

Quantal concept of T-cell activation: adhesion domains as immunological synapses

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Abstract. Adhesion micro-domains (ADs) formed during encounters of lymphocytes with antigen-presenting cells (APC) mediate the genetic expression of quanta of cytokines interleukin-2 (IL-2). The IL-2-induced activation of IL-2 receptors promotes the stepwise progression of the T-cells through the cell cycle, hence their name, immunological synapses. The ADs form short-lived reaction centres controlling the recruitment of activators of the biochemical pathway (the kinases Lck and ZAP) while preventing the access of inhibitors (phosphatase CD45) through steric repulsion forces. CD45 acts as the generator of adhesion domains and, through its role as a spacer protein, also as the promoter of the reaction. In a second phase of T-cell–APC encounters, long-lived global reaction spaces (called supramolecular activation complexes (SMAC)) form by talin-mediated binding of the T-cell integrin (LFA-1) to the counter-receptor ICAM-1, resulting in the formation of ring-like tight adhesion zones (peripheral SMAC). The ADs move to the centre of the intercellular adhesion zone forming the central SMAC, which serve in the recycling of the AD. We propose that cell stimulation is triggered by integrating the effect evoked by the short-lived adhesion domains. Similar global reaction platforms are formed by killer cells to destruct APC. We present a testable mechanical model showing that global reaction spaces (SMAC or dome-like contacts between cytotoxic cells and APC) form by self-organization through delayed activation of the integrin-binding affinity and stabilization of the adhesion zones by F-actin recruitment. The mechanical stability and the polarization of the adhering T-cells are mediated by microtubule–actin cross-talk.

 Online supplementary data available from stacks.iop.org/NJP/13/065013/mmedia

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1. Introduction

A basic process during immune response is the stimulation of freshly born T-lymphocytes (called naïve) by an encounter with antigen-presenting cells (APC), such as dendritic cells (DC) or macrophages [1, 2, 3], which has two consequences:

1. The activation of the genetic expression and the secretion of cytokines (such as interleukin-2 (IL-2)) that stimulate the proliferation of lymphocytes after binding to cell IL-2 receptors (IL-2R), including the generator cell itself.
2. The transformation of the activated T-cells either into helper cells (which support other cells such as the B-lymphocytes residing in the blood) in their fight against pathogens or into cytotoxic cells (which destroy antigen-carrying cells).

This work deals with the physical basis of two essential membrane-mediated events of the immune response: firstly, the process of naïve lymphocyte activation by immunological synapses (ISs), and secondly, the destruction of APC by cytotoxic T-cells (also called killer cells). Both processes are closely related to the physics of cell adhesion and the adhesion-induced reorganization of the actin cortex and its control by actin–microtubule (MT) cross-talk.

The activation of the T-cells by the APC is mediated by a palette of cell surface proteins and associated helper proteins, some of which are shown in figure 1. The adhesion of the T-cell on the APC (see figure 3 below) leads to the assembly of proteins involved in T-cell activation into micro-domains (also called adhesion domains) from which proteins counteracting the activation (such as CD45 in figure 1) are expelled by steric forces. This activation of the T-cell by numerous

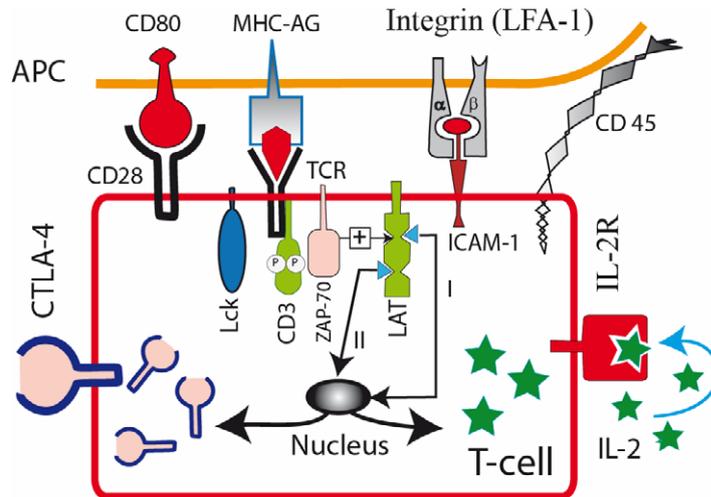


Figure 1. Summary of molecules involved in T-cell activation (see [5], chapter 14, for a detailed presentation). The APC exposes (i) class II MHC (MHC-II) exposing the antigenic peptide and (ii) the intercellular adhesion molecule (ICAM-1), a counter-receptor of the integrins. The T-cell exposes the TCR that recognizes the antigenic peptide bound to MHC-II. It is closely associated with the tetrameric protein complex CD3, which exposes tyrosine segments at the associated ζ -chain that are phosphorylated by the Lck kinase. Other cell surface receptors are CD28 and CD4 (or CD8). The T-cell activation is controlled by the phosphatase CD45, which removes phosphate groups from CD3 and is therefore an inhibitor of the T-cell activation. The lymphocyte–APC adhesion induces the sequential activation of two kinases, Lck and ZAP-70 (synonym for zeta-chain-associated protein), as follows. The ZAP-70 kinase phosphorylates the membrane-bound scaffolding protein LAT (synonym for LATs) that can bind and activate several actuators of the T-cell stimulation (see the bottom of figures 1 and 4). The bottom part of the image points out that binding of the MHC–peptide complex to the TCR triggers the genetic expression of the cytokine IL-2. Binding of IL-2 to the IL-2R stimulates the cell division of the generating cell (autocrine stimulation) as well as other T-cells (endocrine stimulation). The CTLA-4 receptor serves in the down-regulation of the immune response.

adhesion domains formed within the contact zone is reminiscent of the activation of nerve or muscle cells by synapses contacting the target cell. They are therefore called ISs.

After an incubation time of about 10 min, the small adhesion domains reorganize. The T-cell receptors (TCRs) assemble in a large central patch within the T-cell–APC contact zone that is called the central supramolecular activation cluster (c-SMAC). For several years, it was assumed that the T-cell activation is mediated by these long-lived supramolecular complexes. However, more recently, evidence has been provided that the major signalling occurs prior to the SMAC formation [1, 2], while the SMAC serve in the down-regulation of the signalling by internalization (endocytosis) of the TCR–CD3 complexes (as was shown by Varma *et al* [2]).

It appears to be rather well established now that the initially formed micro-clusters act as ISs that trigger the genetic expression.

The APC–T-cell adhesion is mediated by two pairs of cell adhesion molecules (abbreviated as CAM), which are also called the receptor and the co-receptor.

1. The TCRs exposed by the lymphocyte consist of a receptor domain and a closely associated tetramer complex, CD3, which we call the TCR–CD3 complex in the following. It binds specifically to the antigenic peptide, provided that these rather small molecules (frequently peptides of about ten segments) are presented together with the plasma-membrane-bound major histocompatibility complex (MHC-II) (abbreviated as MHC-peptide hereafter).
2. T-cell-bound CAM of the integrin family LFA-1 (synonym of lymphocyte function-associated antigen) binds specifically to ICAM-1 (a CAM of the family of intra-cellular adhesion molecules (ICAM)) exposed by the APC. The ICAM-1 exhibits an extracellular domain composed of five immunoglobulin-like domains, corresponding to an optimal intermembrane distance of about 15 nm.

Three additional sets of cell surface proteins modulate the cell–cell interaction during the later stages of the immune response [5]:

- (a) CD3, a protein complex composed of three pairs of polypeptide chains, is closely associated with the TCR. The supramolecular assembly is called the TCR–CD3 complex below. Two of the chains of CD3 (ζ -chains) extend into the cytoplasmic space. The ζ -chain contains two tyrosine segments that are phosphorylated by the kinase Lck. The kinase ZAP binds to the two phosphate groups and becomes activated (see figure 4).
- (b) CD28 on T-cells mediates co-stimulatory signals that are essential for the immune reaction of naïve T-cells. CD28 is also involved in the T-cell binding to other blood cells. During T-cell stimulation, CD28 is replaced by the co-receptor CTLA-4, which binds more strongly to blood cells, resulting in the down-regulation of T-cell activation. Without this negative feedback mechanism, the cells would produce an excess of cytokines.
- (c) Cell surface proteins of the classes CD-4 and CD-8 control later (secondary) stages of the immune responses. A class of activated T-cells (called helper cells) expressing the membrane protein CD4 helps other immune cells in their fight with intruders. Cells exposing CD-8 become the so-called cytotoxic cells, which kill infected cells exposing a specific MHC-I by a process described below. The genetic expression is mediated by two kinases that are coupled to the inner leaflet of the plasma membrane, namely Lck and ZAP:
 - (i) Lck couples phosphate groups to tyrosine residues at the intracellular tail (called ζ -chain) of the CD3 complex. Lck exhibits two essential tyrosine groups and its activity is controlled by CD45 as follows. One of the groups is located at the COOH-terminal of Lck and the second closer to the amino-terminal. To be fully active, the first group has to be dephosphorylated and the second phosphorylated; CD45 can dephosphorylate both groups. In the absence of CD45, T-cells cannot be activated (see [3] and supplementary data 1, available at [stacks.iop.org/NJP/13/065013/mmedia]).
 - (ii) The second kinase ZAP-70 is attracted by the phosphorylated ζ -chains, resulting in its getting activated by phosphorylation. The phosphorylated ZAP-70 activates a membrane-bound protein linker of activated T-cells (LAT). It acts as a docking station

- (also called a scaffolding protein) for a number of adaptor proteins or actuators that switch on several signalling cascades working in parallel (see supplementary data 1 and figure 4 below).
- (iii) An essential modulator of the activation process is the co-receptor CD45. It exhibits an intracellular domain that acts as phosphatase, which removes phosphate groups from tyrosine residues and can dephosphorylate the ζ -chains CD3. It exposes a very large extracellular domain (of about 40 nm length) that plays a key role in the function of the IS, as we will see below.

Two theoretical models of the IS and c-SMAC formations have been published. In a statistical mechanical study, the nucleation and growth of adhesion micro-domains (ADs) formed by the two sets of CAMs were simulated by the Monte Carlo technique [6]. The basic assumption was that the two CAM–CAM pairs differ in the adhesion strength (w) and bind optimally at different equilibrium intermembrane distances (h). As demonstrated by model membrane studies, this is a prerequisite for adhesion domain formation [4]. The adhesion is further controlled by steric repulsion mediated by glycoproteins acting as repellers. The simulations show that the two sets of CAMs form separate micro-domains by random nucleation, which grow and eventually fuse into two completely separated adhesion zones. The authors therefore predict that the formation of a peripheral (p-SMAC) and central tight adhesion zone (c-SMAC) requires the active transport of the micro-domains, as indeed found in the second phase of the experiments reported in [2]. In a phenomenological model by Qi *et al* [7] based on a complex Landau–Ginzburg theory, it is assumed that the strongly binding integrin–ICAM pair initiates the adhesion of the cell, enabling the formation of a ring of TCR–MHC–AG pairs. The model was refined by Lee *et al* [8] by considering the entropic disjoining pressure generated by the membrane bending excitations.

Neither model addresses the question of activation of genetic expression by ISs. Also, the essential role of the actin cortex and the MT–actin cross-talk during the secondary process of SMAC formation is not considered. Finally, it is assumed that the two sets of CAMs can bind simultaneously. It is well known, however, that the integrins of quiescent cells are initially in a weakly binding state and are activated during the initial phase of the cell–cell interaction [9].

In the present work, we propose a microscopic physical model of IS and c-SMAC formation based on previous model membrane studies of cell adhesion that show that the primary step of cell adhesion is the formation of metastable adhesion domains (such as the IS), which can be stabilized in a secondary process by coupling of the actin cortex to the CAM clusters [3]. The driving force of adhesion domain formation is mediated by the interplay of short-range attraction between the CAMs, medium-range repulsion forces exerted by large cell surface proteins of the glycocalyx acting as anti-adhesive spacers and elastic stresses associated with the local and global bending deformations of the membrane (reviewed in [4]). Prominent examples of spacer proteins (besides the phosphatase CD45) are CD43 and CD44. CD43 exhibits an extended extracellular head group of about 45 nm in length and a high content of negatively charged sialyl groups. CD44 can bind hyaluronic acid, which is known to act as a spacer between cells [4]. In supplementary data 2 (available at stacks.iop.org/NJP/13/065013/mmedia), we also present model membrane studies suggesting that ring-like adhesion zones (such as p-SMAC) can form by self-assembly of strongly interacting CAM–CAM pairs at small CAM concentrations. The rings of tight adhesion are subsequently stabilized by coupling clusters of bound CAM–CAM pairs to the actin cortex.

The cross-talk between actin and MTs stabilizes the long-time co-existence of the central and the peripheral SMAC.

In the first part, I summarize the experimental observations leading to the model of the sequential stimulation of T-cells by adhesion micro-domains (acting as IS). I briefly describe a model of the activation of the T-cell proliferation by genetic expression of quanta of the cytokine IL-2, resulting in the stepwise progression of the T-cell through the cell cycle. It is based on the quantal concept of immunity [10]. The functions of the major players involved in this process are summarized in the supplementary material. Finally, a model of SI formation based on previous model membrane studies of adhesion domain formation is presented.

In the second part, I propose a model of the formation of p- and c-SMAC and the Mexican hat-like adhesion caps formed by adhesion of cytotoxic cells on antigen-exposing cells. In this way, closed reaction spaces for the localized secretion of lytic proteins, such as perforin, are formed. A testable model is proposed, showing that the domes can be stabilized by cross-talk between the actin cortex and the MTs. It is based on a previously developed shell-string model of the mechanical stabilization of soft cells [12].

2. Summary of experimental observations

2.1. Continuous versus sequential activation of T-cells

The primary and secondary steps of the immune response were studied in several types of elegant *in vitro* experiments described below.

1. Dustin and co-workers [1, 2] studied the long-time stimulation of T-cells. The function of APCs is mimicked by solid supported membranes that are doped with MHC-II exposing antigenic peptide (referred to as MHC-AG complex hereafter) and ICAM-1 receptors. Both constituents are anchored in the fluid-supported membrane by the lipid anchor glycosylphosphatidylinositol (GPI anchor) and were thus mobile within the bilayer. T-cells from the spleen of transgenic mice were used, enabling the labelling of the T-cell-based proteins with green fluorescent protein (GFP) or red fluorescent protein (RFP). The interaction of the T-cell with the phantom cell is observed by total internal reflection fluorescence microscopy (TIRF), enabling the analysis of the distribution of fluorescent labelled proteins at the cell-cell interface.
2. In the sequential encounter experiment by Gunzer *et al* [11], the DC were mixed with T-cells from the spleen of transgenic mice and cultured in a three-dimensional (3D) collagen matrix. The T-cells moved rigorously through the matrix, adhered transiently on the DC and moved from APC to APC for several days. The efficiency of activation was verified by the observation of lymphoblast formation and by the analysis of CD25 and CD69 expressions.
3. In the third type of experiment, the molecular organization within the contact zone between the T-cell and the APC is studied by immunofluorescence microscopy [3, 13]. The distribution of proteins involved within the cell-cell contact zone is observed by labelling with antibodies that are visualized by decoration with fluorescent anti-antibodies. The time evolution of the protein distribution is observed by fixing the cells at different times after contact formation. By this technique, the distribution of the constituents involved can be observed both at the cell-cell contact zone and in the cytoplasmatic space. In particular,

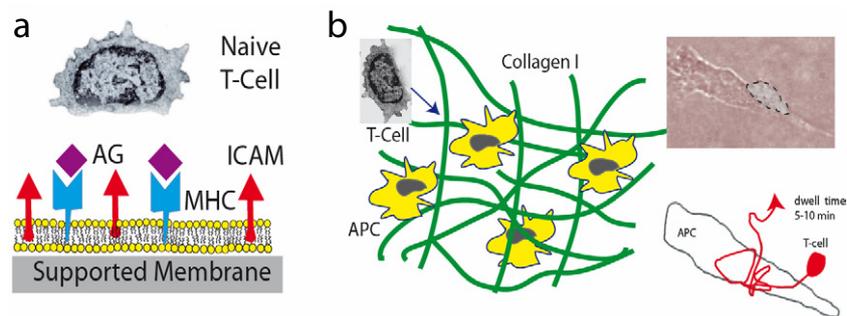


Figure 2. Summary of continuous interaction [1, 2] and kiss-and-run experiments [11]. (a) The continuous interaction of the T-cell with a supported membrane mimicking the APC is studied. The phantom APC is doped with MHC-II exposing the antigen (denoted as MHC-AG) and the CAM ICAM. The distribution of the fluorescent constituents in the contact zone of the cells is evaluated by TIRF that captures all fluorescent components up to a distance of 200 nm above the solid surface. Experiments were performed with Jurka cells, an immortal strain of T-lymphocytes that express IL-2 and are frequently used to study immune reactions. (b) Observation of transient contacts of the T-cell with APCs distributed in a 3D collagen matrix. The contact is observed by phase contrast microscopy. The residence time of a T-cell on an APC is 5–12 min. The upper right panel shows a T-cell (encircled by a broken line) adhering on a DC (image reproduced from a movie in [11], with permission from Elsevier and the authors).

the talin and MT could be visualized, yielding important insights into the reorganization of the actin cortex and the actin–MT coupling during the T-cell stimulation.

3. Visualization of immunological synapse (IS) and supramolecular activation cluster (SMAC) formation

In the experiments described in [1, 2] and [3, 13], the distribution of several of the major proteins involved in the T-cell activation within the adhesion zone was observed as a function of time after contact formation. These included several of the proteins shown in figure 1 (CD3, Lck, ZAP-70 and CD45), as well as the actin–membrane linker talin and the membrane-bound scaffolding protein LAT. After phosphorylation by ZAP-70, LAT recruits several actuators (such as SPL-76 and PLC- γ), which trigger two parallel signalling pathways (see [14–16] and figure 4 for the function).

By using cells from transgenic mice, two sets of the proteins labelled with green (GFP) and red (RFP) fluorescent proteins, respectively, could be observed simultaneously. An example is shown in figure 2(a), where the distribution of CD3 and the ZAP kinase after 3 and 30 min is presented. In figure 3(b), parallel results obtained by the immunofluorescence techniques [3] are shown.

The adhesion process decays in three phases, two of which are shown in figure 2:

1. First, the cell spreads on the support acting as phantom APC (called growth phase). The radius of the contact zone grows linearly with time and reaches a maximum within about

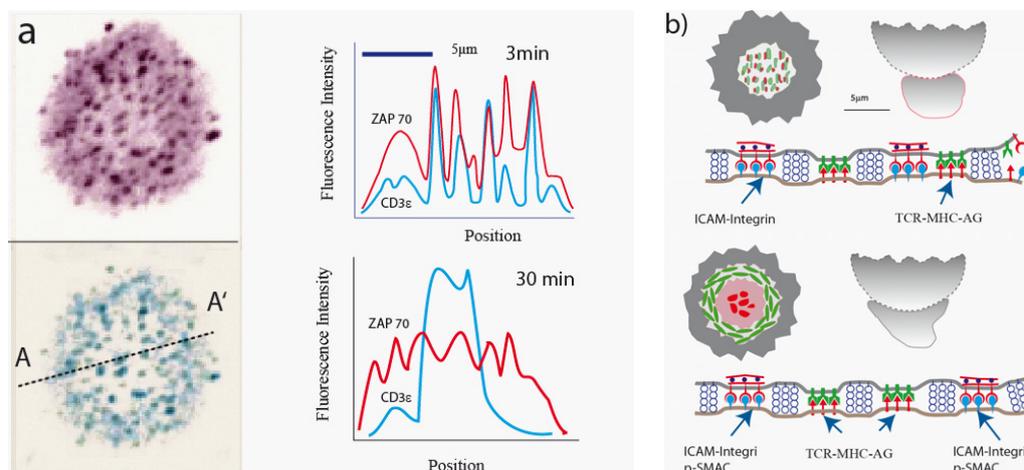


Figure 3. (a) Left side: clusters of co-receptor CD3 and the kinase ZAP-70 labelled with green (GFP) and red (RGP) fluorescent proteins, respectively, are observed (3 min after contact formation) by TIRF. (Copies of fluorescence micrographs in figure 1 of [2], reproduced with permission from Elsevier and the authors.) The TIRF technique captures all fluorescent molecules located up to about 250 nm above the substrate surface. Right side: distributions of the kinase ZAP (red) and the CD3 complex (blue) along a section AA' are shown 3 min (top) and 30 min (bottom) after adhesion of the cell on the supported membrane. Clearly, the domains are in register until about 5 min after onset of adhesion. Thereafter, the TCR-CD3 complexes move towards the centre, whereas the fluorescent ZAP-70 fades, by lateral randomization. (b) Two-stage model of reorganization of the T-cell-APC adhesion zone as suggested by visualization of talin and Lck distribution through immunofluorescence [3]. The top image shows the situation 3 min and the bottom image 23 min after contact formation. Note first that the initially statistically distributed clusters of talin move towards the rim of the contact zone and stabilize the ring-like adhesion domain (called p-SMAC [1-3]). This is mediated by two processes: firstly, the increase in the integrin binding affinity by talin [9], and secondly, the transfer of the co-receptor ICAM from the body of the T-cell to the nascent adhesion ring by active transport [18]. Note secondly that the contact area between the cells grows by about 20%, whereby the originally nearly spherical cell assumes a deformed pear-like shape. The schematic views of the cell contours on the right side are inspired by electron micrographs in figure 1 of [3]. We cannot exclude that the adhesion domains are mixtures of both CAM-CAM pairs.

3 min. Simultaneously, small ($\sim 1 \mu\text{m}$ diameter) domains enriched with the TCR-CD3 complex, phosphorylated Lck (which are not shown) and ZAP-70 are formed. The number of domains increases up to about 20 during the growth phase [1, 2]. Clearly, during the growth phase, the domain-like distributions of Lck, ZAP-70 and CD3 are strictly correlated (cf figure 4(a)).

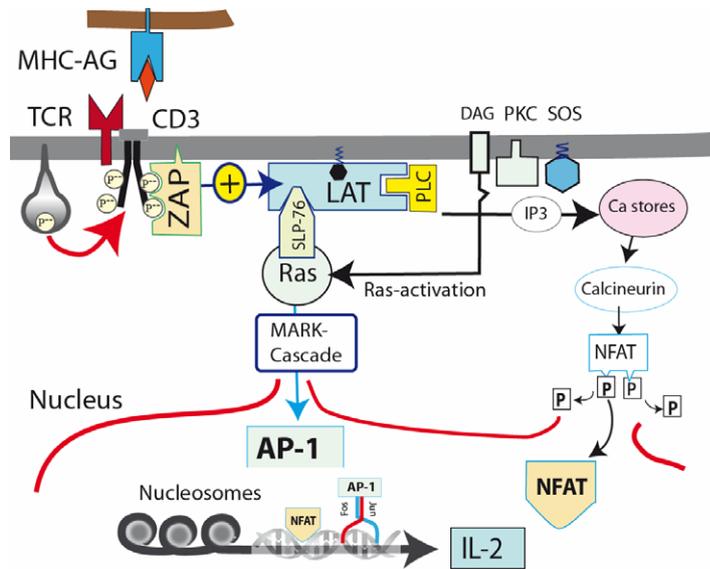


Figure 4. Simplified view of the calcium- and MAPK-mediated pathway of genetic expression of IL-2 triggered by antigen–TCR binding. The ZAP kinase is activated through binding to phosphorylated tyrosin groups of the ζ -chain of the CD3 complex (hence its name zeta-associated protein). It activates several binding sites at the scaffolding protein LAT (synonym for linker for activation of T-cells). LAT is recruited to the immune synapses a few seconds after adhesion (and after attachment of a palmitoyl group). ZAP-70 phosphorylates various binding sites at LAT, which then bind the actuators of the pathways (in a site-specific manner). One example is phospholipase C (PLC- γ). It generates the inositol-triphosphate (IP3), which opens the Ca channels of the Ca-storage vesicles. Together with calmodulin, the second messenger activates the phosphatase calcineurin, which removes phosphate groups from NFAT, enabling the NFAT transfer into the nucleus. The MAPK pathway (triggered by activated Ras) can be activated by binding of the actuator SLP-76 to the scaffolding protein LAT [14, 15]. After decondensation of the chromatin (mediated by NFAT [21]), NFAT and AP-1 form a complex that binds strongly to the DNA and switches on the expression of IL-2 [20]. The role of activation of Ras-GTPase by the guanine nucleotide exchange factor SOS, PKC and DAG (which is indicated by the bent arrow) is discussed in the text. Note that DAG is generated together with Ca^{2+} . The roles of many of the proteins involved are also described in supplementary data 1, available at [stacks.iop.org/NJP/13/065013/mmedia].

2. After about 5 min, the area of the adhesion zone observed by fluorescence microscopy decreases again by about 20% [1]. In this phase, the domains enriched with TCR–CD3 complexes move towards the centre of the adhesion disc, whereas the kinases ZAP-70 and Lck remain stationary but fade slowly. After about 20 min, the TCR and the CD3 complex are assembled in the centre (the c-SMAC), whereas Lck and ZAP-70 become more randomly distributed by lateral diffusion of the membrane-anchored molecules. The diffusivity of the lipid-anchored Lck is $D \approx 0.26 \mu\text{m}^2 \text{s}^{-1}$ (see [17]), suggesting that this

enzyme is randomized in the time scale of minutes. The ZAP-70 protein can randomize by diffusion in the cytoplasm.

3. As shown in figure 3(b) by immunofluorescence, the reorganization of the TCR–CD3 complex is also reflected by the redistribution of the actin–membrane coupling protein talin at the inner surface of the T-cell. Talin plays a twofold role. It is known to activate the initially inactive (sleeping) integrins and simultaneously mediates the coupling between the plasma membrane and the actin cortex. Thus, it reflects the distribution of the integrin (LFA-1). During the growth phase ($t < 5$ min), the talin forms small (slightly elongated) clusters that redistribute into a ring-like organization with radius $R \approx 8 \mu\text{m}$ after 20 min, which agrees well with the radius of the ring-like distribution of ZAP-70 in figure 3(a) (below). This shows that the second phase is controlled by reorganization of the actin network, a process discussed below.
4. Remarkably, the clusters of talin and ZAP-70 appear to be not completely in register, suggesting that the micro-domains enriched with TCR and integrin (LFA-1) are separated. However, this could be a technical artefact.
5. The key regulatory protein CD45 is also dynamically reorganized during the primary steps of the immune response. It is expelled from the adhesion domains generated at the periphery of the contact zone during the growth phase [1–3]. However, the integral membrane protein becomes associated again with the CD3-enriched micro-domains moving towards the centre in the contraction phase. We will show below that the expulsion of CD45 from the small adhesion domains is essential for T-cell activation.

4. Control of signalling pathways

4.1. Two pathways of genetic expression mediating cell proliferation mediated by interleukin-2 (IL-2) (cf figure 4).

In our model, the genetic switching of the IL-2 transcription is assumed to be mediated by cooperative interaction of transcription factors activated by the calcium/calcineurin-mediated [19–22] and the MAPK-activated pathways [21–24] (see also chapter 7 in [5] and supplementary data 1. The Ca^{2+} (liberated by the phospholipase γ) activates the general transcription factor NFAT, whereas the MARK-pathway activates the transcription activators AP-1 and $\text{NF-}\kappa\beta$. The members of the NFAT family play a key role in the activation of most genes of T-cell cytokines, but they bind only strongly to DNA in combination with other TFs such as AP1. Since members of NFAT are involved in many transcription processes, it is assumed that they play a role in chromatin remodelling (see supplementary data and [21, 22]). The heterodimer AP-1 consists of two parts, called c-jun and c-fos. They both exhibit specific DNA-binding domains but can only trigger the transcription of IL-2 in combination with NFAT (see figure 1).

We have to consider next the switching of the transcription factors AP-1 and $\text{NF-}\kappa\text{B}$ through the MAPK pathway, which is induced through activated Ras GTPase (as indicated by the arrow in figure 4). As described below, the activation of the Ras protein is mediated by the signalling molecule diacylglycerol (DAG) and calcium, which are both generated by activation of PLC- γ [22].

Ras switches between an inactive state (with GDP bound) and an active state (with GTP bound). The transition between the two states is mediated by two types of activators: first,

the GTP exchange factor (RasGEF), which releases GDP, enabling the uptake of GTP from the cytoplasm. The lifetime of the excited RasGTP state is rather long; the switching rate of the Ras proteins can be improved by a GTPase-activating protein (RasGAP), which accelerates the RasGTP \rightarrow Ras GDP transition (see supplementary data 1, available at [stacks.iop.org/NJP/13/065013/mmedia]). The activity of Ras is further modulated by calcium [24].

In lymphocytes, the activation of the Ras is regulated by the specific RasGEF SOS (abbreviation for son of sevenless), which exhibits two unique properties. Firstly, it is activated by binding to membrane-bound DAG and by phosphorylation through protein kinase C (PKC). Secondly, SOS exhibits a non-catalytic binding site for RasGTP and its GDP \rightarrow GTP exchange function is greatly accelerated (i.e. by a factor of 75 [22]) by the binding of GTP to this site. By a positive feedback of the Ras activation by SOS, membrane-bound Ras can be switched from the inactive and active states in an all-or-nothing fashion. The transcription of the IL-2 genes could be switched on if the stimulus evoked by ZAP-70 exceeds a certain threshold. Thus signals can be processed by cells in a digital rather than an analogue way [22], similar to the opening of voltage-dependent ion channels. It is thus conceivable that each AD activating a sufficient amount of ZAP-70 can switch on the IL-2 transcription. Moreover, the work of Das *et al* [22] provides evidence that the Ras-SOS-mediated feedback loop exhibits a hysteresis and that the Ras activation is sustained for some time after removal of the stimulus. This could provide a second mechanism for the integration of the responses evoked by the transiently formed adhesion domains during sequential or long-lived encounters.

5. How adhesion domains could act as reactant-selecting platform-mediating ZAP phosphorylation

Comparative studies of the adhesion of vesicles and cells provide strong evidence that cell adhesion is a biphasic process:

1. The initial phase consists in the formation of micro-domains of tight contact (in the following called adhesion domains) that are formed by lateral segregation of bound pairs of CAMs interacting via specific (lock-and-key) forces.
2. The second stage consists in the stabilization of the adhesion domains by coupling of actin gel clusters to the intracellular side of the receptor domains (see figure 6 below). In nucleated cells, such as leukocytes, this coupling is mediated by talin. Most importantly, the binding of talin to the cytoplasmic domains of the receptors increases the adhesion strength in two ways:
 Firstly, the binding affinity of integrin receptors increases by coupling of the talin head group to the intracellular domain of the β -chain, which induces a transition of the extracellular binding pocket from a weak to a high affinity conformation [9]. Secondly, the adhesion strength is increased by stiffening of the cell envelope [25].
 Numerous model membrane studies (reviewed in [4]) suggest that the domain formation is a consequence of the interplay of specific attraction forces between the CAM–CAM pairs and several generic forces that include
3. Medium range steric repulsions exerted by membrane proteins exposing very large head groups (length $>$ 30 nm) called spacer proteins or buffers.

4. Elastic stresses associated with membrane bending deformations between the rim of the adhesion domains and the non-adhering zones that extend over a persistence length ζ (see [26]).
5. Entropic disjoining pressure generated by pronounced bending excitations which generate an entropic repulsion pressure (p_{disj}) between the cell surfaces and which exerts two effects: it inhibits the non-specific adhesion by van der Waals attraction and simultaneously promotes the formation of adhesion domains by pushing the membranes together [4, 27]. It was previously [4] conjectured that the tight adhesion domains may play a key role as confined reaction spaces, enabling the control of the efficiency of the immune response by impeding the access of the Lck inhibitor CD45 (see figure 5). The Lck kinase that initiates the immune response is coupled to the plasma membrane by lipid anchors. It can diffuse freely in the membrane and constitutively phosphorylate the CD3 complex. However, in the non-adhering state, the phosphatase CD45 can also diffuse freely and decouple the phosphate groups again, thus abolishing the effect of Lck.

The extracellular domain of CD45 is about 40 nm long and thus much longer than the inter-membrane distance, enabling the formation of strong TCR–MHC–peptide bonds (~ 15 nm). Therefore, CD45 plays a twofold role: it inhibits the CD3 phosphorylation and acts as a buffer molecule counteracting adhesion (together with other glycoproteins of the glycocalix). It is therefore expelled from the adhesion domains formed by TCR–MHC–peptide aggregation, resulting in the effective phosphorylation of the CD3 ζ -chain and thus the activation of the ZAP-70 kinase. In the following, we summarize recent evidence for this model (first proposed by Springer [28]).

6. The first evidence was provided in the splendid experiments by Choudhuri *et al* [29]. These authors changed the lengths of the extracellular domains of both CD45 and the TCR and showed that the immune response is suppressed if the length of the extracellular part of CD45 is comparable to or shorter than that of the TCR–MHC–peptide complex.
7. Further evidence comes from the experiment by the Dustin group [2] using supported membranes as phantom APC, which show that CD45 is not found in the early formed TCR–MHC–peptide micro-domains. It again becomes associated with the TCR–CD3 complex when the domains enriched with these components are accumulated in the c-SMAC.

In summary, the adhesion domains can play a twofold role. Firstly, they enable cells to adhere strongly by the commitment of a small number (~ 10000) of CAMs [4, 26]. Secondly, they can form platforms for biochemical reactions, which can control the access of a selected fraction of membrane-bound reactants.

The adhesion domain model can reconcile the (seemingly) contradictory observations that lymphocytes can be activated by continuous T-cell–APC contact, as in the experiments by Dustin *et al* [1, 2], and by sequential contacts, as proposed by Gunzer *et al* [11]. We postulate that each adhesion domain formed by TCR–MHC–peptide condensation can act as a signalling platform, which stimulates the genetic expression of a certain number of IL-2. The proliferation of the cells is induced by the binding of the newly expressed cytokine IL-2 to the receptor (see also supplementary data and [10]), which triggers the activation of the cyclins (cyclin D). This member of the cyclin family drives the $G_1 \rightarrow S$ transition (see [5], chapter 20). Thereafter, the IR2-R is internalized with a time

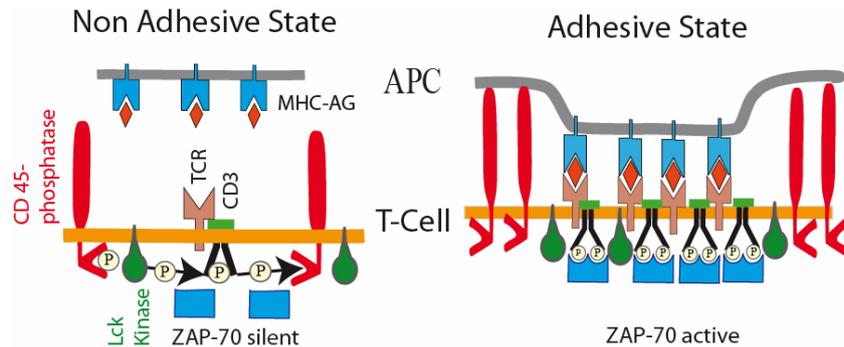


Figure 5. Model of activation of T-cells by micro-clusters formed during the initial phase of T-cell–APC encounters (i.e. before the formation of SMAC). The 70 kDa kinase ZAP-70 is activated by binding to the phosphorylated tyrosine groups of the CD3 ζ -chains. Left side: abolishment of ZAP-70 activation by CD45-mediated continuous dephosphorylation of CD3. Right side: formation of active immune synapse (IS) by lateral segregation of bound TCR–MHC–peptide pairs, resulting in the expulsion of the inhibitor CD45. Note that the Lck kinase can freely diffuse in the membrane [17].

constant of 0.067 min^{-1} . Therefore, the binding of IL-2 has to trigger the synthesis of new IL-2R, which may determine the incubation time.

Experiments summarized by Smith *et al* [10] showed that the signals elicited by the binding of IL-2 to newly expressed IL-2R drive the cell through the G_1 -phase of the cell cycle until the threshold of spontaneous cell division is reached. The experiments also showed that newly synthesized IL-2R are continuously transferred to the cell envelope. After the binding of IL-2, they are internalized again with a time constant of 15 min [10], while the lifetime of the IL-2R bond is much longer (45 min). Each receptor can promote the progression of the cell through G_1 -phase of the cell cycle by a certain increment, which depends on the concentration of IL-2 and the number of IL-2R. The smaller the number of IL-2R, the longer it takes for a cell to progress through the cell cycle to the S-phase [10]. The experiments show further that the threshold of cell division is reached after turnover of $2\text{--}3 \times 10^4$ receptors within 11 h incubation time [10]. Regardless of the number of IL-2R, a minimum of 6 h is necessary before any cell progresses to the S-phase. It should be noted that the production of IL-2 is enhanced by co-stimulation through CD28. This is essential for the stimulation of naïve T-cells via MHC–antigen complexes. Without CD28 co-stimulation, the T-cells are unable to evoke a normal immune response; that is, they are anergic.

Evidence for the sequential stimulation model is also provided by the observation that T-cell activation may be mediated by interrupted signalling. This was shown most clearly in elegant experiments through periodic addition and removal of inhibitors of *src* kinase (such as Lck). Although the ISs were periodically destroyed and reformed, T-cells were eventually activated and produced interferon γ [30].

6. Secondary immune responses: the formation of global reaction spaces can be guided by microtubule (MT)–actin cross-talk

The primary immune response discussed above is a localized process, which is mainly mediated by the composite cell envelope (see figure 7). It involves essentially components of

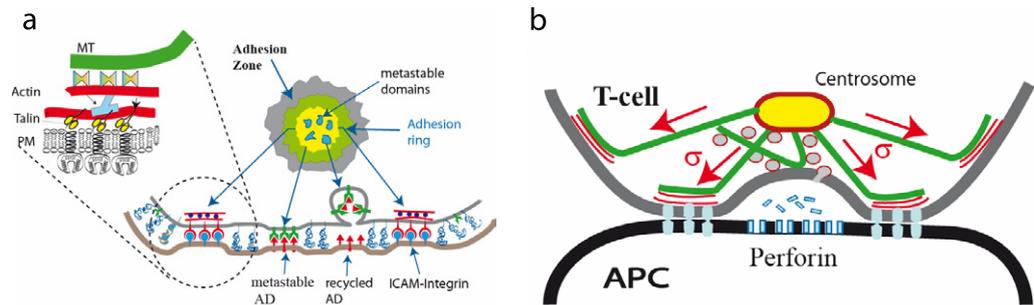


Figure 6. Model of the formation of the peripheral adhesion zone (such as p-SMAC) serving in the long-time stabilization of cell–cell contacts enclosing a central zone (such as a c-SMAC) in which signalling micro-domains of tight adhesion formed by MHC–AG binding (left side) could coexist with areas where mature TCR–CD3 complexes are taken up by endocytosis (right side). The inset on the left shows the coupling of the MT plus end to the actin cortex by MT–actin binding proteins. (b) Mexican-hat-like reaction space formed by cytotoxic cells adhering on antigen-exposing target cells. The reaction space is isolated from the extracellular space by a ring-like zone of tight adhesion acting as a gasket (corresponding to p-SMAC, see also [1, 2]). The global shape is stabilized by MTs which link the actin cortex to the centrosome. A second fraction of MT exhibits dangling ends, which can dynamically shrink and grow. [31, 34]. Note that in this way secretory vesicles can be transported to the site of secretion by dynein and kinesin motors moving towards the minus- and plus-ends of the MT, respectively [31].

the lipid-protein bilayer (such as the proteins of the glycocalyx and the CAMs) and the actin filaments directly coupled to the intracellular domains of the CAMs.

The generation of the supramolecular complex consisting of the p-SMAC and c-SMAC is associated with a global shape change of the lymphocyte, as indicated by the strong deformation of the T-cell after 20 min (see figure 3(b)). This requires the large-scale restructuring of the actin cortex as well as the participation of the MT scaffold [12, 32–35]. The initially (within the first 5 min) formed small micro-domains (that is, the IS) reorganize by active motion of the domains enriched with the TCR–CD3 complex towards the centre of the adhesion zone [1, 2] zone, while a ring-like zone of tight adhesion forms by lateral separation of LFA-1–ICAM-1 pairs. The area of contact between the cells increases by about a factor of two between 5 and 30 min (see figure 3(b)). Moreover, the T-cells undergo a shape change from a quasi-spherical to a pear-like shape, showing that the cell becomes polarized and strongly deformed. As noted above, it is now rather well established that these global reaction spaces serve in the recycling of the TCR–CD3 complexes by endocytosis, while cell activation is mediated by the freshly formed micro-domains [2]. Figure 6(a) shows a model of the global reaction platform that could serve in the simultaneous signalling by newly formed adhesion domains and the recycling of TCR–CD3 complexes.

A similar mechanical polarization is observed during the destruction of antigen-bearing cells by cytotoxic cells (see figure 6(b)). The T-cell envelope contacting the infected cell forms a dome-like shape with the rim tightly fixed to the target cell. In this way, a closed reaction space is formed into which the lytic protein (such as perforin) can be ejected, while the loss of the toxic molecules is minimized.

In this section, I explain the secondary process of immune response outlined above in terms of the cell-string model of cells, combined with the partial wetting model of cell adhesion suggested by numerous model membrane experiments [4, 26].

The adhesion ring formation can be controlled by two processes: the increase in the integrin LFA-1 affinity and the influx of the co-receptor ICAM-1 into the periphery of the adhesion ring. Similar to the centripetal motion of the TCR–CD3 clusters towards the c-SMAC [1], the co-receptor ICAM-1 is transported actively towards the nascent p-SMAC [18].

From the finding that both motions are impeded by dismantling of the actin cortex by cytochalasin or by inhibition of myosin motors, it is concluded that the active transport is driven by actin–myosin-II motors. Interestingly, the movement of ICAM is initiated by the initial fast Ca influx, while no ICAM-1 redistribution occurs before the Ca level of the T-cells becomes elevated, which could explain the delayed formation of the ring of strong adhesion (the p-SMAG). As described in supplementary data 2, model membrane studies show that coexisting ring-like adhesion zones encircling assemblies of small ADs can also form by dynamic self-organization in the presence of two sets of CAM–CAM pairs of different adhesion strengths.

7. Shell-string model of mechanical stabilization of large-scale reaction spaces: on the role of MT–actin cross-talk

The mechanical properties of quiescent and non-adhering lymphocytes (such as naïve T-cells exhibiting a small nucleus) are described by the shell string model developed earlier on the basis of magnetic-tweezer micro-rheometry studies of amoeboid cells (such as *Dictyostelium discoideum*) and macrophages. The cytoplasm is a soft viscoplastic body, which is mechanically stabilized by coupling of a fraction of the MTs (typically 10) with their plus ends to the actin cortex and with their minus end to the centrosome. If an MT of length L is compressed by a force couple ($f, -f$) acting parallel to its axis, it buckles above a critical value F_c (called the Euler buckling force),

$$f \geq F_{\text{cr}} = \frac{4\pi^2 B}{L^2}. \quad (1)$$

The MTs exhibit a bending stiffness of $B \approx 2 \times 10^{-23}$ Nm and the critical force of a $5 \mu\text{m}$ long filament is thus of the order of $F_c \approx 3$ pN.

Under many physiological conditions, forces in cells are two to three orders of magnitude larger. Therefore, cells have to be designed in such a way that the MTs are only subjected to large tensile stresses. This is achieved by the coupling of the several μm long MT filaments with their plus ends to the actin cortex and with their minus ends to the centrosome. Since the actin cortex is a viscoelastic shell, strong external force pulses (of the order of nN) on an MT can be balanced by the interplay of the tensile forces in the other MTs and the shear stress in the viscoelastic actin cortex, as shown in figure 6 [12]. The mechanical coupling between different MTs is mediated by the centrosome, which thus acts both as the force centre and as the global cell organizer (hence the name ‘microtubule organization centre’ (MTOC)). The response of the cell to force pulses on the MT is astonishingly fast. Mechanical equilibrium is re-established within a fraction of a second (~ 0.2 s according to [12]).

It is important to emphasize that only a fraction ($\sim 10\%$) of the MT is fixed to the actin cortex, while the rest exhibits dangling ends and is subject to continuous decomposition and

reconstruction [33–35]. Therefore, the dangling MT could serve in the transport of secretory vesicles towards the TCR–APC contact (see figure 7).

We discuss now the mechanical stabilization of the Mexican hat shape by interplay of the elasticity forces of the composite cell envelope and the MTs bound to the actin cortex. We consider first the role of the membrane tension and the bending elasticity. Many features of adhesion can be explained quantitatively in terms of a simple model based on the consideration of the balance of membrane tension and bending moments at the cell–cell contact zone. The equilibrium of tension at the rim of the adhering membrane (called the contact line L) is determined by the classical Young equation,

$$\Delta g_{\text{ad}} = \sigma(1 - \cos \theta_c), \quad (2a)$$

where Δg_{ad} is the so-called spreading pressure (which is equal to the work of adhesion per unit area). Due to the finite bending modulus κ of the membrane, the transition from the adherent to the free membrane at the contact line L is smooth, exhibiting a finite radius of curvature R_c , which is related to Δg_{ad} by

$$\Delta g_{\text{ad}} = \frac{1}{2} \frac{\kappa}{R_c^2}. \quad (2b)$$

The geometric parameters R_c and θ_c define the contour of the cell envelope. They can be measured by interference microscopy, as described extensively in [4] and [25].

If the number of CAMs is so small that only a ring-like adhesion zone can be formed (cf supplementary data 2), the tensions and the bending moments have to be balanced both at the outer boundary (radius ρ_0 in figure 6) and at the inner boundary (radius ρ_i) of the rim. For that reason, a dome-like shape is formed in the centre of the adhesion zone. The outer boundary condition is determined alone by equation (2a) with the contact angle (θ_c). The boundary condition at the inner contact line is determined by the Young equation and the Laplace pressure $\Pi = 2\Sigma/R_h$, where R_h is the radius of curvature of the indentation. The membrane tension σ and the additional tension Σ determined by the Laplace pressure balance partially, and the inner contact angle θ_{ci} changes as follows,

$$\Delta g_{\text{ad}} = (\sigma - \Sigma)(1 - \cos \theta_{\text{ci}}). \quad (3)$$

This equation predicts that the contact angle at the inner rim of the adhesion zone is smaller than that at the outer one, which is indeed observed in figure 1 of supplementary data 2.

Since the ring-like adhesion zone is also controlled by the tensed MT, we consider now their force balance. The following consideration shows that single MTs may balance the tensions of several tens of pN. To estimate this tension, we use the method above. Owing to the binding to the actin cortex, the MTs tend to maximize the length of coupled segments, which generates the tensile force. The only difference is that we have to consider adhesion energies per unit length and forces instead of forces per unit length. We assume now that the MT is bound to the actin cortex over a length L (see figure 7, right) and that the binding energy per unit length is w (measured in J per unit length). The tensile force Σ in the filament is then determined by the 1D Young equation,

$$w = \sigma(1 - \cos \Theta_c), \quad (4)$$

where w is the binding energy per unit length of MT and Θ_c is the contact angle defined in figure 6. To estimate w , we assume that the average distance between the actin–MT linkers mediating the coupling of an MT to an actin filament is about 10 nm and the binding energy is

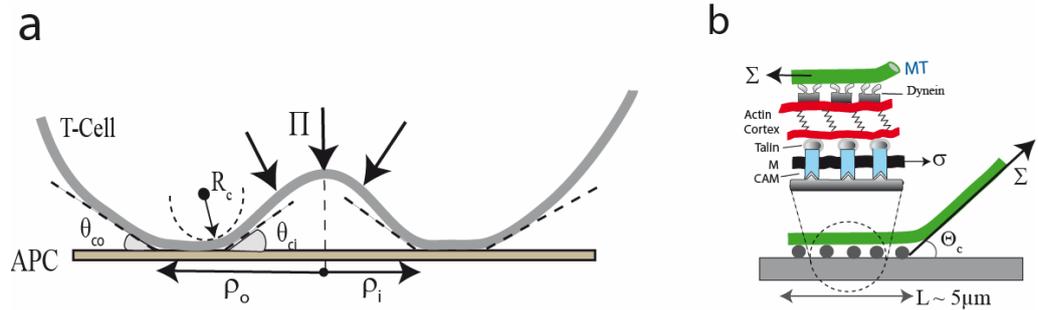


Figure 7. (a) Stabilization of the Mexican-hat-like shape of cytotoxic cells by the balance of the tension of the cell envelope and the Laplace pressure P . (b) Model of mechanical stabilization of cell shape by tangential coupling of MT to the actin cortex. The tensile stress Σ in the MT is determined by the contact angle θ_c and the contact curvature R_c^{-1} , as described in the text. It can be generated indirectly by the binding of the MT to the actin cortex or directly by dynein motors walking in the direction of the MT-minus end. The tensile stress is balanced by the tangential tension σ induced in the actin cortex, as shown in the inset.

about $20 k_B T$ (10^{-19} J); one obtains $w \sim 10^{-11}$ J m $^{-1}$. For a contact angle $\Theta_c \approx 30$, one expects a tension of $\sigma \sim 40$ pN.

Several proteins mediating the coupling of MT to the actin cortex are known. One is the so-called end-protein (END-1) that can also mediate the formation of MT bundles [33, 34]. As shown recently for the case of lymphocytes, the coupling is mediated by dynein through the adaptor protein ADAP, which binds to the MT along the whole length [12]. Since the dynein motor tends to walk towards the minus end of the MT, it generates a tensile force in the MT and a counteracting shear stress on the actin cortex (see figure 7(a) and [12]).

It should be noted that the symmetric MT arrangement in figure 7 is not necessary for establishing mechanical equilibrium. The only stability condition is that the tensile forces of the assembly of MT must be balanced. Thus, the components parallel to the contact zone must cancel $\sum_i \Sigma_i \sin \theta_i = 0$. Moreover, the net force of the MT in the normal direction pulls the centrosome towards the cell envelope. Finally, several force centres can be formed, which are controlled by different centrosomes [12].

8. Concluding discussion

We have developed a model of the activation of T-cells through adhesion-induced domains forming confined biochemical reaction centres, enabling the control of the reactions through the access of activators and inhibitors. The ADs form by the interplay of specific short-range attraction between TCR and antigen-bearing MHC and steric repulsion forces mediated by CD43 and CD45, the inhibitor of T-cell activation [4]. The model is based on the assumption that each micro-domain stimulates the expression of certain quanta of the cytokine IL-2 via the calcium/NFAT and the MAPK/AP-1 mediated pathways of genetic expression. The IL-2 activate the IL-2R and (through these) the signalling pathway that promotes the progress of cell cycle from the G $_1$ - to the S-phase (the DNA replication phase) as proposed by K. A. Smith in his quantal theory of immunity. According to this model, the cells progress from the G $_1$ -phase

to the S-phase if a sufficient number of IL-2R have been triggered. The immune reaction is therefore determined by the quantity of antigenic peptides and not by their quality [10].

An alternative explanation for the sequential activation of T-cells is provided by the recent work of Das *et al* [22], suggesting that the stimuli evoked by the micro-domains could be integrated via the Ras-mediated activation of the MAPK pathway as follows. Owing to the hysteresis of the Ras activation by the allosteric activator SOS, the stimulation of the genetic expression by each micro-domain could be sustained for some time after the removal of the stimulus. If the average time between the sequential stimuli exerted by micro-domains is shorter than the memory lifetime, the signals would be integrated.

The present model was developed to explain the activation of naïve T-cells by sequential, T-cell \leftrightarrow APC encounters within collagen networks lasting several minutes [11], during which small adhesion domains are formed and Ca bursts are generated in the cells. The model would also hold if such stimulating adhesion domains are sequentially formed and recycled in long-lasting contacts between T-cells and DC.

The contact duration and kinetics of IS formation depends critically on the environment and can be influenced by specific factors present under different conditions. The T-cell activation by sequential encounters in collagen networks has been explained on the basis of signals from collagen or associated proteins that inhibit the antigen stop signal [37]. The sequential encounter mode could mediate the T-cell activation in rather stiff tissue (such as in the skin) where the cells are constantly exposed to collagen. In fact, some experiments show that the formation of long-lasting T-cell–APC contacts depends on LFA-1 [39], which is required for the formation of p-SMAC. In dense collagen networks, the formation of ring-like adhesion zones could be impeded by non-specific binding of LFA-1 to the collagen fibres, which depletes the pool of the integrins exposed by the T-cells.

In order to test the physiological relevance of the experiments in the collagen matrix, systematic comparative studies *in vitro* and *in vivo* (i.e. in lymphoid tissue) are required. The micro-anatomy of the lymph nodes is very complex, consisting of mechanically hard and very soft regions. The site where most cells are supposed to meet are local cavities formed by a reticular network of collagen fibres and these areas of resting lymph are filled by many cells with little contact to the collagen [36]. Owing to technical difficulties, systematic studies of the kinetics of the T-cell–APC contacts in lymphatic tissue are still missing. Mempel *et al* [38] showed that the priming of T-cells in the lymph node occurs in three successive steps: (i) transient serial encounters during the first activation for about 8 h; (ii) the formation of stable contacts associated with cytokine production for about 12 h; and (iii) transition to a state of high motility and rapid proliferation.

Gunzer *et al* [39] studied the activation of T-cells by naïve and activated B-cells in collagen tissue and the intact lymph nodes. The naïve resting B-cells formed long-lasting contacts with T-cells both *in vivo* and *in vitro*, which was, however, dependent on integrin LFA-1. In contrast, activated B-cells also formed short-time contacts that are not LFA-1 dependent. Irrespective of the character of the 3D environment, the T-cells respond faster if the contacts with the APC are short lived and sequential.

Stoll *et al* [40] studied the T-cell–DC interaction in intact explanted lymph nodes and observed long-lived contacts. They analysed the distribution of CD43 (a sialoglycoprotein exposing a long extracellular domains similar to CD45) and showed that it is mostly removed from the cell–cell contact zone forming a peripheral ring. A close inspection of the contact zone (the equatorial plane of SMAC) shows coexisting dark red and yellow patches (cf figure 4

of [40]). The latter are obviously enriched with CD43. It may therefore well be that the contact area inside the CD43-enriched ring consists of micro-domains formed by tightly bound TCR–MHC–peptide pairs that are separated by regions in which the cell distance is determined by the repulsive force mediated by the spacer protein CD43, such as shown in figure 3(b). It is conceivable that the dark red domains correspond to the ADs which trigger the T-cells activation and which may be continuously recycled. Hopefully, more powerful optical methods will be developed in the future enabling high-resolution studies of T-cell–APC interaction in different parts of the lymph node.

One main purpose in this work was to show that systematic studies of the physics of cell adhesion may help us to gain insights into T-cell stimulation by cross-talk with other cells. Adhesion domains formed by interplay of specific and generic interfacial forces may help us to assemble specific adaptor enzymes and inhibitors within multicomponent membranes. Owing to the fluidity of the cell plasma membrane, such local biochemical platforms are expected to be short lived. Long-lasting cell–cell contacts are stabilized by high-affinity CAM–CAM pairs coupled to the actin cortex. Global reaction spaces (associated with the cell polarization) form by ring-like tight adhesion zones (such as p-SMAC), whereas the global mechanical stability of the cell is mediated by interplay of the actin and MT networks. The inner zone can be highly dynamic. Adhesion domains acting as IS may continuously form and be recycled by endocytosis, or cells may be killed by lytic proteins.

9. Supplementary materials

The roles of the receptors and other proteins involved in the genetic transcription are summarized in supplementary data 1. Model membrane studies showing that ring-like adhesion zones coexisting with adhesion micro-domains may form by dynamic self-assembly are presented in supplementary data 2. The supplementary data are available at stacks.iop.org/NJP/13/065013/mmedia.

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