

The role of microtubules in the immune system and as potential targets for gut-based immunotherapy

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ABSTRACT

Microtubules (MTs) are tubular polymers of tubulin that are highly dynamic and found throughout the cytoplasm. MTs are involved in maintaining cell structure and, together with microfilaments and intermediate filaments, form the cytoskeleton. Recent findings on MT structure and function contributed to the understanding of their potential role as players in the innate and adaptive immune systems. Additionally, studies suggest an essential role for these cellular structures in the gut. Here, we review recent data on interactions between MT and various arms of the immune system and propose a model that represents gut MTs as potential targets for immunotherapy, and specifically for oral immunotherapy.

1. Introduction

Many current immune-based therapeutic methods target lymphocytes or alter the secretion of mediators (e.g., cytokines) from immune cells. Recent findings on microtubule (MT) structure and function contribute to the understanding of their potential roles as players in the innate and adaptive immune systems (Ilan, 2019). Additionally, studies suggest a potential role for these cellular structures in the gut. This review summarizes data regarding the role of MT in the immune system and suggests a model in which they can serve as potential novel targets for immunotherapy and specifically for gut-immune-system-based oral immunotherapy.

1.1. Microtubules

MTs, tubular polymers of tubulin, are highly dynamic and found throughout the cytoplasm (Pilhofer et al., 2011). MTs are involved in maintaining cell structure and, together with microfilaments and intermediate filaments, form the cytoskeleton. MTs also comprise the internal structure of cilia and flagella (Vale, 2003), and are involved in chromosome mitosis and meiosis as constituents of mitotic spindles (Derivery et al., 2015). Additionally, MTs provide platforms for intracellular transport, movement of secretory vesicles, organelles, and intracellular macromolecular assemblies. Proteins that bind to MTs include kinesin, dynein, and katanin (Howard and Hyman, 2007). In polarized interphase cells, MTs are disproportionately oriented from MT-organizing centers (MTOCs) toward the site of polarity (Luders and

Abbreviations: MT, microtubules; MTOCs, microtubule-organizing centers; MAPs, microtubule-associated proteins; IS, immune synapse; APCs, antigen-presenting cells; TCR, T cell receptor; DCs, dendritic cells; IFT, intraflagellar transport; TGFβ, transforming growth factor-β; STIM1, stromal interaction molecule 1; CRAC, Ca²⁺ release-activated Ca²⁺; Hh, Hedgehog; TH2, T helper 2; RAC1, Ras-related C3 botulinum toxin substrate 1; MTOC, MT-organizing center; cSMAC, supramolecular activation cluster; dSMAC, distal SMAC; PKCδ, protein kinase Cδ; WASP, Wiskott–Aldrich syndrome protein; PLCγ1, phospholipase Cγ1; PLCγ1: phospholipase Cγ1; HS1, hematopoietic lineage cell-specific protein 1; STIM 1, stromal interaction molecule 1; WAVE, WASP-family verprolin homologous protein; IQGAP1, IQ motif-containing GTPase-activating protein 1; Cdc42, control protein 42; GIMAP, GTPase of the immunity-associated protein; EB1, end-binding 1; DAG, diacylglycerol; ERK, extracellular signal-regulated kinases; SPAG6, sperm associated antigen 6; TCR-MCs, TCR microclusters; LAG3, lymphocyte activation gene-3; PD1, programmed cell death 1; SLOs, secondary lymphoid organs; BCR, B cell receptor; ITAMs, immunoreceptor tyrosine-based activation motifs; ABP1, actin-binding protein; TNTs, tunneling nanotubes; ILK, integrin-linked kinase; DOCK8, dedicator of cytokinesis 8; DCs, dendritic cells; NK, natural killer; Miro-1, mitochondrial Rho GTPase-1; TLR, Toll-like receptor; ERCs, endosomal recycling compartments; APCs, antigen-presenting cells; mDia1, diaphanous-related formin; LFA-1, lymphocyte function-associated antigen 1; TEM, transendothelial migration; PECAM, platelet/endothelial cell adhesion molecule; LBRC, lateral border recycling compartment; CGRP, calcitonin gene-related peptide; RhoA, Ras homolog gene family member A; CTGF, cytokine connective tissue growth factor; JNK, Jun N-terminal kinase; PGCs, primordial germ cells; LPS, lipopolysaccharide; FA, food allergy; SCFA, short chain fatty acids; CDM, cholesterol-dependent membrane

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Stearns, 2007). Dynamic MTs regulate levels of G proteins that regulate cell contractility and spreading and are required to trigger focal-adhesion disassembly, which is necessary for migration (Ezratty et al., 2005).

Several drugs that bind tubulin also modify its properties (Ganguly et al., 2010). These drugs are capable of stopping the cell cycle in order to promote programmed cell death or apoptosis, although interference with MT dynamics is insufficient to block cells undergoing mitosis (Ganguly et al., 2010). However, suppression of MT dynamics inhibits cell migration (Yang et al., 2010). Drugs that alter MT dynamics include anti-neoplastic agents (paclitaxel and docetaxel) that block dynamic instability by stabilizing GDP-bound tubulin in the MT, epothilones (e.g., ixabepilone, nocodazole, vincristine, and colchicine) that have an opposite effect and prevent tubulin polymerization into MT, and eribulin, which binds to the favorable growing end of MTs and triggers cancer-cell apoptosis (LaPointe et al., 2013).

MT-associated proteins (MAPs) are involved in regulating MT dynamics (Barsegov et al., 2017). The rate of MT polymerization and depolymerization varies depending on the type of MAPs that are present and classified based on their molecular weight. Tau (τ) proteins bind to MTs to promote nucleation, prevent disassembly, and induce the formation of parallel arrays (Sayas and Avila, 2014). Another class of MAPs is separated into MAP-1, MAP-2, MAP-3, and MAP-4. MAP-1 proteins are involved in the retrograde transport of vesicles and can associate with cytoplasmic dynein, an MT motor. MAP-2 proteins are located in neuronal dendrites and bodies, where they bind other cytoskeletal filaments. MAP-4 proteins are found in a majority of cells and stabilize MTs (Halpain and Dehmelt, 2006; Maiato et al., 2004; Cassimeris and Spittle, 2001). In addition to MAPs that have a stabilizing effect on MT structure, other MAPs destabilize MTs either by cleavage or through induction of MT depolymerization (Ghosh et al., 2012; Salinas et al., 2007), with the proteins katanin, spastin, and fidgetin capable of regulating the number and length of MTs through their destabilizing activities.

1.2. Microtubules and immune cells

1.2.1. The role of MTs in the immune synapse: polarization during T cell activation

An essential feature of T cell recognition is the formation of an immunological synapse (IS) between a T cell and the cell that it recognizes (Dustin and Baldari, 2017). Synapses rely upon cell-cell contacts formed between T lymphocytes and antigen-presenting cells (APCs). The formation of an IS correlates with cytotoxicity in the case of killer cells (mostly CD8⁺), T cell activity, robust cytokine release, and proliferation in cases of a longer-lived IS formed by CD4⁺ T helper (Th) cells. In the IS, T cells release chemokines via a multidirectional pathway directed away from the synapse (Huse et al., 2008).

Intracellular organelles are polarized and highly constrained by the cytoskeleton. This relationship is established for neurons and epithelial cells and also occurs in T lymphocytes (Martin-Cofreces et al., 2014). T cell receptor (TCR) signal transduction initially directs cytoskeletal- and vesicle-traffic polarization, which establishes IS design to regulate T cell activation (Soares et al., 2013; Ritter et al., 2013). The function of the IS depends upon an active T cell polarization process, which results from cross-talk between antigen-receptor signal-transduction machinery, actin, and MT cytoskeletons (Soares et al., 2013). Electron microscopy studies show that the initial interaction between T cells and target cells occurs through projections from the T cell that form an inter-digitated contact area between cells (Sanderson and Glauert, 1979), with the initial stage of IS formation involving invasive T cell pseudopodia that penetrate deeply into the APC, almost to the nuclear envelope. T cell projections also predominate in new synapses formed by CD4⁺ T cells (Ueda et al., 2011).

Several dynamic changes in MT organization occur below the IS (Ueda et al., 2011). Organelle polarization occurs in the early phase of

T cell activation, with the IS determining the rhythm of organelle motion and the spread of signal activation to the nucleus (Martin-Cofreces et al., 2014). Centrioles and the Golgi complex are located beneath the synapse, with centrioles shifted toward the late contact zone with either B lymphocytes or bone-marrow-derived dendritic cells (DCs). Thin, lengthy processes on the surface of the T cells originate predominantly from the area next to the Golgi apparatus and are involved in orientation during the initial phases of attachment, which precede IS formation (Arkhipov and Maly, 2015). The primary cilium is a sensory organelle that integrates multiple signals from the environment into a cellular response (Singla and Reiter, 2006). The IS and the primary cilium may facilitate contact between the centrosome and the plasma membrane during ciliogenesis (de la Roche et al., 2016); however, the centrosome, which communicates with the plasma membrane, and the Golgi apparatus are polarized toward the plasma membrane at the site of centrosome contact (Hildebrandt and Otto, 2005). Dynein drives centrosome repositioning in T cells via MT end-on capture-shrinkage operating at the center of the IS (Yi et al., 2013). Centrosome repositioning can be impaired by inhibiting dynein or MT depolymerization (Yi et al., 2013; Martin-Cofreces et al., 2008). The actin cytoskeleton controls the organization and activation of signaling microclusters at the IS in a nitric oxide (NO) dependent manner. NO generated by endothelial nitric oxide synthase (eNOS) controls the coalescence of protein kinase C- θ (PKC- θ) at the central supra-molecular activation cluster (c-SMAC) of the IS (Garcia-Ortiz et al., 2017). Nitrosylation of actin through eNOS centrosomal/Golgi-resident enzyme plays a role in this process.

Immune cells lack a cilium, with the IS representing a type of surrogate cilium that utilizes machinery associated with ciliogenesis, including MT nucleation at the centrosome (Cooley et al., 2016). Many similarities exist between the IS and cilium (de la Roche et al., 2016), and these functional similarities raise the possibility that the adaptive immune system has modified an ancient organelle to create a sensory and signaling structure to focus secretion and provide a regulated mechanism for communicating between cells. Both structures have a membrane-docked centrosome that reduces cortical actin in a region where the centrosome attaches at the plasma membrane (Francis et al., 2011; Cooley et al., 2016; Francis et al., 2011; Ritter et al., 2015; Stinchcombe et al., 2006). Intraflagellar-transport proteins essential for the assembly and maintenance of cilia and flagella are found in CD4⁺ T cells (Rosenbaum and Witman, 2002; Finetti et al., 2009), and ciliary transforming growth factor- β (TGF β) signaling is regulated by endocytosis at the ciliary pocket (Clement et al., 2013). In addition to their roles in signaling, both primary cilia and IS form specialized Ca²⁺-signaling structures. The efficiency of energy supply by the mitochondria depends on its proper positioning within the cytosol. In lymphocytes, mitochondria preferentially localize into the vicinity of the IS, a process which is regulated by motor-based cytoskeleton-mediated transport (Quintana and Hoth, 2012). The IS as a critical cellular compartment for Ca(2⁺) dependent lymphocyte activation (Quintana et al., 2011, 2009; Kummerow et al., 2009). Ca²⁺ influx is locally generated at the synapse through a stromal-interaction molecule 1 (STIM1)-activated Ca²⁺-release-activated Ca²⁺ (CRAC) channel in the plasma membrane and is required for effective TCR signaling (Hogan et al., 2010). IS formation induces the accumulation of CRAC/ORAI1 Ca(2⁺) channels, the CRAC/ORAI1 channel activator STIM1, K(+) channels and plasma membrane Ca(2⁺) ATPase (PMCA) within the IS (Quintana and Hoth, 2012). The primary cilia play roles in different signaling pathways (Huangfu et al., 2003; Rohatgi et al., 2007). The Hedgehog (Hh)-signaling pathway is involved in T cell development in the thymus during the differentiation of naive CD4⁺ T cells into Th2 cells, as well as in the CD8 synapse (Crompton et al., 2007). Hh signaling is required for upregulation of Ras-related C3 botulinum toxin substrate (RAC)1, which plays a role in actin and MT dynamics (Wittmann et al., 2003).

Similar to primary cilium that shed vesicles (Dubreuil et al., 2007),

T cells secrete vesicles at the IS, cytotoxic granules, and microvesicles carrying bioactive molecules, microRNAs, and TCRs (Choudhuri et al., 2014; Mittelbrunn et al., 2011). There are differences between synapses and cilia. Cilia are stable structures that persist for days, whereas IS are transient, existing only for a few minutes in cytotoxic T lymphocytes (CTLs). Several studies challenge the idea that the centrosome directly contacts the plasma membrane in the IS, and suggest that the centrosome polarizes toward the synapse with granules being delivered by short MTs that bridge the gap between the centrosome and the secretion site at the plasma membrane (Bertrand et al., 2013). The mother centriole docks at the plasma membrane of the IS through its distal appendages, and MTs radiate outward from the subdistal appendages to provide a mechanism for granule delivery directly to the synapse membrane (de la Roche et al., 2016).

CTLs are triggered to undergo rapid, polarized secretion of cytolytic granules to promote TCR signaling within minutes. Once CTLs recognize their target cells on the periphery, a synapse is formed, and the cytolytic granules are secreted. CTLs build a cytolytic IS with their target cell following actin and MT-cytoskeleton restructuring, which orients the centrosome near the plasma membrane at the point of TCR signaling (de la Roche et al., 2016). CTLs kill target cells via the polarized secretion of cytotoxic granules recruited around the polarized MTOC at the IS. In a dynein-dependent transport process, the granules move along the MTs toward the MTOC in the minus-end direction and in a kinesin-1-dependent process (Kurowska et al., 2012), whereas secretory granules move toward the centrosome and are delivered to the point of secretion. CTLs destroy pathogen-infected cells by polarized exocytosis of secretory lysosomes containing the pore-forming protein perforin, which involves movement of the centrosome in order to contact the plasma membrane at the center of the IS formed between killer and target cells. However, other studies suggest that MTOC polarization is not essential for efficient, lethal hit delivery (Bertrand et al., 2013). In CTL/target-cell conjugates, lytic granule secretion precedes MT polarization, and inhibition of MTOC and centrosome polarization impairs neither lytic granule release at the CTL synapse nor killing efficiency. Disorganization of the T cell MT cytoskeleton abrogates cytotoxic granule exocytosis and the synaptic secretion of chemokines (Franciszewicz et al., 2014).

IS formed by naive CD8 + T cells and fully differentiated CTLs with their APCs are similar. Both cell types form a central supramolecular activation cluster (cSMAC) of TCRs, polarize their centrosomes (the MTOC in T cells) to the contact site with the APC, and reorganize their actin cytoskeleton to form a distal SMAC (dSMAC) (Stinchcombe et al., 2006). TCR signaling is triggered at the IS by the generation of signaling complexes that associate into dynamic TCR microclusters (TCR-MCs). For TCR signaling, TCR-MCs coalesce in the center of the IS to form the cSMAC (Grakoui et al., 1999), which is dependent upon F-actin flow and MT movement driven by dynein motors (Hashimoto-Tane et al., 2011). F-actin flow regulates TCR-MC movement at the IS periphery, with dynein-mediated transport of TCR-MCs along the MT occurring in the center of the IS. Dynein and the MT plus-end binding 1 (EB1) protein, which recruits dynein to the plus-end of the MT, co-immunoprecipitate with the TCR-complex component CD3 (Zyss et al., 2011; Martin-Cofreces et al., 2012; Liu et al., 2013). The minus-end-directed MT motor protein dynein is recruited to the dSMAC downstream of diacylglycerol (DAG) and controls centrosome translocation (Lin et al., 2009). It is possible that dynein pulls MTs from the IS periphery using a cortical sliding mechanism to translocate the centrosome to the activation site (Kuhn and Poenie, 2002). By contrast, another study suggests that centrosome translocation to the IS occurs by a capture-shrinkage mechanism, in which pioneer MTs that extend between the centrosome and plasma membrane at the synapse use the dynein motor force together with depolymerization to shorten (Yi et al., 2013).

When T cells recognize a peptide–MHC complex on APCs, TCR-MCs are generated and move along MTs toward the center of the IS and in

the direction of the T cell–APC interface in a dynein-dependent manner in order to form cSMAC. This movement regulates T cell activation (Hashimoto-Tane et al., 2011), and the dynein motor complex reportedly co-immunoprecipitates with the TCR complex. T cells with impaired cSMAC formation exhibit enhanced cellular activation, including protein phosphorylation and IL-2 production (Hashimoto-Tane et al., 2011).

To control centrosome polarization, TCR activation triggers actin reorganization and initiates centrosome polarization and translocation of the associated secretory organelles to the IS. Rapid actin reorganization occurs as the cytolytic synapse forms, with actin flow creating a mechanical force required to activate the integrin and ensure tight adhesion between T cells and the APC as they meet (Comrie et al., 2015). In the first 20 s after contact is made between a CTL and a target cell, actin accumulates at the IS; however, within the next 20–40 s, actin is depleted across the synapse (de la Roche et al., 2016). Simultaneous with this membrane reorganization, plasma-membrane-associated TCRs cluster at the center of the actin-depleted area to form a cSMAC within 2 min of contacting the target cell (Ritter et al., 2015). The initial contacts in the IS occur via actin-rich protrusions at the leading edge of migrating T cells, which are regulated by protein kinase C δ (PKC δ), RAC1, and downstream effectors of actin polymerization, including Wiskott–Aldrich syndrome protein (WASP) (Wei et al., 2014). These protrusions form the first point of contact between cells as the IS forms, and they correspond to the actin foci reportedly required for activation of phospholipase C γ 1 (PLC γ 1) via WASP (Kumari et al., 2015). Contact mapping microscopy was used to study the fractal organization of microvilli showing that the majority of opposing surfaces occur within 1 min. Stabilization of the microvilli was independent of tyrosine kinase signaling and the actin cytoskeleton, suggesting a specific selection of TCR microclusters (Cai et al., 2017).

CD4 + T cell activation and IS formation are associated with migration of the MTOC and organelles toward the APCs. MTs are essential for directing cytokines into the IS, but are not involved in the number of cytokines produced early after its formation. MTs also play a role in mobilizing organelles toward the IS during T cell activation and in stabilizing organelles against a force generated through actin polymerization to ensure their movement toward the APCs (Ueda et al., 2015). CD4 + T cells treated with an MT-destabilizing agent (vinblastine) following IS formation display dispersed MTOCs along with the movement of other major cellular organelles away from the IS. Cytokines are not directed toward the IS, but are randomly secreted in quantities similar to those observed in IS secretion (Ueda et al., 2015).

The close interdependence between cytoskeletal dynamics in T cells and Ca²⁺ signaling provides positive feedback for T cell activation (Babich and Burkhardt, 2013). Ca²⁺ channels regulate both ciliary activity and exocytosis via an influx of extracellular Ca²⁺ and the release of Ca²⁺ from specialized alveolar sacks, which is reminiscent of Ca²⁺ release from secretory lysosomes found in T cells (Plattner, 2015). Inositol cleavage by PLC γ 1 simultaneously triggers both the release of endoplasmic reticulum stores and DAG-dependent MTOC reorientation, thereby depleting the pool of phosphatidylinositol-4,5-bisphosphate, an activator of multiple actin-regulatory proteins (Huse et al., 2008). Cytoskeletal dynamics promote Ca²⁺ signaling in two phases. During the first phase, Ca²⁺ is released from endoplasmic reticulum stores, and the actin cytoskeleton encourages mechano-transduction and serves as a dynamic scaffold for microcluster assembly. Proteins that drive actin polymerization, such as WASP and hematopoietic lineage cell-specific protein 1, promote signaling through PLC γ 1 and Ca²⁺ release from endoplasmic reticulum stores (Huse et al., 2008). The second phase involves STIM1 clustering and CRAC-channel activation. In this phase, the WASP-family verprolin homologous protein complex and the MT cytoskeleton promote STIM1 clustering at sites of plasma-membrane apposition, thereby opening membrane channels. Conversely, elevated intracellular Ca²⁺ activates cytoskeletal remodeling (Huse et al., 2008).

Several molecules are involved in the regulation of T cells via an

effect on the cytoskeleton. IQ motif-containing GTPase-activating protein 1 (IQGAP1) is a cytoskeleton-interacting scaffold protein that mediates chemokine receptor 4 cell-surface expression and signaling (Bamidele et al., 2015). The Rho GTPase cell-division control protein 42 (Cdc42) coordinates regulation of the actin and MT cytoskeletons by binding and activating WASP. Cdc42 plays a role in the motility of mature B cells, their interaction with T cells, and their differentiation into antibody producing cells (Gerasimcik et al., 2015). Deletion of Cdc42 in B cells is associated with the formation of smaller germinal centers and reduced antibody response, as well as impaired formation of protrusions that contain F-actin, MTs, and Cdc42-interacting protein 4. GTPase, a member of the immunity associated protein (GIMAP) family, is highly expressed on immune cells. Additionally, GIMAP4 exhibits GTPase activity, and its transcription is regulated during early human CD4 + Th differentiation. GIMAP4 localizes to cytoskeletal elements and controls cytokine secretion in early differentiating human CD4 + Th lymphocytes and, in particular, the secretion of interferon- γ (Heinonen et al., 2015). GIMAP5 functions in T lymphocytes are associated with facilitation of MT-dependent mitochondrial buffering of Ca^{2+} (Chen et al., 2013). Additionally, GIMAP5 deficiency in T cells impairs Ca^{2+} entry via plasma-membrane channels. Disruption of MTs, but not the actin cytoskeleton, abrogates mitochondrial Ca^{2+} sequestration in T cells.

Activated interleukin (IL)-7 receptors embedded in membrane microdomains induce actin-microfilament meshwork formation, anchoring MTs that grow radially from rafted receptors to the nuclear membrane. Phosphorylated signal transducer and activator of transcription 5 is loaded onto kinesins and slides along MTs across the cytoplasm to reach the nucleus following IL-7 stimulation (Tamarit et al., 2013).

T cell activation requires the growth of MTs mediated by the plus-end-specific protein EB1, interaction of which with TCR is essential for controlling TCR sorting (Martin-Cofreces et al., 2012). Activated extracellular signal-regulated kinases (ERKs) are localized at the IS, with their activation critical for MTOC polarization (Filbert et al., 2012). Taxol (which inhibits microtubule depolymerization) and ciliobrevin (a dynein inhibitor) block centrosome polarization when used together, whereas taxol alone slows polarization. Pioneer MT, which plays a role in the formation of protrusions at the leading edge of migratory cells, is regulated by RAC1 and stathmin and forms complexes involved in centrosome polarization (Wittmann et al., 2003; de la Roche et al., 2013; Gomez et al., 2007). Stathmin is an ERK substrate and a regulator of the MT network during T cell activation, at which time ERK is recruited to the IS to allow its phosphorylation of stathmin molecules (Filbert et al., 2012). The cytoskeletal-adaptor protein paxillin localizes to the MTOC in T cells and upon target-cell binding is recruited to the SMAC. Paxillin is recruited to sites of integrin engagement and contributes to MTOC reorientation required for degranulation (Robertson and Ostergaard, 2011).

Ezrin is a membrane-microfilament linker that regulates IS architecture and T cell activation via interaction with the scaffold protein discs large homolog 1 (Dlg1). Ezrin plays a role in microcluster dynamics and TCR signaling through its ability to establish MT-network organization at the IS. Along with Dlg1 and MTs, it promotes the organization of the IS and TCR-signal downregulation (Lasserre et al., 2010). Aurora A is a serine/threonine kinase that contributes to mitosis progression by inducing MT nucleation and plays a role in antigen-driven T cell activation. Aurora A is phosphorylated at the IS during TCR-driven cell contact, and inhibition of Aurora A disrupts MT dynamics and CD3 zeta-bearing vesicles at the IS. Additionally, absence of Aurora A activity impairs activation of early signaling molecules downstream of TCRs, as well as expression of IL-2, CD25, and CD69 (Blas-Rus et al., 2016). The MT-damaging agent aruncin B exerts apoptogenic activity, with its exposure to T cells causing apoptosis, MT damage, G_2/M -arrest, B cell lymphoma 2 (Bcl-2) phosphorylation, Bcl-2 homologous antagonist/killer activation, loss of mitochondrial

membrane potential, cytochrome c release, activation of multiple caspases, and poly(ADP-ribose) polymerase degradation (Han et al., 2012). Sperm-associated antigen 6 is a component of the central apparatus of the axoneme, plays a role in ciliary and flagellar motility, and regulates IS function (Cooley et al., 2016). It is expressed in lymphoid tissues and is associated with the centrosome in lymphocytes, where its deficiency results in IS disruption due to loss of centrosome polarization and actin clearance at the synaptic cleft, defective CTL functions, impaired humoral immunity, reduced germinal centers, and decreased production of class-switched antibodies (Cooley et al., 2016).

Antibody blockade of co-inhibitory receptor lymphocyte activation gene-3 (LAG3) and programmed cell death 1 (PD1) or knockout of LAG3 and PD1 enhances T-effector function. LAG3 and PD1 co-localize in activated CD8 + T cells at trans-Golgi vesicles, early/recycling endosomal compartments, lysosomes, and MTOCs. The association of LAG3 with PD1 contributes to their trafficking to the IS, leading to synergistic inhibition of T cell signaling (Huang et al., 2015). These data support a close association between several molecules associated with MT-lymphocyte interconnections in the IS.

1.2.2. MTs and B cell function

The ability of B cells to capture external antigens and present them as peptide fragments on major histocompatibility complex (MHC) class II molecules to CD4 + T cells is vital to the adaptive immune response (Yuseff et al., 2013). The interaction of B cells with antigens presented on the surface of neighboring cells in secondary lymphoid organs triggers the formation of an IS that facilitates efficient processing of membrane antigens. The establishment of an IS is coupled to an arrest in B cell migration, which enables antigen acquisition. B cells use cell polarity to coordinate the events that lead to efficient humoral responses (Yuseff et al., 2013).

The MT network is essential for B cell synapse formation. The mechano-sensing ability of B cells is dependent upon MTs and linked to the actin cytoskeleton (Wan et al., 2013), which is important for the trafficking of B cell receptor (BCR)-antigen complexes (Yuseff et al., 2013). The BCR comprises a plasma membrane immunoglobulin coupled to a signaling module formed by the Ig α -Ig β dimer, which contains immunoreceptor tyrosine-based activation motifs, in which tyrosine residues are phosphorylated by SRC family kinases upon antigen engagement (Reth and Wienands, 1997). This results in recruitment and activation of SYK, followed by induction of calcium signaling, involved in initiating gene transcription required for B-cell function (Baba and Kurosaki, 2011). The early events of B-cell activation induce a rapid actin-dependent membrane-spreading response at the antigen-contact site, which increases the number of BCR-antigen encounters (Harwood and Batista, 2010). Antigens are gathered into BCR microclusters, followed by a contraction phase in which antigen-BCR complexes join into a central cluster. This contraction is caused by the concerted actions of rearrangements of the cortical actin cytoskeleton and is mediated by ERM proteins that link plasma membrane proteins to the actin cytoskeleton, and the MT-based motor protein dynein (Treanor et al., 2011).

The actin cytoskeleton is also required for internalized BCR-antigen complexes to be stored within non-terminal lysosomal compartments in which antigen degradation is limited. BCR-dependent actin remodeling also controls the trafficking of MHC class II molecules, stimulating the formation of the antigen-processing compartment. Following BCR engagement, actin-associated motor myosin II is activated and regulates the polarized transport of MHC class II molecules towards internalized antigens (Weber et al., 2008). B cell polarity is essential for B cell activation. B cells rapidly polarize their MTOC, together with MHC class II + lysosomes, towards the antigen contact site (Yuseff et al., 2013). Following antigen acquisition, B cells continue to show a polarized phenotype.

Dynein is required to concentrate BCR microclusters at the IS. Similar to IS established CTLs, B cells rapidly relocate their MTOC to the site of antigen encounter (Yuseff et al., 2011). The MT network

guides lysosome trafficking to the IS that forms upon BCR engagement with the immobilized antigen. BCR engagement initially induces fast depolymerization of the actin cytoskeleton, followed by a polarized re-polymerization. Coupling of BCR signaling and dynamic actin cytoskeletal reorganization is required. BCR engagement triggers tyrosine phosphorylation of actin-binding protein which promotes dynamin recruitment and actin rearrangements that enable receptor internalization into late-endosome compartments, where antigen processing occurs (Onabajo et al., 2008).

Tunneling nanotubes (TNTs) are extended intercellular connecting structures providing a particular transport route between two neighboring cells and have been reported in T cells, natural killer (NK) cells, DCs, and macrophages. Mature B cells form extensive TNT networks under conditions resembling the physiological environment. Spontaneous growth of TNTs is either reduced by BCRs or increased by LPS signals, supporting the role of cytoplasmic Ca^{2+} in the regulation of TNT formation. Transport of GM1/GM3+ vesicles, lysosomes, and mitochondria inside of TNTs and intercellular exchange of MHC-II and B7-2 (CD86) molecules might serve as pathways of intercellular communication and immunoregulation (Osteikoetxea-Molnar et al., 2016). Downstream BCR effectors, including the Rho GTPases RAC1 and RAC2, regulate actin-cytoskeletal rearrangements that promote actin polymerization, after which actin-cytoskeleton reorganization occurs through activation of the actin-severing protein cofilin (Freeman et al., 2011). These data support a role for MTs and MT-related proteins in B cell function and synapse formation.

1.2.3. MTs and innate cells: NK, NKT, and DCs

The cytoskeleton also acts as a central regulator of innate immune cells (Laguerre et al., 2013). NK cells discriminate between target cells by using activating and inhibitory signals at the IS and similarly polarize their centrosome to CTLs (Stinchcombe et al., 2006); however, integrin activation alone triggers centrosome and granule polarization in NK cells, but not in CTLs (March, 2011). The integrin signal in NK cells is mediated by integrin-linked kinase, which forms a heterotrimeric complex involved in cell adhesion, spreading, and polarity (Legate et al., 2006). The dynamics of filamentous actin are essential for organizing NK-cell receptors, establishing cellular polarity, and coordinating immune receptor and integrin-mediated signaling, as well as directing secretion of lytic granules and cytokines (Laguerre et al., 2013).

Centrosome polarization is a mechanism involved in secretory lysosome delivery to the IS in NK and NK-T cells (Stinchcombe et al., 2011). The MT network determines the distribution of lytic granules and vesicles containing cytokines to the synapse. Exocytic and endocytic organelles polarized toward the centrosome at the plasma membrane form a focal point for exocytosis and endocytosis within the IS. Patients with loss-of-function mutations in the dedicator of cytokinesis-8 protein display low T cell number, and their NK cells have reduced cytotoxicity (Zhang et al., 2009). Additionally, patients lacking coronin 1 A, an actin-regulatory protein, display loss of NK-cell activity linked to a failure to reorganize synaptic cortical actin, which inhibits granule release (Mace and Orange, 2014).

DCs rapidly extend their class II MHC-positive late endosomal compartments into tubular structures, a process induced by Toll-like receptor (TLR) triggering. Tubular endosomes within DCs polarize toward antigen-specific CD4^+ T cells, with TLR triggering inducing late-tubular endosomes in DCs. TLR triggering was insufficient for tubulation of transferrin-positive endosomal recycling compartments (ERCs) in DCs, as tubulation of ERCs within human DCs requires antigen-specific CD8^+ T cell interactions. MT disintegration abolished tubular ERCs, which coincided with reduced antigen-dependent CD8^+ T cell activation (Compeer et al., 2014).

These findings demonstrate the importance of cytoskeleton-related proteins in the function of innate immune cells.

1.2.4. MTs and lymphocyte movement

Diapedesis of leukocytes across endothelial cells is a crucial step involved in both the innate and adaptive immune responses. MTs are required for persistent migration and chemotaxis, and endothelial MTs are essential for diapedesis (Mamdouh et al., 2008). Transendothelial migration (TEM) involves a series of distinct interactions regulated sequentially by molecules concentrated at the endothelial-cell border, including platelet/endothelial-cell-adhesion molecule (PECAM), poliovirus receptor (CD155), and CD99. These molecules are components of the lateral-border recycling compartment (LBRC), with targeted recycling of LBRC requiring kinesin to move the membrane along MTs (Muller, 2014). The interaction between PECAM on leukocytes and at the endothelial border triggers focused recycling of the membrane from reticulum localization close to the lateral border of the endothelial cell. Additionally, targeted recycling from the LBRC is required for diapedesis and mediated by kinesin-family molecular motors, which usually require functioning endothelial MTs. Disruption of MTs blocks targeted recycling and monocyte diapedesis.

$\text{mDia1}^{-/-}$ T cells exhibit impaired lymphocyte-function-associated antigen 1 (LFA-1)-mediated T cell adhesion, migration, and in vivo trafficking. These are related to impaired MT polarization and stabilization, altered MT dynamics, and reduced peripheral clustering of the MT plus-end protein adenomatous polyposis coli and results in T cell migration following LFA-1 engagement (Dong et al., 2013). DCs present lipidated peptides through an endocytosis-independent pathway in order to promote potent antitumor effects in vivo (Song et al., 2011). The uptake of non-lipidated peptides by DCs is inhibited by depolymerization of actin filaments or MTs. By contrast, internalization of lipidated peptides is not inhibited when actin filaments or MTs are depolymerized. The mammalian diaphanous-related formin (mDia1), a Rho-regulated cytoskeletal modulator, promotes T lymphocyte chemotaxis and interaction with APCs (Dong et al., 2013).

Effector memory CD4^+ T cells transmigrate across endothelial-cell monolayers in response to inflammatory chemokines or TCR recognition of antigens presented on the surface of endothelial cells. The MTOC and cytosolic granules follow the nucleus across the endothelium during chemokine-driven TEM. MTOC reorientation to the contact region between the T cell and the endothelial cell accompanied by dynein-driven transport of granzyme-containing granules to the contact region, as well as exocytosis at the contact region, are early events in TCR-driven, but not chemokine-driven, TEM. In the final stages of TCR-driven TEM, the MTOC precedes the nucleus across the endothelium (Manes and Pober, 2014).

Regulation of MT stability and Ras homolog gene family member A (RhoA)/Rho-associated protein kinase activity alters T cell migration from lamellipodium-based persistent migration to bleb-based migration (Takesono et al., 2010). Nocodazole-mediated disruption of MTs in T cells prevents the formation of a stable uropod or lamellipodium, with T cells instead moving via membrane blebbing with reduced migratory persistence. MTs are explicitly required for uropod stability. IQGAP1 is an adaptor protein that binds to filamentous actin and MTs at inter-endothelial junctions during lymphocyte TEM. IQGAP1 contributes to MT stability at endothelial junctions and is involved in junction remodeling required for efficient lymphocyte diapedesis (Nakhaei-Nejad et al., 2010).

During leukocyte adhesion to the activated endothelium, lymphocyte mitochondria redistribute to the adhesion zone together with the MTOC in an integrin-dependent manner. Mitochondrial redistribution and efficient lymphocyte adhesion to the endothelium requires the function of mitochondrial Rho GTPase-1 (Miro-1), an adaptor molecule that couples mitochondria to MTs (Morlino et al., 2014). Mitochondria accumulate around the MTOC in a process regulated by Miro-1, suggesting that Miro-1 controls lymphocyte adhesion and migration through the regulation of mitochondrial redistribution (Morlino et al., 2014). These findings support a role for MTs in lymphocyte migration.

1.2.5. MTs and immune-associated disorders

The cytoskeleton plays a role in immune-associated diseases. In multiple sclerosis, the most prevalent autoimmune disease of the central nervous system, polarized CD4 Th1, Th17, CD8 T cells, and NK cells induce MT destabilization within neurites, which occurs prior to signs of apoptosis. MT destabilization is restricted to axons, thereby sparing dendrites. Lymphocytes with cytolytic activity directly drive MT axonal destabilization in a bystander manner that is independent of neuronal death (Miller et al., 2013). In fibrosis, RhoA activation is dependent upon an intact MT network. Upregulation of the pro-fibrotic cytokine connective-tissue growth factor by mechanical strain is dependent upon RhoA activation and inhibited by MT disruption. Colchicine, an MT-depolymerizing agent, inhibits glomerular RhoA activation and attenuates glomerular sclerosis and interstitial fibrosis (Guan et al., 2013). Additionally, MT disruption decreases renal infiltration of lymphocytes and macrophages.

1.3. MTs and the gut

1.3.1. MTs and the gut immune system, neuroimmune interactions, and the gut microbiome

The cytoskeleton plays a role in several gut functions. The dynamic process of organ elongation of embryonic structures requires a mechanism that modulates intercellular adhesion to allow cells to change position without compromising structural integrity. Jun N-terminal kinase (JNK) is necessary for tissue elongation of the gut tube and regulates MT architecture to preserve adhesive contacts between gut cells and the establishment of MT stability and tissue cohesion (Dush and Nascone-Yoder, 2013). Tao-1 can destabilize MTs at the actin-rich cortex, thereby controlling the cytoskeletal architecture of cells. Knockdown of Tao-1 causes disordered migration of primordial germ cells out of the gut epithelium and subsequent cell death (Pflanz et al., 2015). As gut epithelial cells are associated with the immune system in the bowel, it indirectly implies an association between Tao-1 and immune response (Cukrowska et al., 2017; Kurashima and Kiyono, 2017).

Disruption of the gut cytoskeleton is relevant to several systemic disorders. Oxidant injury to epithelial cells and gut-barrier disruption are vital factors in the pathogenesis of inflammatory bowel disease. Oxidants induce disruption of epithelial-barrier integrity by disassembling the cytoskeleton largely through activation of the PKC-lambda isoform, which is sufficient for disruption of the cellular cytoskeleton and monolayer-barrier permeability (Banan et al., 2005). Additionally, actin and MTs are essential for *Klebsiella pneumoniae* invasion into colonic epithelial cells (Hsu et al., 2015). Macrophages are activated upon exposure to proinflammatory cytokines and pathogenic stimuli, such as LPS (Xu and Harrison, 2015). Stathmin, an MT-catastrophe protein, is reduced in activated macrophages, which contain significantly more stabilized MT, as part of an LPS-specific response that induces proteasome-mediated degradation of stathmin (Xu and Harrison, 2015). Deletion of the endogenous *ppkl* gene in the malaria parasite *Plasmodium* causes abnormal ookinete development and differentiation and dissociation of apical MTs from the inner-membrane complex and was associated with generation of an immotile phenotype and a failure to invade the mosquito mid-gut epithelium. These observations were substantiated by changes in localization of cytoskeletal tubulin and actin, and the micronemal circumsporozoite- and TRAP-related protein in knockout mutants (Guttery et al., 2012).

Neuroimmune interactions in the gut are associated with maintenance of intestinal immune homeostasis and the pathogenesis of several immune-mediated diseases. Calcitonin gene-related peptide (CGRP) is a neurotransmitter of intrinsic enteric sensory neurons and that is found at elevated levels near one another in the colon of mice used as a food-allergy (FA) model. CGRP augments MT reorganization in resting and A23187-activated mouse bone-marrow-derived mast cells. CGRP-enhanced MT reorganization augments IgE-independent/non-antigenic stimuli-induced mucosal mast-cell degranulation,

contributing to FA development (Kim et al., 2014). Enteric neurons controlling gut functions are prone to oxidative insult that damages mitochondria during intestinal inflammation, with mitochondrial transport in guinea pig myenteric neurites blocked by colchicine-mediated MT disruption and alteration of actin-filament stabilization (Vanden Berghe et al., 2004).

MT dynamic instability is characteristic of their cellular activity (Kilner et al., 2016). Short-chain fatty acids (SCFAs), acetate, propionate, butyrate, and valerate are produced by the gut microbiome (Kilner et al., 2016), and odd-chain SCFAs exert an anti-mitotic effect in colon cancer cells by disrupting MT structural integrity via tubulin dysregulation. Simulations of untreated and butyrate-treated cells reflected MT behavior in interphase or untreated control cells, with propionate and valerate simulations displaying increased frequencies and more extended periods of MT shrinkage. The enhanced dynamicity of the MTs was dissimilar to that observed in mitotic cells, but paralleled that induced by MT-destabilizing treatments (Kilner et al., 2016). These data support an essential role for MTs in various gut functions, inflammatory conditions, and associations with the microbiome.

1.3.2. Oral immunotherapy and gut MTs

Oral immunotherapy is a novel method for altering the systemic immune system by generating an immune signal in the gut (Ilan, 2009a, 2016a,b) and reportedly effective in animal models of various immune disorders (Ilan et al., 2016; Israeli et al., 2015a; Mizrahi et al., 2012; Israeli and Ilan, 2010; Shibolet et al., 2004; Nagler et al., 2000; Ilan et al., 1998, 2017). Preliminary data support the potential use of this method in humans with immune-associated diseases (Ilan, 2016a,b; Israeli and Ilan, 2010; Lalazar et al., 2015; Halota et al., 2015; Margalit et al., 2006; Lalazar et al., 2017). This new class of drugs includes several compounds being developed as potent immune-modulatory agents targeting immune-associated inflammatory, metabolic, infectious, and malignant disorders. Among these compounds are those associated with viral proteins used to treat chronic hepatitis B and C virus infections (Ilan, 2004; Israeli et al., 2004; Safadi et al., 2003); liver-extracted proteins, glycosphingolipids, soy-derived extracts, and oral anti-CD3, anti-tumor necrosis factor (TNF), and anti-LPS antibodies (Ilan, 2016b; Ilan et al., 2016; Lalazar et al., 2015; Halota et al., 2015; Lalazar et al., 2017; Khoury et al., 2015; Zigmond et al., 2014; Ilan, 2013; Elinav et al., 2006; Ilan et al., 2010); non-absorbable 6-mercaptopurine, gut-derived proteins, glycosphingolipids, and an oral anti-TNF antibody for treating inflammatory bowel disease (Israeli et al., 2015a; Israeli and Ilan, 2010; Ilan et al., 2017; Ben Ya'acov et al., 2015; Israeli et al., 2015b); and non-absorbable disease-related antigens used to treat liver cancer and graft-versus-host disease (Ilan et al., 2007, 2005; Zigmond et al., 2007). These methods show efficacy as modulators of the immune system without inducing immune suppression.

A role for innate immune cells, including NK T cells and DCs in the gut, was described as underlying the mechanism associated with this mode of immune modulation (Ilan, 2016a,b; Ilan et al., 2010). Activation of these cells promotes the activation of regulatory cells, thereby enabling systemic immune modulation in a target-organ-specific manner. A potential role for glycosphingolipids was demonstrated in this process partly through effects on NK T cells, as well as by affecting lipid rafts in cell membranes (Lalazar et al., 2009, 2008a; Lalazar et al., 2008b). NK T cells are subsets of lymphocytes that are activated by glycosphingolipids (Heymann and Tacke, 2016; Marrero et al., 2015), several of which were recently described as playing a role in enabling NK T cell type II lymphocytes (Nair et al., 2015), with oral glucosylceramide exerting an immunomodulatory effect via alteration of NK T cells (Shuvy et al., 2009; Ilan, 2009b; Zigmond et al., 2009, 2008; Adar and Ilan, 2008; El Haj et al., 2007; Lalazar et al., 2006). Cell membranes comprise discrete protein and lipid domains with various functions, with the structural and biological properties of these domains represented as cholesterol-dependent membrane (CDM) domains (i.e., membrane rafts) (Byrum and Rodgers, 2015). Glycosphingolipids are

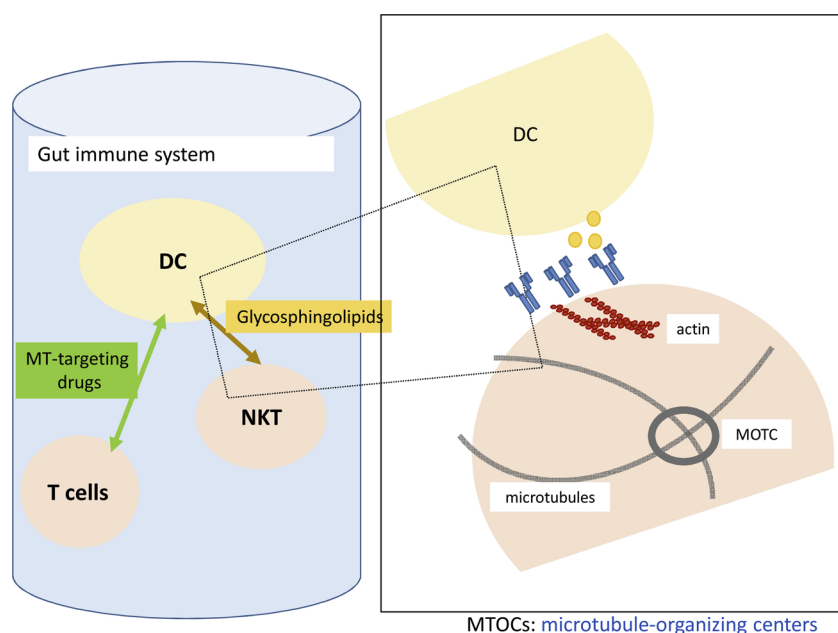


Fig. 1. Potential associations between the gut immune system, the cytoskeleton, and intracellular glycosphingolipids. Drugs that target the MT system, as well as glycosphingolipids in the gut, can potentially improve the efficacy of oral immunotherapy through alterations in cross-talk occurring in the cytoskeleton between NKT cells, T cells and DCs.

constituents of membrane rafts, and oral immunotherapy using glycosphingolipids reportedly alters raft structure (Lalazar et al., 2009, 2008a; Lalazar et al., 2008b).

In T cells, CDMs regulate the Src family kinase Lck (p56lck) by sequestering it from its activator CD45. The actomyosin cytoskeleton plays an integral role in the formation of CDM domains (Byrum and Rodgers, 2015), and alteration of MTs changes raft membranes. One of the first events occurring upon antigen activation of mature T cells is failure of TCR-stimulated $CD4^+CD8^+$ thymocytes to polarize their MTOC (Cunningham et al., 2011). Unstable MTs are a feature of immature murine $CD4^+CD8^+$ thymocytes, which exhibit higher levels of glycogen synthase kinase 3 (GSK3) activity, a known inhibitor of MT stability. $CD4^+CD8^+$ thymocytes acquire the ability to polarize their MTOC in response to TCR signals upon GSK3 inhibition (Cunningham et al., 2011). Altering glycosphingolipids results in cytoskeleton modification. Additionally, glycosphingolipids can serve as mediators of MT alteration in innate NK T cells, thereby enabling immune modulation via the gut immune system.

Treatment with MT stabilizers, such as colchicine, efficiently prevents bacterial internalization and translocation of nonpathogenic *Escherichia coli*, suggesting that epithelia under metabolic stress increase their endocytotic activity to promote MT-dependent bacterial internalization and transcytosis (Nazli et al., 2006).

Targeting MTs in gut immune cells as a form of oral immunotherapy is advantageous, because it requires a relatively low dose of drugs. As for most oral immunotherapeutic agents, no absorption is required to achieve a therapeutic effect, thereby allowing a more moderate treatment modality that is less toxic and exhibits fewer side effects. Fig. 1 shows a potential scheme whereby drugs targeting the MT system in the gut can improve oral immunotherapy. This mechanism suggests a potential role for glycosphingolipids to promote cross-talk in the NKT cells, T cells and DC cytoskeletons.

2. Summary

Much data has accumulated in recent years concerning the importance of MTs as players in both the innate and adaptive immune systems. Studies also suggest an essential role for these cellular structures in the gut and the gut immune system. Interactions in the gut between the cytoskeleton and cell membranes of innate immune cells represent attractive targets for new therapeutic interventions involving

immunotherapy and specifically for oral immunotherapy. This mode of therapy can potentially provide a means of modulating deleterious immune responses by targeting the cytoskeleton of cells associated with the gut immune system.

Disclosure

T. Ilan-Ber is the CMO of Oberon Sciences; Y. Ilan is the medical director of Exalenz Biosciences; Oberon Sciences, and a consultant for Teva; Enzo Biochem; Protalix; Therapix; Nasvax; Immuron; Immunepharm; Tiziana; and Natural Shield.

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