and genetic tools that enable the systematic overexpression, knock-down and knock-in of specific, individual genes, using different high-throughput and high-content screening approaches. Modern robotic systems, combined with automated imaging technologies, enable systematic genetic perturbation, followed by detailed quantitative analysis of multiple cellular responses, including changes in cell shape, gene expression profile, cell locomotion, survival and proliferation. Moreover, the interaction of adherent cells with the matrix can also trigger "inside-out" modulation of the ECM on which they grow, which in turn, affects "outside-in" processes (see Fig. 1).

2. Towards the development of an engineered "multi-stimulatory" immune niche

One major research field that greatly benefits from synthetic systems is that of adaptive immunity. Adaptive immunity involves complex sets of multicellular interactions and paracrine stimulations, most of which take place in specific sites within the lymphatic system, commonly referred to as "immune niches" [33–36]. One emerging approach involves the mimicry of such niches through the engineering of a synthetic immune niche (SIN). The lymph node provides just such a niche, a well-orchestrated microenvironment that enables naïve lymphocytes and antigen-loaded dendritic cells (DCs) to meet, scan each other for matching epitopes and, in the case of positive recognition, support the survival, activation and proliferation of antigen-specific T cells [37] (Fig. 2).

The physical, cellular and molecular structure of the lymph node uniquely suits facilitation of such cellular interactions. It consists of two main, histologically distinguishable regions; namely, the

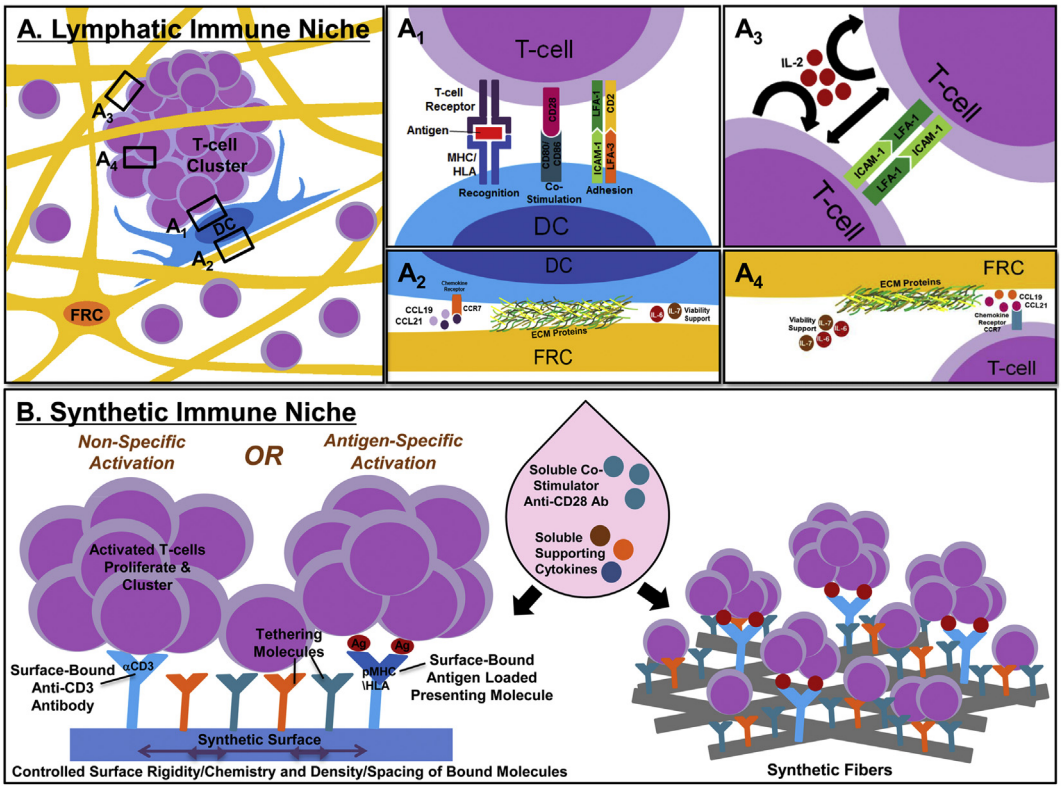

Fig. 2. The complexity of the natural immune niches within lymph nodes. A) Lymph node-residing and infiltrating T-cells and dendritic cells (DC) migrate on a 3D network of fibroblastic reticular cells (FRCs) and their associated ECM. A_1) T cells can also interact with, and be activated by, DCs presenting via MHC/HLA a specific antigen matching the T-cell receptor, and accompanied by co-stimulatory (CD80/CD86 DCs, and CD28 T cells) and adhesive molecules (ICAM-1/LFA-3 DCs, and LFA-1/CD2 T cells). $A_{2,3}$) Both DCs and T cells interact with FRCs and their secreted chemokines (CCL19, CCL21), as well as cytokines (IL-6, IL-7), which promote their migration and survival. A_4) Activated T-cells form intercellular contacts with each other and cluster through ICAM-1-LFA-1 binding. Secretion of IL-2 by activated T-cells induces an autocrine- and paracrine-induced proliferative effect on adjacent T-cells. B) In a novel approach for construction of a synthetic immune niche, many of the cell-bound and ECM-associated ligands are transferred to a synthetic 2D (left) or 3D scaffold (right), to which T cells can bind. In the synthetic niche, interactions with the functionalized scaffold are expected to provide the signals needed for T-cell activation (with or without antigen specificity), lineage selection, proliferation, and survival.

medulla, which contains many plasma cells, and the cortex, further subdivided into the B cell area and the paracortical T cell area, where DCs and T cells meet and interact [38]. In addition, porous, sponge-like stromal tissue, composed of elongated fibroblastic reticular cells (FRCs) and associated reticular fibers, extends throughout the entire lymph node [38]. FRCs are the main conditioners of the microenvironment in the lymph node, which support the various aspects of the immune process. Their activities subdivide the node into numerous narrow channels and interstices (Fig. 2A), which provide spaces for cells to meet, and surfaces on which cells can interact (Fig. 2A_{1-4}); meanwhile, the ECM proteins they secrete facilitate the adhesion and random crawling of immune cells on the FRC surface [39]. In addition, FRCs produce chemokines, cytokines, and growth factors, which co-localize and, in tandem, promote the survival and proliferation of immune cells [40,41] (Fig. 2$A_{2,3}$).

Altogether, the lymph node is anatomically and functionally subdivided into various microenvironments, supporting stroma, extracellular components, and several immune cell types, each with its own phenotypes and regulators (Fig. 2). Consequently, the natural *in vivo* immune niches, and the adjacent microenvironments that reside within the lymph node, are highly complex and dynamic, supporting multiple interactions within a confined space measuring only a few micrometers (see discussion of spatial confinement and microfluidics in Section 4, below). This complexity makes SIN engineering a challenge, with numerous factors to be considered, coupled with multiple combinations of components and properties of the desired system [42–45] (shown in Table 1).

The main components "setting the stage" for synthetic analogs of the immune niche fall into several categories: First come diverse scaffolds, which may be either synthetic [46,47], or of biological origin [48–51]. Due to the huge progress made in recent years in materials science, enabling researchers to produce highly ordered and fine-tuned "smart surfaces", with precise positioning of multiple stimulatory ligands, the fully synthetic approach has lately attracted much attention (see Section 3, below). The other "classical" components include T cell stimulators, both antigen-specific (peptide/MHC complex) and non-specific stimulators (anti-CD3 and anti-CD28). Important elements of the niche are soluble stimulatory molecules (e.g., cytokines, chemokines), which may also be attached to the surface, and exert their effects in the immobilized state. The synthetic scaffold should also support the supply of nutrients and oxygen to the immune cells by means of microfluidic systems, or the growth of different types of supporting stromal cells [52–55]. With all these components in place, the stage is set for the key players: namely, T cells (CD4/CD8) [56] and B cells, as well as antigen-presenting cells (APCs-dendritic cells and macrophages), unless they were already replaced by an immobilized MHC/αCD3 complex.

The precise design and fabrication of a synthetic immune niche, enabling simulation of specific cell-fate decisions, an increase in cell survival and proliferation, and support of antigen-targeted responsiveness, require development of novel types of surfaces –

Table 1
The diversity of components and properties of synthetic immune niches.

The Diversity of Components and Properties of Synthetic Immune Niches			
Physical and Chemical 2D/3D Structure	**Specificity of Immune Activation**	**Immobilized and Soluble Regulators**	**Integration of Stromal Cells**
▪ Artificial Materials ➢ Electro-spun polycarbonate fibers ➢ Synthetic sponges and polymers ➢ Synthetic hydrogels ▪ Biological Materials ➢ Agarose ➢ Laminin ➢ Fibrin ➢ Collagen gels (Matrigel) ➢ Collagen sponges ➢ De-cellularized tissue ➢ Cell printing	▪ Antigen-Specific Activation ➢ Antigen-loaded APCs ➢ Surface-bound peptide-loaded recombinant MHC ▪ Non-Specific Activation ➢ Surface-bound activating antibodies (such as α-CD3) ➢ Soluble recombinant co-stimulatory molecules (CD28).	▪ Adhesion Molecules ▪ Extracellular Proteins ▪ Activation Ligands and Receptors **Regulating Oxygen and Nutrient Supply Systems** ▪ Microfluidic Systems ▪ Synthetic and Cellular Micro-Vascularization ▪ Bioreactors and Membrane-Based Perfusion Systems	▪ Immortal Fibroblastic Cell Lines from Various Tissues ▪ Primary Stromal Cells Isolated from Lymph Nodes ▪ Differentiated Lymph Node Stroma from Mesenchymal Progenitor Cells **Cellular Immune Composites** ▪ T cells (CD4/CD8) ▪ B cells ▪ Macrophages ▪ Dendritic Cells

a major challenge, yet one that carries with it great potential for future adaptive immunotherapy. Among the expected benefits are synthetic *ex vivo* activation and expansion of antigen-specific effector immune cells, which might be applied to adoptive *in vivo* transfer for vaccination, treatment of infections and malignancies, or specific regulatory T cell (Treg)-mediated suppression of autoimmune processes [57,58]. Moreover, SINs could provide novel tools for basic research into immunological processes by enabling the regulation, perturbation and isolation of various factors potentially involved in cell–cell or cell–matrix interactions in immune cells. Finally, these fine-tuning features could perhaps be used as an alternative, *in vivo* testing method for large-scale screening of potential immunomodulatory drugs, or for cytotoxicity assays routinely performed in the development of novel cancer immunotherapies.

3. Multi-scale and multi-dimensional synthetic bio-interfaces: towards a better understanding of our immune system

In recent decades, the control of environmental parameters through artificial bio-interfaces has become a fundamental part of cell biological research. The development of novel biomaterials, featuring impressively finely-tuned mechanical, chemical and structural properties, has led to a wide variety of synthetic cellular environments. Moreover, the ability to pattern and arrange bioactive molecules in a highly ordered and even hierarchical manner, has paved the way toward the creation and design of cellular environments at length scales with relevance to individual cells, or even subcellular structures. These synthetic bio-interfaces can contribute significantly to our understanding of the mechanisms underlying the cell's ability to acquire, process, and respond to environmental information, pointing toward novel therapeutic strategies for a variety of diseases.

In the field, synthetic surface substrates have accompanied researchers almost since they first began to investigate the behavior of individual immune cells. In the case of 2D planar antigen-presenting analogs, the most common technology was based on supported lipid bilayers (SLBs), which provide a suitable model system for mimicking the cell membrane (reduced from three to two dimensions), because the lateral mobility of lipids and proteins partially resembles the *in vivo* situation [59,60]. SLBs containing fluorescent-labeled APC ligands enabled the discovery of the immunological synapse (IS), and have since been heralded as a powerful model for investigating the dynamically coordinated formation of micrometer-scale spatial patterns of cellular surface molecules, so-called supra-molecular activation clusters (SMACs) [61–63]. Moreover, different semiconductor fabrication strategies have been adapted to define spatial constraints within SLBs, with the aim of altering the mobile fraction and the diffusion of incorporated proteins, as well as cognate receptors present on the T cell surface, to better mimic membrane properties *in vivo* [64–70]. Such partitioned bilayers provide a source for locally mobile ligands confined to a micrometer-sized area, enabling the investigation of important biophysical concepts that are essential for effective dynamic T cell stimulation. For example, TCR signaling can be controlled by maintaining TCR in the periphery and preventing its clustering at the center of the synapse, by means of continuous linear barriers that increase TCR-associated phosphorylation, as well as intracellular Ca^{+2} levels [66]. Furthermore, in an attempt to provide a dynamic portrait of TCR complex transport in the IS, DeMond et al. [64] used electron-beam patterned SLBs containing molecular mazes composed of short, periodic linear fences, to observe that TCR clusters are deflected by maze fences, when they encounter them. Following deflection, TCR clusters continue to move parallel to the fences, at speeds that scale with the relative angle of motion to the preferred centripetal direction. Moreover, detailed examination of the centripetal movement of TCR microclusters, and their capacity to navigate around small obstacles, revealed that a frictional drag force determined by the coupling chemistry (e.g., via talin) to the dynamic actin cytoskeleton, drives the movement of protein microclusters.

An alternative strategy to that of SLBs involves the precise arrangement of proteins of interest on solid substrates, then

utilizing these molecular patterns as artificial antigen bio-interfaces. In contrast to SLBs, here cells are unable to change the ligand location during their interactions with the substrates. Indeed, immobilized ligands do not mimic native physiology; however, such approaches provide more precise insights into receptor organization at the micro- and nanoscale levels, and enable distinct manipulation/control of the immunological cell response [25]. For instance, Doh and Irvine [71] patterned different micrometer-sized lateral ligand distributions on solid substrates. They utilized photolithography to create activation sites of various shapes, each consisting of anti-CD3 patches surrounded by adhesive molecules (ICAM-1), and simulating the synaptic pattern between $CD4^+$ T cells and artificial APCs. $CD4^+$ T cells seeded on these surfaces assemble into an IS whose structure is modulated by the anti-CD3 ligand pattern. They also found that ligand distribution significantly influenced T cell response. For example, when anti-CD3 ligands were patterned so as to prevent TCRs from forming a central cluster, T cells failed to form stable contacts with the activated sites, resulting in a significant reduction in cytokine secretion.

Higher-resolution patterning, down to single ligands (i.e., less than 10 nm) may be obtained using block copolymer self-assembly at interfaces, in combination with domain-specific functionalization using proteins [72]. The method is based on self-assembly of amphiphilic macromolecules, which create periodic patterns of nanometer-sized gold particles (Fig. 3A). Such nanoparticle arrays serve as templates in which each individual gold particle serves as an anchor point for the selective binding of biomolecules (Fig. 3D). The final substrates feature periodic molecular patterns, and constitute an ideal platform for regulation of the number and nanoscale arrangement of ligands [73]. Moreover, a standard photolithography technique was applied, to superimpose additional structures onto the self-assembled pattern. Such laterally separated, micrometer-sized nanopatterned islands (Fig. 3B) enabled investigation of clustering effects that depended on high local or low global ligand density, respectively [74]. Nanopatterned as well as micropatterned ligand arrays have successfully been used to investigate cellular spreading and adhesion forces, as well as the polarization and migration behavior of fibroblasts, at the single-receptor level [75–79].

Very recently, this well-established nanolithography technique was employed by Matic et al. [80] and Deeg et al. [81] to present T cell ligands, with nanometer-scale accuracy. Matic et al. fabricated nanopatterns with anti-CD3 presentation, and investigated the activation of primary human $CD4^+$ T cells, depending on the specific anti-CD3 arrangement. They demonstrated that activation-

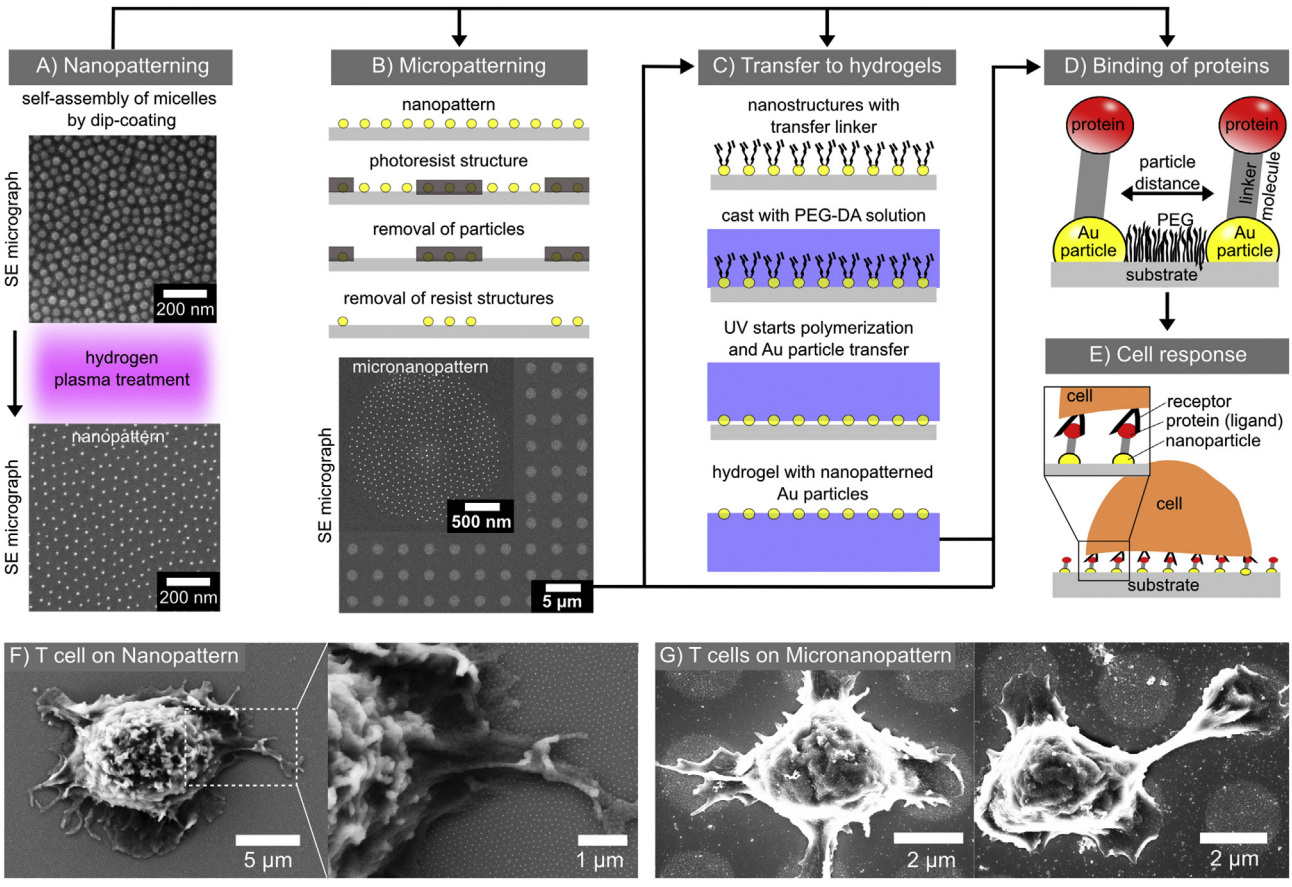

Fig. 3. Fabrication of nanoscale protein patterns on rigid and soft substrates for cell studies. A) Gold-loaded micelles self-assemble on rigid substrates during a spin- or dip-coating process. Hydrogen plasma removes the polymer shell, and leaves quasi-hexagonally ordered gold nanoparticles on the surface. B) An additional photolithographic technique creates micrometer-sized islands of nanopatterns, separated by empty areas. C) Gold nanoparticles can be transferred to soft hydrogels, in order to create elastic, nanopatterned substrates. D) Gold nanoparticles can be used as anchor points, to selectively bind biomolecules such as stimulating or adhesive ligands. The distance between adjacent gold nanoparticles can be varied, from 20 to 300 nm. The space between the gold nanoparticles is coated with a bio-inert PEG layer, to prevent non-specific cellular interactions. E) These substrates enable researchers to present ligands to cells with extraordinary precision, and investigate cell–matrix interactions at the single-receptor level. F) Scanning electron micrograph of a T cell on a nanopattern presenting stimulating peptide-loaded MHC molecules (particle spacing is approximately 90 nm). G) Scanning electron micrographs of T cells on micronanopatterns presenting stimulating peptide-loaded MHC molecules. T cells preferentially establish contacts with the nanopatterned patches presenting stimulating peptide-loaded MHC molecules.

related effects strongly correlated with anti-CD3 density on nanopatterned surfaces. In essence, immobilization of anti-CD3 via nanopatterning techniques had two effects: (i) Each assessed activation read-out (CD69 up-regulation, IL-2 production, cell proliferation) was significantly elevated for T cells on nanopatterned surfaces, compared to T cells on anti-CD3-coated plastic dishes; and (ii) T cell response could be even more finely-tuned, by varying the density of the nanopattern. Cellular activation-related effects reached a plateau at a 60 nm inter-particle distance, and declined significantly at inter-particle spacings of between 60 and 100 nm.

In a similar manner, Deeg et al. replaced the APC surface with nanopatterned antigen arrays. Instead of antibodies, they used stimulatory peptides (antigens) loaded on major histocompatibility complex class II proteins (pMHC), to simulate the physiological antigenic stimulation of mice $CD4^+$ T cells. In agreement with Matic et al., they could show that these nanopatterned antigen arrays induce T cell responses in a dose-dependent manner. Furthermore, they demonstrated that the overall number of presented pMHCs dominates over local pMHC density. This finding implies that subcellular-sized patches of high pMHC density are unable to induce T cell activation. Instead, the presence of pMHC throughout the entire cell-substrate contact area was found to be essential, at least in the absence of additional adhesive molecules. An area-wide pMHC density of approximately 110 molecules per μm^2 was identified as the threshold for T cell activation solely based on pMHC. The authors assumed that on substrates where pMHC molecules are more sparsely scattered, pMHCs are unable to compensate for the absence of adhesive molecules. In the latter case, the presence of adhesive ligands would be pivotal to supporting the clustering of T cell receptors, resulting in further activation events. In contrast, if the pMHC density is above the threshold value, additional co-stimulatory and adhesion-mediating molecules may only serve as signal amplifiers, and may not be a crucial requirement.

We described above the advantages of using multi-component protein micro- and nanopatterned surfaces to control ligand composition and distribution for T cell activation studies. We also highlighted the powerful role of SLBs in modulating the spatial dynamic organization of IS components to direct T cell activation. Nonetheless, the major disadvantage of these systems lies in their inability to enable T cells to exert force in all three dimensions. Unlike biological membranes, planar bilayers do not permit any pushing or pulling of proteins through force. Instead, proteins are trapped on the glass surface, causing rigidity in the vertical dimension.

As previously mentioned, IS formation upon interaction with APCs is also characterized by a dynamic cytoskeleton, and integrin (LFA-1) interactions. This suggests the involvement of mechanical forces in T cell activation. Recent research supports that theory, showing that TCRs are sensitive to forces in the pN range, and indicating an important role for cytoskeletal assembly and contractility in APCs during T cell activation [82,83]. In another study, Hosseini et al. [84] employed single-cell force spectroscopy to quantify the degree of force between different interacting T cell-APC pairs. Dynamic analysis of antigen-specific T cell-APC interactions revealed a strong correlation between the kinetics of IS formation and the interaction forces.

Inspired by these observations – and considering that T cells are unlikely to encounter stimulatory surfaces with the stiffness of glass *in vivo* – much effort has been put into the development of biocompatible compliant supports that more closely mimic the physiological elasticity ranges found in living organisms. For instance, viscoelastic materials such as polyacrylamide (PA) [85] and poly(ethylene glycol)-diacrylate (PEG-DA) hydrogels [86–88] (Fig. 3C), or polydimethylsiloxane (PDMS) [89] elastomers, have been applied as extracellular bio-interfaces to answer questions such as these: 1) which range of stiffness values can T cells sense; and 2) to what degree can changes in the substrate Young's modulus (E_Y) determine T cell fate?

A recent study by O'Connor et al. [89] revealed the important, previously unrecognized influence of the APC's mechanical properties on T cell fate. In this study, the impact of PDMS substrates with altered mechanical properties (E_Y < 2 MPa) on the *ex vivo* activation, expansion and differentiation of human naïve $CD4^+$ and $CD8^+$ T cells was examined. It was shown that soft (E_Y < 100 kPa) substrates support, on average, a fourfold increase in overall T cell expansion, compared to stiffer (EY ~ 2 MPa) substrates. This effect was observed in both CD4+ and CD8+ T cells. Moreover, CD4+ T cells expanding on soft substrates yield an average threefold greater proportion of IFN-γ-producing type 1 helper (Th1) T cells. This observation could indicate that naïve T cells expanding on soft materials can function more effectively following adoptive therapy in cancer [90]. In the case of clinical-grade culture systems for *in vitro* T cell expansion, these findings could contribute to more rigorous material selection, with an eye to mechanical property control.

Mono-disperse (4 or 5 μm diameter) polystyrene or magnetic iron oxide rigid bead-based APCs endowed with anti-CD3 and anti-CD28 antibodies were qualified as clinical-grade culture systems for T cell expansion in adoptive therapy (Fig. 4A) [91–95]. Bead-based APC systems combine the advantages of 2D configuration with surfaces of well-defined molecular composition, together with the possibility of effectively contacting a large number of T cells at once [94]. Establishing contact between T cells and beads in suspension is considered to represent *in vivo* conditions more realistically than those mimicked on solid planar systems. Moreover, ease of preparation and bio-functionalization made these beads the first choice for many interesting investigations that discovered important biophysical concepts. For example, the co-stimulatory effects of ICAM-1 and anti-CD28 differed, depending on the cell type used: CD8+ cells were similarly co-stimulated by ICAM-1 and anti-CD28, while $CD4^+$ cells were co-stimulated only by ICAM-1, in addition to anti-CD3 [96]. Another study examined the effect of cytokine delivery on long-term stimulation. It was shown that without addition of exogenous cytokines, the expansion of T cells in response to stimulatory and co-stimulatory signals provided by TCR and CD28 or LFA-1 was limited in time (up to 4 days) [93]. Bead-based APC surrogate systems can provide adequate control over signal delivery, but the approach has a remarkable limitation. The ability of these systems to serve as optimal APC analogs is mainly hindered by their inability to dynamically remodel their protein composition, in stark contrast to natural APC-T cell interactions.

More recently, Platzman et al. [97] synthesized and developed a novel approach to form gold-nanostructured, specifically bio-functionalized droplets of water-in-oil emulsions, with the potential to serve as 3D APC surrogates. By means of a drop-based microfluidic device (Fig. 4B), they succeeded in generating stable droplets (30 μm in diameter) with gold nanoparticles at various densities, then synthesized gold-linked surfactants, and mixed them with other surfactants (not containing gold) at different concentration ratios. To establish specific interactions with Jurkat E6.1 T cells – cells that express α4β1 and α5β1 integrins and exhibit activation-dependent regulation of integrin-mediated adhesions – the nanostructured droplets were functionalized with cyclic arginine-glycine-aspartic acid peptide (cRGDfK). Remarkably, more than 90% of encapsulated T cells were found to be in contact with the inner periphery of the droplets, compared to less than 10% in the case of non-functionalized droplets. Moreover, the cells proliferated in the functionalized droplets and remained viable for up to 5 days of incubation, without the addition of any further supplements [86]. This study highlighted the advanced

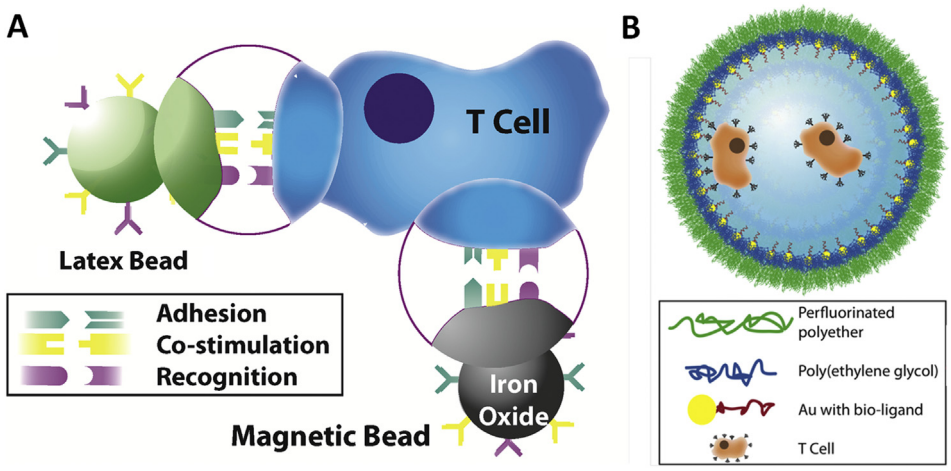

Fig. 4. A) Schematic representation of latex and magnetic bead-based APCs. Note: for the sake of clarity, the scale presentation is such that two types of bead-based APCs, in contact with the same T cell, are illustrated. B) Schematic representation of two encapsulated T cells inside a gold nanostructured and bio-functionalized water-in-oil emulsion droplet.

properties of emulsion droplets for use in T cell stimulation. The combination of flexible bio-functionalization and pliable physical droplet properties can play a crucial role, because it results in a flexible and modular system that closely models *in situ* APC-T cell interactions.

4. Monitoring of cellular behavior and fate in confined synthetic niches

Synthetic cellular microenvironments enable the precise characterization of cell state, phenotype, and behavior, at a level of resolution that is difficult to obtain in natural cellular environments within tissues. The well-defined geometries and composition of synthetic devices, as discussed above, and their compatibility with a number of imaging technologies, enable high-resolution monitoring of static and dynamic aspects of cellular behavior, over a range of spatial and temporal scales. These properties are of utmost importance for studies of heterogonous cell populations such as immune cells, for which methods for scrutinizing cell state dynamically, and at single-cell resolution, are desired.

Monitoring the dynamic state of individual immune cells in their natural, *in vivo* environment using intra-vital imaging is highly challenging, for a number of reasons. First, cell motility makes it difficult to track the same cell over a sufficient period of time to enable the monitoring of individual cells during prolonged processes such as immune cell activation and differentiation. Secondly, a low signal-to-background ratio currently precludes *in vivo* measurements of gene expression levels, using fluorescent reporter proteins or fluorescently labeled antibodies. Finally, it is very hard to control or manipulate the cellular environment in a well-defined manner, obscuring assessment of the relationship between environmental signals, and the cellular responses measured. Synthetic immune niches provide control over many of these parameters, and progress is being made toward dynamic, single-cell measurements of primary immune cells in synthetic environments of ever-growing complexity. Such studies would be of great importance to promote investigation of the mechanisms underlying cell-to-cell variability, and provide insights into the ways in which intercellular interactions influence the collective behavior of immune cells.

Live-cell imaging of primary lymphocytes *in vitro* presents a different challenge, as these cells are non-adherent, and display high motility upon activation and differentiation. One promising method to avoid such difficulties is to utilize microfluidic devices to trap, culture and monitor immune cells over long periods of time. Microfluidic devices have been used, for example, to study quantitative gene expression [98] and signaling responses [99–102] in single mammalian cells, following their exposure to various stimuli. In addition, the use of microfluidics enables the measurement of immune cell migration [103,104], Ca^{2+} influx upon stimulation [105,106], and secretion of signaling molecules [107–109]. Using hydrodynamic cell trapping structures [110] in microfluidic channels, we dynamically monitored IL2-GFP expression, as well as cell proliferation following activation of primary murine T cells (Fig. 5A). Microfluidic chambers also enable continuous flow and medium exchange during an experiment [111], or generation of precise chemical and thermal gradients [112]; thus, microfabricated devices can be used to precisely define an extracellular chemical microenvironment at high temporal and spatial resolution. Further examples of the use of microfluidic devices for immune cell characterization may be found in two current reviews [113,114].

In recent years, we and others have demonstrated the use of microwell arrays to capture and culture primary lymphocytes, and to investigate gene expression and cell differentiation [115–117], cytokine secretion [118], and asymmetric cell division [119], as well as the timing of cell proliferation and death [116,120,121], all at the single-cell level. This method, which does not rely on (but is compatible with) cell adherence, benefits from rich statistics, and is broadly applicable in single-cell studies of cell survival, cell differentiation, and isolation of rare cells that cannot be distinguished by other methods [122]. Microwell arrays are compatible with sensitive fluorescence detection [123], and enable high-throughput experiments for statistically significant analysis of heterogeneous cell populations. In addition, such arrays enable cell trapping with minimal perturbation, as cells are not tethered to the surface. The microwells themselves are separated, but growth medium is shared, allowing for cytokine-mediated communication between cells in different microwells.

Furthermore, microwells can be patterned, using microcontact printing of active molecules onto their surface. For example, microwells were patterned with fibronectin, using subtractive microcontact printing and surface passivation, for the long-term culture of marrow stromal cells [124]. In another study, supported lipid bilayers that carried protein ligands were integrated into microwells to activate human T cells, followed by analysis of cytokine secretion from these cells at later time points [125]. These studies exemplify the versatile nature of these devices, which can

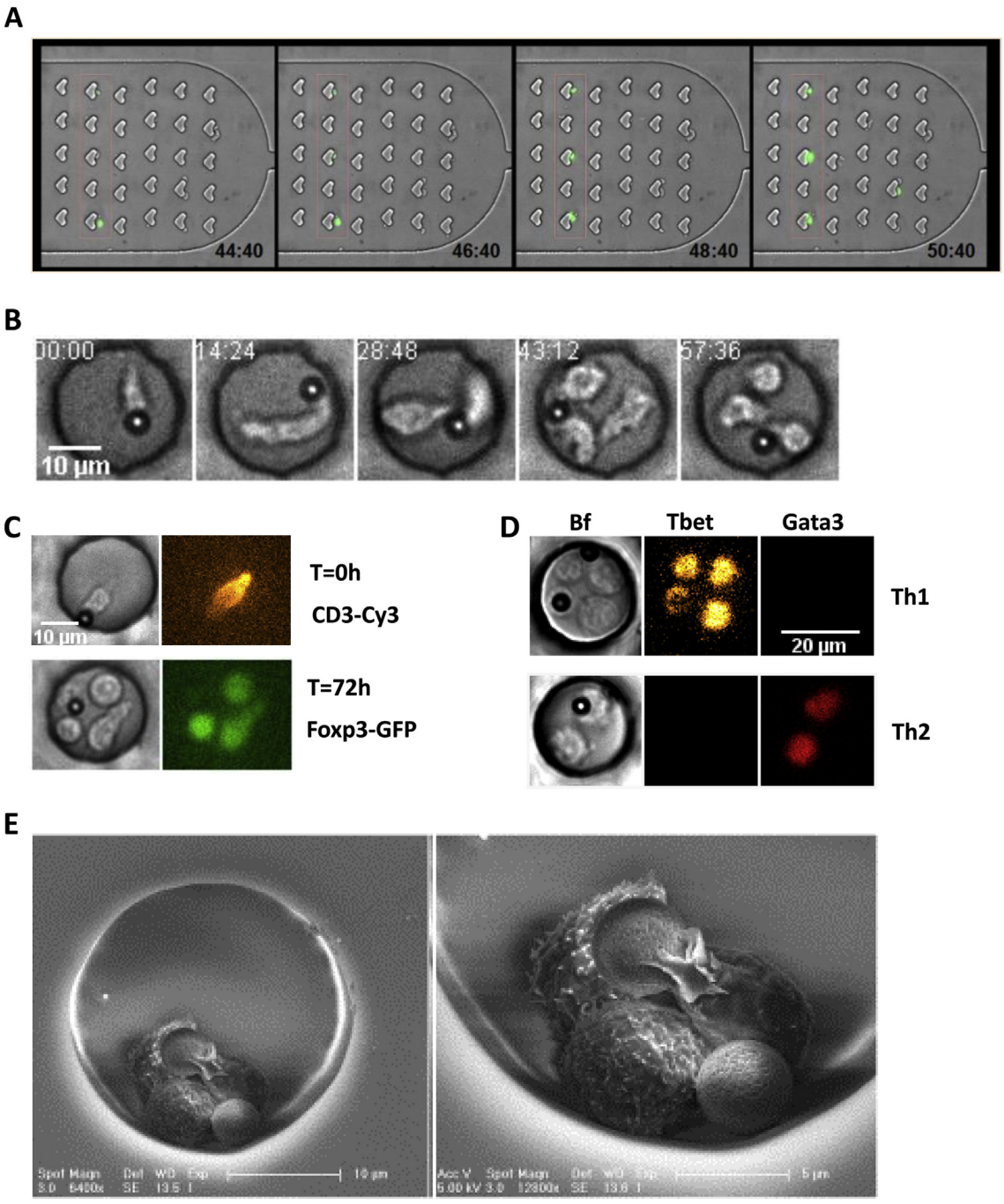

Fig. 5. Monitoring cellular state and fate using microwell arrays and microfluidic devices. A) Representative microscope images of primary mouse T cells that were activated and monitored in a microfluidic device with cell traps, over the course of a 72 h experiment (courtesy of Nir Waysbort). Cells express GFP as a reporter for IL2 promoter activity. B) Dynamics: Representative microscope images of a typical microwell within a microwell array, sampled over the course of a 60 h experiment. A primary T cell is stimulated and proliferates, upon interaction with an activation microbead. C) Correlating initial cell state with cellular fate: The initial level of TCR expression is evaluated by αCD3-PE (top − bright field and fluorescence channels). The same cell is monitored for 72 h under conditions that drive Treg differentiation, and Foxp3-GFP levels are measured (bottom− bright field and GFP channels). D) CD4 T cell differentiation: Microscope images of microwells containing T cells cultured for 96 h with TCR activation, co-stimulation, and either IL12 + IFNγ (driving Th1 differentiation; top) or IL4 (driving Th2 differentiation; bottom). Cells were monitored for 4 days, and were then fixated and stained within the microwells with αTbet-PE (orange) and αGATA3-APC (red). Patterns of expression of these lineage-specifying transcription factors confirm the expected cell states (courtesy of Inbal Eizenberg). E) Scanning electron microscopy: Representative images of T cells stimulated by activation microbeads in a microwell array. Cells were cultured for 2 days, and then fixated and prepared for SEM imaging within the microwell array.

accommodate different methods for surface modification, combined with long-term culture and a variety of cell characterization techniques.

In addition, microwell arrays enable monitoring of a number of cellular characteristics simultaneously, and over long periods of time, covering a large number of cells at single-cell resolution (Fig. 5B). Indexing of microwells in the array provides registration, such that measurements of an individual cell obtained by different imaging approaches, can be combined. We have demonstrated the dynamic characterization of a large number of cellular responses at

the single-cell level, using live imaging of primary lymphocytes in microwell arrays [116,126]. Cell viability and cell death events can be continuously monitored and timed, using continuous propidium iodide staining [116]. The degree of cell differentiation can be traced through single-cell analysis of lineage-specific transcription factors in cells obtained from genetically altered mice (e.g. Foxp3-RFP [127] for monitoring regulatory T cells, or RORγ-GFP [128] for Th17 cells). Dynamic information on levels of protein expression can also be obtained by live staining of cell state-specific surface markers with fluorescently labeled antibodies, which are added at low concentrations to the culture medium. Upon expression of the target protein on the cell surface, cells are decorated with the specific antibodies to generate a detectable signal, which can be used to monitor changes in levels of the studied protein over time. This strategy was used for studying the dynamics of immunoglobulin class switching in B cells [117], as well as for monitoring the dynamics of T cell activation [116].

Using registration, the initial cell state can be evaluated, and correlated with later cellular process. As an example, naïve T cells isolated from the spleen of a Foxp3-GFP mouse were loaded into a microwell array in which they were stained with αCD3-PE antibody, to evaluate initial levels of expression of the T cell receptor (TCR). The cells were then cultured in the microwells for 3 days, in the presence of cytokines promoting regulatory T cell (Treg) differentiation. Cell state was then evaluated by dynamically monitoring the expression of the Treg transcription factor Foxp3-GFP, as well as the degree of cell proliferation (Fig. 5C). Hence, the initial variability in TCR levels between the naïve progenitor T cells (indicated by an αCD3 stain) could be correlated to the dynamics of consequent Treg differentiation (evaluated by an increase in Foxp3-GFP levels, and cell proliferation).

Similarly, cells can be evaluated at an experimental end-point, relating, for example, expression levels of specific transcription factors, to the preceding history of the same cell. This was demonstrated by experiments in which naïve T cells were cultured in microwells for 4 days, in the presence of Th1/Th2 promoting cytokines, and were then fixed and stained for lineage-specifying transcription factors (Tbet and GATA3, respectively, Fig. 5D). Microwells were also shown to be compatible with high-throughput detection of mRNA levels within single cells [129], further extending the ability to characterize cell state.

In addition to fluorescent microscopy, microwell arrays enable multi-parameter analysis of single cells, using different imaging modalities. For example, T cells can grow within surface-coated microwell arrays, followed over time with live-cell fluorescence microscopy, and then fixed within the array and imaged using scanning electron microscopy (SEM), to provide subcellular-level details such as the morphology of interacting T cells during activation (Fig. 5E). Another example concerns the co-culturing of T cells with dendritic cells (DC) that present a cognate antigen. T cell activation, clustering and proliferation can be monitored by live-cell imaging for a few days, followed by fixation and imaging of 3D cell clusters, by means of confocal microscopy.

Finally, different functional assays can be performed within microwell arrays, and their outcome related to other cellular characteristics and history. Thus, measurements of cytokine secretion by micro-engraving were followed by a killing assay, to correlate patterns of cytokine expression with cytotoxic T cell activity at the single-cell level [118]. Microwells were also used to study cytolytic activity of natural killer cells at the single-cell level [130,131], and to monitor the suppression of effector T cells by regulatory T cells [116].

To summarize, the combination of surface patterning with microfluidic devices and microwell arrays creates new possibilities for monitoring cellular behavior under controlled conditions, at single-cell resolution, and over long periods of time. Cells can be characterized using a variety of imaging approaches, and data can be combined to generate dynamic molecular profiles of individual cells in conditions that mimic physiological settings.

5. In conclusion

In this review, a synthetic approach for studying the complex encounters between T cells and other diverse cell types and extracellular matrices within the "immune niche" of the lymph node was discussed. We primarily addressed the notion that in view of the huge complexity of the natural niche, the use of a precisely engineered "synthetic immune niche" might provide novel insights into the mechanisms underlying specific T cell activation. Key advances enabling the development of this synthetic approach arose out of recent progress made in materials science, nanotechnology, molecular genetics, quantitative automated imaging, and computational biology. Together, these advances enable identification of defined extracellular, environmental cues that drive T cell activation and differentiation along a desired pathway, the nature of the genes that are involved in sensing these cues, and specific physiological cellular responses that are induced by them. A deeper understanding of the underlying mechanisms should pave the way toward the development of novel, *ex vivo* procedures for the stimulation and modulation of T cell function. Finally, it is with gratitude and appreciation that we provide this contribution to a special issue of the Journal of Autoimmunity that honors the distinguished careers of Ruth Arnon and Michael Sela. This special issue is part of a series that honors distinguished immunologists which have included such figures as Abul Abbas, Pierre Youinou, Noel Rose, and Ian Mackay and attempts to highlight subjects that are uniquely important to immunologists [132–134]. It is particularly noteworthy that the work described herein is a direct reflection of the classic paper in 1960 by Michael and Ruth on studies of the chemical basis of the antigenicity of proteins [1].

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