High-resolution imaging of the immunological synapse and T-cell receptor microclustering through microfabricated substrates

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T-cell activation via antigen presentation is associated with the formation of a macromolecular membrane assembly termed the immunological synapse (IS). The genesis of the IS and the onset of juxtacrine signalling is characterized by the formation of cell membrane microclusters and the organization of such into segregated microdomains. A central zone rich in T-cell receptor (TCR)–major histocompatibility complex microclusters termed the central supramolecular activation cluster (cSMAC) forms the bullseye of this structure, while the cellular interface surrounding the cSMAC is characterized by regions enriched in adhesion and co-stimulatory molecules. In vitro, the study of dynamic TCR microcluster coalescence and IS genesis in T-cell populations is hampered by cell migration within the culture system and resolution constraints resulting from lateral cell–cell contact. Here, we detail a novel system describing the fabrication of micropit arrays designed to sequester single T-cell–antigen presenting cell (APC) conjugates and promote IS formation in the horizontal imaging plane for high-resolution studies of microcluster dynamics. We subsequently use this system to describe the formation of the cSMAC in T-cell populations and to investigate the morphology of the interfacial APC membrane.

Keywords: micropits; microclusters; CD3; T-cell; immunological synapse

1. INTRODUCTION

Juxtacrine signalling and cell–cell interactions involve multiple adhesion and regulatory molecules, the collective interaction of which can direct cell activation and differential cell function. In particular, T-cell recognition and activation is mediated by clonotypically distributed αβ and γδ T-cell receptor (TCR) molecules that interact with peptide-loaded major histocompatibility complexes (MHCs) presented on the antigen-presenting cell (APC) membrane [1]. The antigen-specific chains of the TCR do not possess signalling domains per se but instead are coupled to the multi-subunit signalling apparatus CD3, to form the TCR–CD3 receptor complex [2,3]. The mechanism by which TCR ligation can directly regulate the T-cell signalling apparatus remains elusive in immunology. It seems clear however that a sustained T-cell response involves the engagement of multiple co-stimulatory and adhesion membrane receptors, TCR oligomerization and a high-order arrangement of TCR–MHC complexes at the T-cell–APC interface.

Coined by C. Sherrington from the Greek ‘syn’ (together) and ‘haptein’ (to clasp) to signify neuronal cell–cell junctions [4,5], the term immunological synapse (IS) was first extended to T-cell biology by M. Norcross to describe the interfacial interaction that occurred between a T-cell and an antigen-presenting B-cell [6,7]. Later, Kupfer and colleagues revealed a compartmentalization of the interactions at the interface of the T-cell and antigen-presenting membranes [8], whereby signalling and adhesion molecules self-organize into concentric regions at the IS. A central TCR–MHC-rich zone termed the central supramolecular activation cluster (cSMAC) forms the bullseye of this structure, while the cellular interface surrounding the cSMAC, termed the peripheral supramolecular activation cluster (pSMAC), denotes an outer region enriched in cell–cell adhesion molecules. Parallel studies with supported planar bilayers have led to the proposal that the active organization of segregated

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adhesion molecules and antigen receptors constitute the hypothesized IS [9,10], and that this segmented organization is important in the regulation of lymphoid juxtacrine signalling processes. Studies to date suggest that the macrostructure of the cSMAC is formed from the centripetal streaming of plasma membrane microclusters, TCR aggregates which measure approximately 1 μm in diameter [11–13]. Further to this, evidence suggests that the spatial organization of the clustering within the IS plays an active role in regulating the signalling state of individual molecular components, and thus can alter T-cell activation [14–17].

In vitro study of the genesis of the IS and the dynamic process of TCR microcluster coalescence in T-cell populations is hampered by cell migration within the culture system as well as by resolution constraints resulting from lateral cell–cell contact relative to the normal trajectory of the incident light (figure 1a,b). At present, high-resolution imaging of the IS in a live cell system relies on the use of several stratagems to facilitate the study of TCR microcluster dynamics, all of which are associated with specific advantages and drawbacks.

Confocal volume rendering is commonly used in biological confocal imaging and uses a combination of optical and computational techniques to acquire a series of x, y-plane images, which are recorded along the z-axis and the signal volume rendered for quantitative analysis. Computer algorithms are used to compensate the absence of interplane detail and to reconstruct a three-dimensional image from a rendered z-stack [18]. This method is commonly employed to study the dynamics of IS formation in lateral T-cell–APC conjugates [8,19], and can be used to rapidly acquire three-dimensional information of the IS morphology and structure. Deconvoluted and three-dimensional rendered images however suffer from artefactual distortion, and a loss of resolution in the z-dimension. As a result, reliable interpretation of these images may be difficult or even impossible, particularly in dynamic live-cell systems [20].

Laser tweezer or laser trap technology has recently been implemented for the manipulation of T-cell–APC conjugates in order to resolve dynamic microcluster localization to the IS [21]. Importantly, this system allows vertical cell orientation, placing the IS within the horizontal x,y-plane for high-resolution imaging experiments [22]. This system suffers however from extremely low throughput owing to the manual intricacies needed for cell capture and alignment, which is carried out in single cell conjugates. A further limitation of this method arises from cellular phototoxicity complications, resulting from prolonged cellular exposure to the trap beam or thermotoxicity owing to local heating of the medium.

As mentioned above, supported lipid bilayers formed on glass surfaces have driven current models for IS formation and sustained T-cell signalling [23]. The continuous fluid lipid bilayer forms from the spontaneous self-assembly of liposomes or proteoliposomes on a clean glass surface, and imitates the phospholipid bilayer of the APC membrane [24]. Membrane proteins can subsequently be inserted into the bilayer and undergo the free lateral mobility that is inherent to native cell membrane proteins [25–27]. Additionally, the well-defined planar interface facilitates high-resolution imaging, particularly by total internal reflection microscopy [28,29]. While the bilayers provide a powerful approach, testing the predictions from this model system requires high-resolution imaging of the T cell–APC interface. Further, other phenomena requiring membrane dynamics of the APC are lost when the APC is replaced by a supported lipid bilayer.

Recently, Maus et al. [30] have described an artificial APC (aAPC) system derived from the chronic myelogenous leukaemia line K562 and subsequently transduced to express an array of T-cell stimulatory ligands. K562 cells do not naturally express MHC or T-cell co-stimulatory ligands, yet do retain many other attributes that make dendritic cells (DCs) such effective APCs, such as cytokine production, expression of the adhesion molecules ICAM-1 and LFA-3 that enhance T-cell–APC interactions, and macrophagocytosis. These cells can be readily transduced with a library of lentiviral vectors for the customized expression of stimulatory and co-stimulatory molecules that can be used to activate and expand different subsets of T cells.

Here we detail a novel system describing the fabrication of micropit arrays designed to sequester a single T-cell–aAPC conjugate and promote IS formation in the horizontal imaging plane for the high-resolution study of TCR microcluster dynamics (figure 1c). We used K562-based aAPCs which expressed multiple gene inserts, including human lymphocyte antigen (HLA)-A2, CD64 (the high-affinity Fc gamma type 1 receptor) CD80, CD83, CD86, CD137L (4-1BBL) and CD252 (Ox40L), and have proved to be more efficient in activating and expanding CD8+ and CD4+ T-cells than the magnetic bead-based

![Image](http://rsif.royalsocietypublishing.org/)

Figure 1. Imaging the immunosynapse with micropits. (a,b) Lateral orientation of the T-cell–APC interface prevents detailed analysis of microcluster dynamics. Green, Jurkat T-lymphocytes; blue, immune synapse (TCR). (c) Ideal plane of imaging to study the dynamic rearrangement of microclusters at the IS in a cell–cell system.
Figure 2. The fabrication regime for micropit substrates (a) An array of circles is written in a 3 μm thick resist layer via photolithography. (b) The pattern is developed and etched via reactive ion etching (RIE), forming a master mould in silicon. (c) The master pillar mould is used to transfer an array of pits into a PDMS-coated cover-glass. (d) Experimental micropit substrates were 40 μm in depth and 20 μm in diameter. (e) aAPCs and T-cells were sequentially loaded into the pits forming cell conjugates for imaging. (Online version in colour.)

aAPC system [31]. APCs were loaded with a fluorescently tagged anti-CD3 marker and T-lymphocytes were subsequently loaded onto the sequestered aAPC cells to study the dynamics of microcluster genesis and the recruitment of the TCR to the IS. The system successfully isolated and allowed large-scale analysis of the immunological interface in thousands of T-cell–APC conjugates, facilitating both fixed and live-cell imaging.

2. MATERIAL AND METHODS

2.1. Micropit fabrication

Master moulds for pit substrates were made by etching silicon wafers patterned with arrays of circles in photoresist. The SPR 220-3 photoresist (Shipley, MA) was spin-coated at 3000 r.p.m. to a thickness of 5 μm and soft-baked for 90 s at 115 °C. The resist was patterned with a square array of circles with a series of defined widths ranging from 15 to 23 μm and a 50 μm pitch. Patternning was carried out with a GCA Autostep 200 DSW i-line Wafer Stepper with a 200 mJ pattern exposure dose. Samples were developed in 300 MIF developing solution (AZ Electronic Materials, NJ, USA) for 2 × 30 s at room temperature (RT). Samples were then rinsed in ultrapure H2O. The resist was then used as an etch mask in a Bosch etch UNIAXIS 770 reactive ion-plasma etcher tool. Samples were etched in a C4F8/SF6 atmosphere for approximately 11 min to a depth of 40 μm to form pillars. The residual resist was subsequently removed using a YES CV200RFS Asher oxygen plasma for 60 s at 80°C and 70 mT (Yield Engineering Systems, CA, USA).

Master pillar substrates were placed at 5 Torr in a vacuum chamber (Nalgene, NJ, USA) with 200 μl of 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (Gelest, PA, USA) for 12 h, to form an anti-adhesive coating. Microscope cover-glasses (Corning, NJ, USA) (22 mm² No. 1.5) were cleaned for 12 h in a 1 per cent v/v solution of the detergent MICRO-90 (International Products, NJ, USA), rinsed in reverse osmosis water (ROH2O) and blown-dry in a stream of filtered nitrogen. Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning, MI, USA) was mixed with the supplied accelerating agent at a ratio of 10:1 for 5 min and degassed under vacuum for 10 min at 5 Torr. PDMS of 0.5 ml was applied to the microscope cover-glasses and spin-coated for 45 s at 2000 r.p.m. and an acceleration of 400 r.p.m. s⁻¹ to form a uniform film. PDMS-coated cover-glasses were applied to the master pillar substrates at 50 N on a hotplate, and the temperature was ramped to 215°C over 1 min. Substrates were cured for 5 min, then allowed to cool and separated from the master moulds.

Experimental substrates were sealed with vacuum grease onto aluminium microscopy slides, which were fabricated with a central square-cut viewing window. Substrates were immersed in a 10 per cent w/v pluronic F68 solution (Sigma-Aldrich, MO, USA) for 30 min and then rinsed in phosphate-buffered saline (PBS; pH 7.4) for 3 × 5 min and covered with 1 ml of serum-free RPMI 1640 medium. Substrates were then placed within an evacuation chamber for 5 min at 5 Torr to remove any residual air trapped within the individual pits. Figure 2 shows a schematic description of micropit substrate fabrication.

2.2. Cell culture and labelling

The K562 erythromyeloid cell line was used as a cellular scaffold and was engineered to stably express CD64, the human high-affinity Fc-gamma type I receptor, the CD28 ligand CD80 and 4-1BB ligand (for more information see [30]). The Jurkat subclone E6.1, originally derived from a CD4⁺ T-lymphocyte leukaemia cell line was generously provided by Prof. Michael Dustin, NYU. Both cell lines were maintained in suspension in RPMI 1640 Medium (Gibco, CA, USA) supplemented with 10 per cent FCS and 0.3 g l⁻¹ L-glutamine in a 5 per cent CO₂ atmosphere at 37°C. Cell medium was replaced every 2 days.

Prior to substrate loading, Jurkat E6.1 cells were pulsed with Fluo-4 direct calcium detection label (Invitrogen, CA, USA), according to manufacturer’s instructions. Briefly, 10 ml of a cell suspension was
centrifuged at 1500 r.p.m. and 9 ml of the medium was removed. Cell pellets were subsequently re-suspended in 1 ml of Fluo-4 to yield a final cell density of 5 × 10⁶ cells ml⁻¹. Cells were incubated at 37°C for 1 h, then the PBS and Fluo-4 dye were removed as previously described and the cell pellet re-suspended in serum-free RPMI 1640 medium.

The therapeutic monoclonal antibody (mAb) OKT3 (Biolegend, CA, USA) [32] was labelled with Cy3 dye (GE Healthcare, NJ, USA) to yield a fluorescently tagged anti-CD3 construct. Then 100 μl of 1 mg ml⁻¹ OKT3 was added to 2.5 ml of Cy3/DMSO solution to give a final dye/antibody ratio of 1:10 m²/m. The labelled antibody was transferred to a Mini Dialysis Unit (Thermo Fisher Scientific, IL, USA) and incubated at RT for 30 min with agitation every 10 min. The 20 000 molecular weight cut-off membrane used ensured that the antibody was retained inside the dialysis unit. Tubes were floated in ultrapure H₂O overnight with agitation at 4°C. The labelled antibody was recovered from the dialysis unit and stored at 4°C. For visualization of the K562 cytoskeleton, a DNA construct coding for human β-actin with an EGFP insert was transfected into K562 cells using the Amaxa Nucleofector System (Lonza, MD, USA). Approximately 5–10 μg of DNA was used per reaction (10⁶ cells). Efficiency, confirmed with a GFP marker, was greater than 50 per cent after 3.5 days.

The aAPCs were seeded onto the micropit array substrates at a density of 2 × 10⁴ in 1 ml of serum-free RPMI 1640 medium. Cells were allowed to settle for 30 min at 37°C before substrates were rinsed three times in PBS to remove non-sequestered cells. Sequestered cells were then pulsed with the OKT3 antibody at a concentration of 1 μg ml⁻¹ in PBS for 10 min at RT, followed by rinsing in PBS for three times for 1 min. Fluo-4-labelled Jurkat E6.1 cells at a density of 5 × 10⁶ ml⁻¹ in serum-free RPMI 1640 were pipetted onto the micropit arrays, which contained entrapped aAPCs and allowed to form conjugates over 1 h.

Cells were rinsed for 1 min in PBS, then stabilized in 4 per cent paraformaldehyde with 1 per cent sucore in PBS for 5 min at RT and rinsed three times for 2 min in PBS to remove unreacted aldehyde. Substrates were wet-mounted in PBS to a second cover-glass and viewed. For live cell imaging, the standard RPMI 1640 medium was replaced with phenol red-free CO₂ independent media (Gibco, CA, USA).

2.3. Scanning confocal microscopy

Cell–substrate and cell–cell interaction were examined by scanning confocal microscopy on a stage maintained at 37°C (live cell imaging). Imaging was performed on an Olympus IX81 confocal microscope with an argon-ion laser (wavelengths 457, 488 and 514 nm) fitted with an Olympus 100 × PLAN Apochromat objective having a numerical aperture of 1.35. Image stacks consisted of 8–15 confocal planes spaced by 0.40 μm.

2.4. Scanning electron microscopy

Cells were rinsed for 1 min in 0.1 M 1,4 piperazine bis(2-ethanosulphonic acid) (PIPES) buffer (pH 7.4). Cells were then stabilized in 4 per cent paraformaldehyde with 1 per cent sucrose in 0.1 M PIPES (pH 7.4) buffer for 5 min at RT and rinsed three times for 2 min in 0.1 M PIPES buffer. Samples were fixed permanently in 2.5 per cent glutaraldehyde for 5 min at RT in PIPES buffer and rinsed three times for 2 min in PIPES buffer. Additional contrasting of the cell was accomplished by staining the cells with 1 per cent osmium tetroxide in PIPES for 1 h at RT.

Cell samples were dehydrated through a graded alcohol series (50, 60, 70, 80, 90, 96 and 100%) for 5 min, followed by an acetone/ethanol series (25, 50, 75 and 100%). The samples were dehydrated in CO₂ with a BAL-TEC CPD 030 critical point dryer (Leica, IL, USA) for 1.5 h, and mounted on aluminium stubs. Substrate and biological samples were sputter-coated with a 12 nm layer of Au at 10 mA and 0.1 mbar using a Croessing 108 sputter coater (Cressington, UK). Samples (both with and without cells) were imaged using a Hitachi S-4700 field-emission scanning electron microscope (FESEM) fitted with an Autrata yttrium aluminium garnet (YAG) back-scattered electron scintillator-type detector. The images were taken in secondary electron mode, with accelerating voltages between 2 and 20 kV. Images were taken with an emission current of 20 μA, an aperture of 100 μm (apit1) and working distances of 10–12 mm.

2.5. Image analysis

The time series started 5 min after adding T cells to the sequestered aAPC. Cells were fixed at 5, 10, 15, 20, 30, 40, 50 and 60 min following conjugation. The images were analysed using Fluoview (Olympus) and ImageJ (National Institutes of Health). Because of the curved nature of the cell–cell interface, acquired stacks consisting of 8–15 confocal planes were rendered using standard deviation image intensity to produce a single image, effectively transforming the curved cell interface into a planar surface. TCR cluster area, frequency and location were analysed in cells from three separate experiments (20 cells each) using ImageJ. The TCR cSMAC was identified in cell conjugates as being the cluster with the greatest area when greater than 3 cells (20 cells each) using ImageJ. The TCR cSMAC was identified in cell conjugates as being the cluster with the greatest area when greater than 3 μm² in a single group. Fluorescence intensity of both microcluster coalescence and calcium flux was recorded with ImageJ via the plot profile function. A transecting line was drawn through each individual cell and the grey value plotted along this line. A cross-correlation analysis by shifting the green image in x-direction pixel per pixel relative to the red image and calculating the respective Pearson’s coefficient was used to analyse the colocalization of actin to TCR microclusters. The Pearson’s coefficient was then plotted as the function of dx (pixel shift) to obtain a cross-correlation function. See van Steensel et al. [33] for more information.

3. RESULTS

3.1. Master substrate fabrication and imprint fidelity

In order to sequester single aAPCs and analyse the IS in a horizontal imaging plane, we fabricated micropit
substrates onto microscope cover-glass via a micropillar master mould. Master micropillar substrates contained square arrays of orthogonally aligned cylindrical pillars, which were verified by SEM to be approximately 40 μm high and possessed defined widths of 15–23 μm. Imprinting of the pillar substrates resulted in the formation of patterned pit arrays as defined by the master substrate pillar dimensions. Micropit substrates were fabricated on microscopy cover-glasses laminated with a 50 μm deep spin-coated film of PDMS.

3.2. Sequestering of aAPC into micropit substrates

Scanning laser confocal and differential interference contrast microscopy revealed that viable cells became localized within the pits 30 min post cell seeding (figure 4a,b). The aAPCs were readily washed away from the hydrophobic PDMS interpit surface, ensuring that cell conjugation occurred exclusively within the pit system. Incubation periods greater than 30 min however resulted in increased aAPC adhesion to the planar interpit substrate (data not shown). Varying the pit
diameter influenced the density of occupied pits (figure 4c), yet a diameter of more than 20 μm introduced an unacceptable increase in peri-cell area, resulting in increased cell mobility within the pits. Substrates greater than 23 μm in diameter were unable to retain cells during rinsing protocols. For this reason, the 20 μm wide pit substrate was identified as optimal for the application of aAPC trapping, and were employed for subsequent imaging experiments.

3.3. T-cell activation

In order to assess the efficiency of T-cell activation by OKT3-loaded aAPCs, the intensity of calcium flux was measured in both conjugated and unconjugated T-cells via a fluorescent indicator (figure 4d). Calcium levels were observed to peak rapidly in T-cells following cell conjugation yet were observed to undergo random fluctuation. This rapid influx of calcium was observed to decline over 1 h, yet fluorescence intensity still remained above that of unconjugated cells (see electronic supplementary material, movie S1).

3.4. Cell conjugate formation and microcluster analysis

T-lymphocytes formed stable and long-lasting conjugates with pit-sequestered aAPCs, facilitating long-term imaging experiments of the IS (figure 5a). The engagement of TCR by the aAPC-bound OKT3 induced the formation of microclusters which measured approximately 1.5 μm² and were observed to coalesce at the IS centre, resulting in the formation of a clearly defined cSMAC following 1 h of culture (figure 5b).

Anti-CD3–TCR microclusters became recruited to the T-cell–aAPC interface 5 min post T-cell seeding. Both total and cSMAC-associated anti-CD3 cluster area increased in a nonlinear manner, producing a cSMAC approximately 20 μm² in area 1 h post conjugation. The proportion of engaged TCR localized at a continuous cSMAC was observed to increase following cellular conjugation, with a maximum cSMAC area of approximately 22 μm² occurring after 50 min. However, only approximately 43 per cent of total anti-CD3-bound TCR was associated with a defined, yet segregated cSMAC, the majority being localized to the pSMAC region (figure 5c). Microcluster frequency was observed to increase following initial cell conjugation and reached a maximum after 15 min. Here, mean microcluster frequency was 14.1 clusters per cell. Microcluster formation frequency declined with time, as single microclusters aggregated and coalesced towards the cSMAC (figure 5d; see electronic supplementary material, movie S2).
3.5. Imaging the dynamics of cSMAC coalescence

In order to determine the dynamics of TCR clustering in T-cell–aAPC conjugates, we conducted imaging experiments of the cSMAC in a live cell system. Live cell imaging revealed the dynamic translocation of punctuate anti-CD3–TCR microclusters, which were visible following 5 min of T-cell seeding. Microclusters were observed to coalesce centripetally and form a discontinuous central microcluster aggregation or cSMAC; however, punctuate TCR microclusters persisted throughout the IS. Following 40 min of cell conjugation, the cSMAC coalesced into a complex geometry, consisting of a network of dense TCR clusters interspersed with regular circular TCR voids. This structure was observed to persist 60 min post cell conjugation (figure 6).

3.6 Morphology of the aAPC interface

To further study the cellular interface, and in particular, the morphology of the aAPC membrane during the formation of the IS, we investigated the structure of the cell–cell interface with both confocal microscopy and SEM of fixed samples.

Following 1 h of cell–cell conjugation, elongated actin-rich cellular processes were observed to extend from the aAPC at the intercellular interface to form a dense complex at the IS (figure 7a,b). This extension network was identified to form an investing ring around the cSMAC (figure 7c,d). This peripheral ring was correlated to the cSMAC using Pearson’s coefficient of correlation test, which determined an absence of colocalization (figure 7e).

4. DISCUSSION

In this paper, we have described a novel high-throughput method for the high-resolution analysis of microcluster dynamics and IS formation in an aAPC–T-cell coculture system. The IS as a concept has evolved from a description of the junction between T-cells and their antigen-presenting partners, to a sophisticated model detailing a dynamic signalling site of receptor and co-stimulatory molecules. The exact mechanisms of microcluster dynamics are still not yet resolved, however the entire process of IS formation and degradation is now known to entail a dynamic reorganization of membrane domains and clustered proteins within and adjacent to those domains, as corroborated by this study.

A number of therapeutic strategies have been shown to modulate T-cell activation by targeting TCR signalling; in particular, the anti-human CD3 mAbs are widely used clinically in immunosuppressive regimes to reduce rejection following organ transplantation [34,35]. In this study, the CD3-specific mouse mAb OKT3 was employed both as an activator of TCR signalling and an immunofluorescent marker of TCR clustering. Although the mode of action of OKT3 involves multiple mechanisms, its activity depends on the specific interaction with the CD3e subunit of the TCR complex [36].

Here, we used fabricated micropits to align and image the dynamic rearrangement of the anti-CD3–TCR complex at the T-cell–aAPC interface. aAPC extensions were observed to coalesce into a circumscribing extension complex, thought to represent a network of podosomes rich in cell–cell adhesion molecules formed at the pSMAC. Although the role of this structure in T-cell function has yet to be defined, the presence of cellular extensions at the IS have been noted in previous studies of T-cell morphology [37,38]. Interestingly, these elongated membranous structures were shown to be involved with the formation of a multifocal IS, similar to those observed in this study, as well as with the transfer of IS-associated protein.

The aAPC podosomes were observed to form peripheral contacts at the T-cell membrane, which seemed to converge towards a peripheral investing ring at the IS.
One intriguing hypothesis for this phenomenon is that this network represents a sealing zone-like structure, a feature of osteoclast adhesion to the bone during resorption. Osteoclasts, also of myelogenic origin, form an actin-rich ring-like adhesion zone circumscribing an area of bone resorption. Formation of a sealing zone (also known as the sealing ring) is considered to be a marker of osteoclast activation and is marked by the presence of a podosomal network [39], actin-rich extensions commonly observed in DCs [40,41]. It may be speculated that this podosome ring can stabilize the IS and further, the idea that the aAPC cytoskeleton plays an important role in the generation of the IS is consistent with the observations that the DC cytoskeleton contributes to T cell activation [42,43].

Interestingly, the role of the APC cytoskeleton in the modulation of microcluster dynamics and T-cell activation is reportedly absent in the most highly studied model for IS formation, namely the synaptic interactions between T-cells and antigen-presenting B-cells [10,44–46]. Studies with B-lymphoma APCs have identified the rearrangement of surface receptors on the APC as a passive event and that B-cell cytoskeletal disruption does not modulate T-cell activation [47], however, studies are emerging on the importance of the APC cytoskeleton in IS formation, particularly in DC-mediated T-cell activation [37,42]. Here, the K562 aAPC, being of myeloid origin may share homologies with both DCs and osteoclast cells, also of myeloid lineage [48]. It is tempting to suggest that apart from providing an adhesive ring around the IS, costimulation of T-cell activation through the transfer of external forces to the IS through contractile APC podosomes may enhance T-cell activation. Several candidate molecules are currently being investigated as mediators of mechanotransduction at the IS; however, recent studies indicate that mechanotransduction in T-cells is mediated through the cell adhesion molecule, LFA-1 [49,50] in a SLP-76-dependent manner [51], a model that allows for the modulation of cell activation by APC-mediated forces.

In APC-engaged T-cells it is thought that sustained TCR signalling is mediated through the continued formation of new TCR microclusters at the periphery of the IS [12,13]. According to this model, TCR microclusters signal as they migrate through the pSMAC but cease signalling activity and are targeted for downregulation upon accumulation at the cSMAC. It can be hypothesized that the disruption to the free diffusion of MHC–TCR complexes within the cell membranes, as induced by a podosome-rich sealing ring, may perturb TCR microcluster flow and subsequent deactivation at the cSMAC. Data acquired in this study suggest that TCR migration velocity may be significantly reduced relative to T-cells cultured on planar bilayers or conjugated to antigen-presenting B-cells. This observation may provide an insight into how these APCs may regulate TCR signalling and T-cell activation by modulating cluster deactivation. In addition, recent evidence suggests that mechanical TCR entrapment into segregated microdomains is required for optimal T-cell activation [17]. Additional experiments to address the specific role of TCR signalling, ligand binding and the role of juxtacellular...
forces on T-cell activation will be required to address this issue.

5. CONCLUSIONS

Here, we describe a system of micropit fabrication coupled with laser confocal microscopy for high-resolution imaging of the IS in T-cell–APC conjugates. This system has many advantages over conventional methodology for imaging the IS, in particular, microfabricated pits allowed the vertical orientation of cell–cell conjugates, facilitating planar imaging of microcluster dynamics at the IS. Importantly, cell conjugates are not substrate adhered, or influenced by the interactions of adjacent cells, factors which may influence microcluster dynamics and cell signalling. Micropit substrates allowed for the simultaneous analysis of multiple cell conjugates within a single imaging field. Further, following master fabrication, the PDMS embossing process is a relatively simple and quick technique requiring little specialized equipment.

Data presented within this paper indicate that active APC processes may play a role in regulating the temporospatial mechanisms of IS formation and T-cell activation through the formation of cellular podosomes, structures that converge at the pSMAC in a specialized ring structure. Future studies will concentrate on a molecular breakdown of signalling pathways along with sophisticated real-time imaging studies to define the specific roles of TCR, the APC membrane and co-stimulatory molecules in T cell activation.

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Imaging the immunosynapse with microtubumps M. J. P. Biggs et al. 1471


