

# Les liaisons dangereuses: immunological synapse formation in animals and plants

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**The immunological synapse in vertebrates describes a specialized junction between a T cell and a target cell, enabling execution of immune responses through focal secretion. Recent insights in the plant immune system suggest that plant cells assemble a pathogen-inducible machinery at the cell surface that shares several features with the immunological synapse. Apparent mechanistic commonalities include co-stimulatory non-self alarm signals as triggers, cell polarization driven by actin cytoskeleton remodeling, protein concentration into ring-shaped assemblies at the cell periphery and focal exocytosis mediated by soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins that are core factors for vesicle fusion. Although in plants, execution of immune responses by polar secretion seems to be a cell type-independent property, its confinement to T cells in the vertebrate immune system might reflect a greater division of labor.**

## Introduction

In vertebrates, lymphocytes play critical roles in cell-mediated immunity against pathogens. On the detection of non-self molecular structures on cell surfaces, a subset of these, including cytotoxic T cells (CTLs), natural killer (NK) cells and natural killer T cells (NKTs), release cytolytic and other cell killing molecules to destroy their target cells, but spare themselves from being killed [1]. The delivery of cytotoxic cargo occurs at contact sites of CTLs and NK cells with their respective target cells, where a specialized and localized structure, the immunological synapse, is formed (see below). Immunological synapses are also established between T helper cells that express the CD4 glycoprotein (CD4<sup>+</sup> cells) and professional or nonprofessional antigen presenting cells. However, cargo secreted by the T helper cells is not cytotoxic but is made up of cytokines that stimulate further immune cells such as macrophages and cytotoxic T cells. Although the molecular composition of both types of immunological synapses is distinct, they share biochemical and functional similarities [2]. Plants lack specialized immune cell patrols and rely on the capacity of each cell to recognize and mount effective immune responses against potential invaders. Plants evolved two radar systems for non-self recognition: one comprises pattern recognition receptors (PRRs) on the

cell surface, which detect conserved microbe-associated molecular patterns (MAMPs). A second class of largely intracellular sensors, called resistance (R) proteins, recognize polymorphic, typically isolate-specific pathogen effectors. Unlike PRR-triggered immune responses, immunity conferred by R proteins is frequently associated with a rapid suicide of the host cells. Although structural similarities between plant pattern receptors and animal innate immunity receptors have been described before [3–6], potential mechanistic parallels in the execution of immune responses in the two kingdoms have not been explored.

Cell autonomous immune responses are widespread in plant–microbe interactions and often terminate a pathogen attack at the cell surface, without affecting host cell viability. Here we discuss recent findings on the formation of a pathogen-induced secretion machinery in plant cells that stops parasite growth at the cell surface. We contrast these events with processes underlying T cell–mediated immunological synapse formation in vertebrates. Although the former involves the formation of a cellular junction between a plant and pathogen cell and the latter a junction between two host cells, we argue that these processes can be considered as a realization of cell-mediated immunity in the two kingdoms that share fundamental mechanistic features through localized recruitment of functionally similar molecules.

## Non-self recognition triggers immune responses in plants and animals

The critical contact leading to CD4<sup>+</sup> T-cell activation is provided by binding of the plasma membrane–resident T cell receptor (TCR) to a short antigen peptide presented by major histocompatibility complex proteins (MHC) on the surface of another cell [e.g. a professional antigen presenting cell (APC)] [7]. This interaction is enhanced by the plasma membrane–resident CD4 co-receptor that binds to other portions of peptide-presenting MHC molecules [7,8]. Activation of naïve T cells needs a co-stimulatory second signal provided by the CD28 membrane receptor, which binds CD80 (B7.1) and CD86 (B7.2) proteins on the APC surface [9,10]. CD80 and CD86 are expressed at low levels on resting APCs and are upregulated after activation [9,10]. The signal triggered by the TCR–peptide–MHC interaction is transduced by a tightly controlled phosphorylation cascade through tyrosine phosphorylation of

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the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR complex [11]. Additionally, integrins such as leucocyte function associated antigen (LFA-1) seem to stabilize the immunological synapse [12]. Ultimately, this leads to the activation of transcription factors including nuclear factor- $\kappa$ B (NF- $\kappa$ B), AP-1, and nuclear factor of activated T cells (NFAT) to induce activation, differentiation, proliferation and effector function of T cells [13,14].

Two plant PRRs, *Arabidopsis* flagellin sensing 2 (FLS2) and Elongation factor Tu receptor (EFR), have been identified, each composed of structural modules permitting perception of extracellular MAMPs and subsequent initiation of intracellular signaling. Both receptors contain extracellular leucine-rich repeats (LRRs), a single transmembrane-spanning helix, and an intracellular kinase domain. FLS2 directly binds to the flg22 peptide, the most conserved part of the bacterial motor protein flagellin, whereas EFR recognizes elf18, an acetylated 18-mer peptide derived from the N terminus of bacterial elongation factor EF-Tu [15–17]. Because both receptors contain an intracellular kinase module, signal transduction is likely initiated through phosphorylation cascades [18] after binding of the cognate MAMPs to the extracellular LRR region. Recent findings have shown that flg22 signal transduction through FLS2 involves *in vivo* complex formation of FLS2 with BRI1-associated receptor kinase 1 (BAK1), an LRR-containing receptor-like kinase that was originally identified as an adaptor or co-receptor of the brassinosteroid hormone receptor BRI1 [19]. This suggests that BAK1 might function as an adaptor or co-receptor for the function of various surface receptors. In rice, a chitin elicitor binding protein (CEBiP) binds one of the major constituents of fungal cell walls, *N*-acetyl-chitooligosaccharide oligomers. The plasma membrane-anchored CEBiP contains two extracellular LysM domains, a module implicated in peptidoglycan-binding, but lacks an intracellular kinase domain, whereas the recently identified *Arabidopsis* chitin elicitor receptor kinase 1 (CERK1) contains both three extracellular LysM domains and a cytoplasmic kinase motif [20–22]. One possibility is that rice CEBiP functions as co-receptor by binding to a yet unidentified receptor-like kinase.

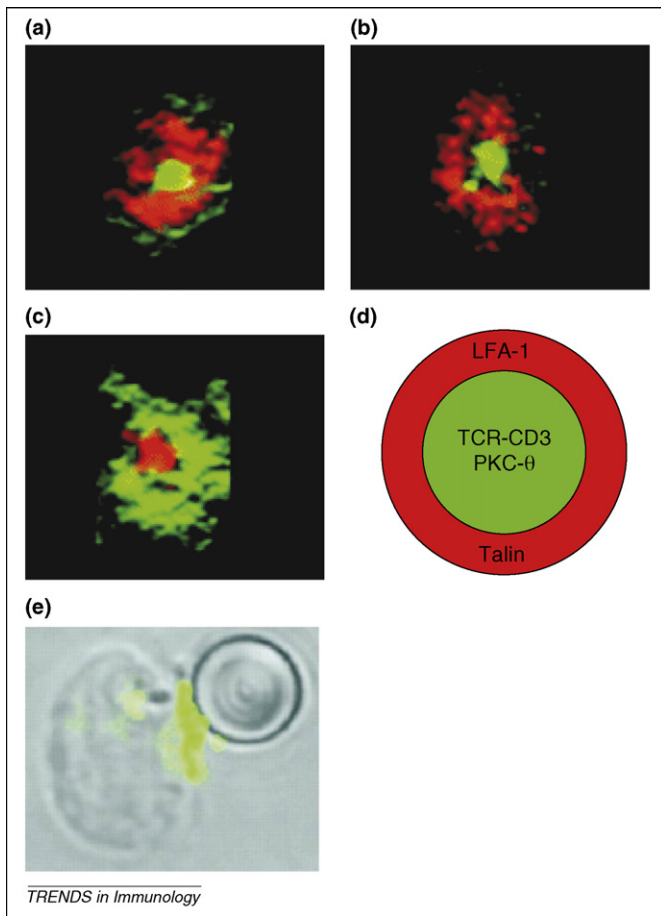
Application of the above described MAMPs changes the expression profile of ~3% of tested *Arabidopsis* genes within 30 min. These encode a disproportionate number of transcription factors, protein kinases and phosphatases, receptor-like kinases, and proteins regulating protein turnover. Because these changes are also seen in the presence of cycloheximide (an inhibitor of protein biosynthesis), MAMP-triggered transcriptional reprogramming likely involves rapidly turned-over repressors [23]. Of note, elf18, flg22 or chitin treatment each change the expression of a largely overlapping set of genes, suggesting convergence of signaling pathways triggered by different MAMP receptors [17,23,24]. Thus, analogous to T cells, extracellular non-self signals are in plants rapidly processed to reprogram the transcriptional machinery for immune responses. A highly diversified family of WRKY DNA binding domain-containing transcription factors has been implicated in MAMP-triggered transcriptional

reprogramming events [18,25–29]. Recent findings suggest that a disease resistance response that is triggered by an intracellular R sensor involves potentiation of PRR-triggered immune responses. This requires nuclear activity of the R sensor and its ligand-dependent direct association and interference with WRKY repressors that exert negative feedback control on PRR-triggered defense responses [29].

### Establishment of the immunological synapse

Although CD4<sup>+</sup> T cells are capable of detecting a single agonist-peptide MHC complex on a target cell, approximately ten complexes are needed to stop T-cell migration and to initiate the formation of a stable APC contact site [30]. Ligated TCR complexes become, within minutes after an initial APC encounter, relocalized and accumulate together with the co-stimulatory CD28 receptor at the nascent synapse [31]. This protein relocalization in the plasma membrane is part of a complex molecular segregation process leading to the formation of spatially distinct supramolecular activation clusters (SMACs) with a ‘bull’s-eye’ zone pattern at contact sites between T cells and target cells (Figure 1a–d) [32–35]. The segregation process of distinct SMACs (called central and peripheral SMACs) requires both TCR ligation and co-stimulation [36,37], is energy-dependent and involves mass transport of randomly distributed lipid raft-like microdomains on the T-cell surface to APC contact sites through cytoskeletal linkage and molecular motors (Figure 1e) [35,37,38]. Formation of SMACs and maturation of the nascent synapse seems to be driven by cytoskeletal polarization, which is accompanied by the movement of the microtubule-organizing center (MTOC) and Golgi apparatus (GA) beneath contact sites, thereby enabling directed release of cytotoxic granules and cytokines as well as localized TCR internalization [39–41]. Numerous TCR proximal signaling molecules are needed for actin accumulation and F-actin dynamics at the immunological synapse [34,41]. The guanine-nucleotide-exchange factor (GEF) VAV1 plays a key role in this process and is thought to catalyze the exchange of GDP for GTP on the RHO GTPases CDC42 and RAC1, as well as recruiting the large GTPase dynamin-2 to the immunological synapse [42,43]. Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin homologous protein (WAVE) are key proteins needed for actin cytoskeleton re-organization by activating Arp2/3 complex activity, which in turn nucleates actin filaments [44–46]. CDC42 activates WASP by direct interaction with the auto-inhibitory domain of WASP, and RAC1 activates WAVE1 by association with an inhibitory protein complex, thereby releasing active WAVE1 [47–50]. Despite these advances, the exact roles and action sites of actin-regulatory and actin-nucleating proteins during T-cell activation and their spatial distribution during maturation of the immunological synapse are still ill-defined.

Although TCR–peptide–MHC complexes are highly concentrated in the center of the immunological synapse, initial signaling to induce the formation of the immunological synapse is transient [33,35]. Rather than amplifying the signal, the synapse controls the signal strength by recruiting and degrading signaling molecules to fully



**Figure 1.** Spatial rearrangement of proteins at contact sites between T cells and target cells. At the immunological synapse of a CD4<sup>+</sup> T cell with an antigen presenting cell, T cell receptor (TCR)-CD3 complex [green in (a) and (b)] is centrally concentrated and differentially localized from talin [red in (a)] and leucocyte function associated antigen (LFA-1) [red in (b)]. Protein kinase C  $\theta$  (PKC- $\theta$ ) [red in (c)] also accumulates in the center of the synapse [talin, green in (c)] [32]. (d) Molecular segregation results in a 'bull's-eye' pattern at the immunological synapse. TCR-CD3 complex and PKC- $\theta$  are localized in the central supramolecular activation cluster (cSMAC, green), and talin and LFA-1 accumulate in the peripheral SMAC (pSMAC, red). (e) Co-stimulation-dependent clustering of sterol-rich lipids raft (green) at the contact site between a CD4<sup>+</sup> T cell and a polystyrene microbead coated with anti-CD3 and anti-CD28 antibodies. The lipid raft was visualized by staining GM1 glycosphingolipid with fluorescein isothiocyanate-labeled cholera toxin subunit b [37]. Adapted, with permission, from Refs. [32,37].

activate T cells [51–53]. Toward this end, the polarized cytoskeleton plays a key role in the recruitment of signaling components to the synapse because inhibition of actin polymerization in preformed CD4<sup>+</sup> T cell–APC contacts abrogates T-cell activation [51–53].

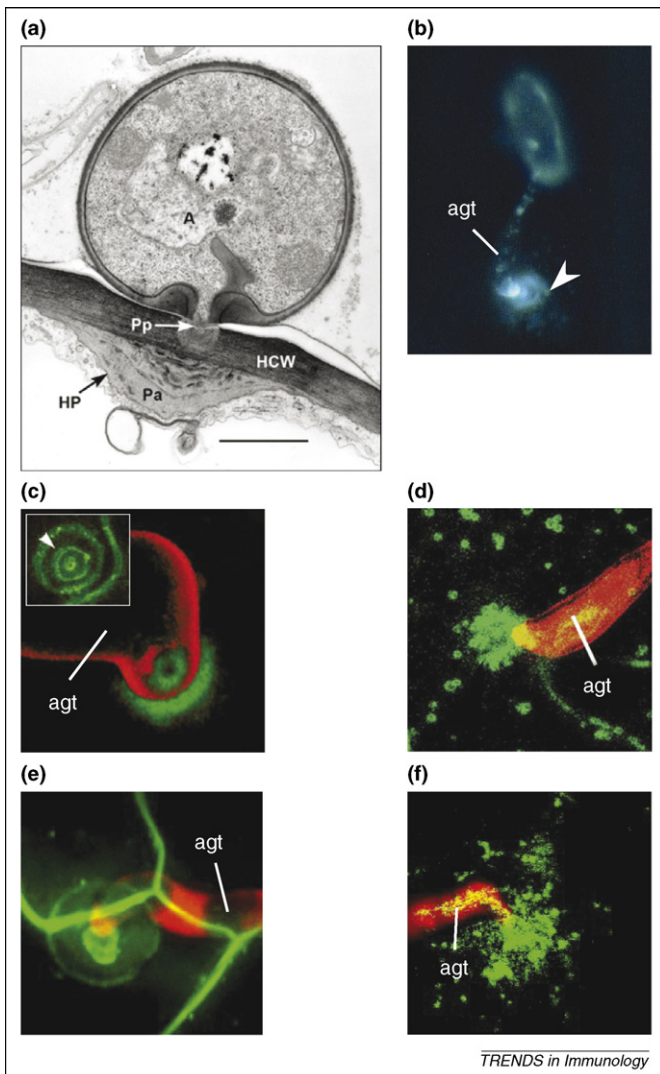
### Pathogen-induced cell polarization and focal secretion in plants

Plant cells respond to surface colonization by phytopathogenic bacteria or attempted entry of filamentous parasites such as fungi or oomycetes (filamentous protists) with rapid cell polarization and secretion, including polar alignment of the actin cytoskeleton and directed movement of organelles to pathogen contact sites [54–57]. Visualization of plant actin cytoskeleton dynamics by green fluorescent protein (GFP) fused to the actin-binding domain of human talin in interactions between *Arabidopsis* and oomycete parasites showed extensive host cytoskeleton remodeling,

leading to the formation of large polarized actin bundles beneath pathogen entry sites [56]. Drug-mediated or genetic perturbation of actin dynamics permitted effective parasite entry [58,59]. Expression of a constitutively active Rac/Rop GTPase modulates actin cytoskeleton rearrangements beneath pathogen contact sites and increased fungal entry [60,61]. Although plants contain homologs of ARP2/3, WASP and WAVE complex components, their potential contribution to pathogen-induced cell polarization remains to be tested.

Pathogen-triggered cell polarization typically leads to the formation of a ring-shaped and stable subcellular structure of ~50- $\mu$ m diameter, designated papilla (Figure 2a) [62]. Papillae are unique to plant cells because they are generated by focal deposition of *de novo* synthesized cell wall material, including callose, a (1 $\rightarrow$ 3)- $\beta$ -D-glucan, between the inner cell wall and the plasma membrane (paramural space) [63]. The multilayered appearance of papillae is indicative of a sequential order of cell wall deposits generated by the focal release of cell wall precursors. One hypothesis is that pathogen-inducible cell wall appositions provide a structural scaffold for antimicrobial compounds and contribute to structural reinforcement of the cell wall against parasite ingress. However, specific depletion of papillary callose in *pmr4/gsl5 Arabidopsis* mutants, carrying null mutations in 1 of 12 callose synthase-like family members, results in constitutive activation of the salicylic acid (SA)-dependent defense pathway and enhanced disease resistance responses [64,65]. This suggests a more subtle role of papillary cell wall components such as negative regulation of the SA defense pathway by plasma membrane-resident *PMR4/GSL5* callose synthase or by its enzymatic product.

The isolation of *Arabidopsis* mutants that are partially immunocompromised against ascomycete powdery mildew fungi revealed novel components required for focal immune responses at the cell periphery. The penetration 1 (PEN1) syntaxin, peroxisomal PEN2  $\beta$ -glycosyl hydrolase and the PEN3 ABC transporter are each necessary to restrict powdery mildew entry coincident with the formation of papillae [66–68]. PEN2 and PEN3 act in the same pathway and are implicated in the cytoplasmic synthesis and transport of small antimicrobial compounds across the plasma membrane, respectively [67,68]. The plasma membrane-resident PEN1 syntaxin acts in a second pathway [66,67] and has recently been shown to mediate vesicle fusion processes by forming secretory ternary SNARE complexes together with the adaptor protein synaptosomal-associated protein of 33 kDa (SNAP33) and vesicle-associated membrane protein (VAMP) 721 or VAMP722 [69]. Each of these proteins is subject to dynamic relocalization on parasite attack and becomes concentrated beneath incipient pathogen entry sites. This includes concentration of PEN1 syntaxin and associated SNAP33, as well as of the PEN3 ABC transporter in a seemingly sterol-enriched lipid raft-like plasma membrane micro-domain (Figure 2b, c and e) [68–71]. Residence of the PEN1 syntaxin in lipid raft-like microdomains is further supported by a partial, sterol-dependent association of the SNARE protein with detergent-resistant membranes (N. Zappel and R.P., unpublished), which are widely



**Figure 2.** Focal concentration of secretion-related gene products of plants at incipient pathogen entry sites. (a) Transmission electron micrograph showing penetration of a cucumber epidermal cell by an appressorium (A) of the fungus *Colletotrichum lagenarium*. The fungal penetration peg (Pp) emerging from the base of the appressorium has breached the plant cuticle but has become restricted within the host cell wall (HCW), and a callose papilla (Pa) has been deposited by the host cell at the site of attempted penetration. HP, host plasma membrane. Bar = 1  $\mu\text{m}$  [62]. (b) Filipin-mediated fluorescence at the tip of the fungus *Blumeria graminis* appressorial germ tube (agt) on the leaf surface and underneath in the attacked host epidermal cell (arrowhead). Filipin staining indicates sterol accumulation in membranes and might represent clusters of lipid rafts (indicated by the arrowhead) [71]. Focal accumulation of penetration 1 (PEN1) (c), PEN2 (d), PEN3 (e) and VAMP722 (f) (green) at the contact site between plant and fungal cells (red) [67,70]. Transgenic plants expressing functional green fluorescent protein (GFP)-fused proteins were inoculated with *B. graminis* conidiospores, and the interaction sites were analyzed by confocal microscopy. A typical 'bull's eye' pattern of GFP-PEN1 accumulation beneath a fungal attack site is shown in the inset of (c). The arrowhead points to a presumed endomembrane compartment [70]. agt, appressorial germ tube. Adapted, with permission, from Refs. [62,67,70,71].

considered as a biochemical approximation of lipid rafts [72]. In addition, PEN2-associated peroxisomes and VAMP722-containing vesicles directionally move to and congregate under pathogen portals (Figure 2d and f) [67]. However, unlike in animals where peroxisomes shuttle on microtubules, plant peroxisomes move exclusively along actin filaments, and pharmacological interference with the actin cytoskeleton leads to a rapid arrest of peroxisome movement [73–75].

Functional GFP-PEN1 and PEN3-GFP each accumulate in concentric 'bull's-eye' rings in the region of the host plasma membrane under a fungal appressorium (the tip of the fungal germ tube that adheres tightly to the plant cell wall and produces a needle-like peg to puncture the host cell wall; Figure 2a), suggesting a supramolecular organization that is reminiscent of T cell SMACs (Figure 2c and e) [32,68,70]. GFP-PEN1 was additionally located in the apparent interior of cell wall containing papillae [70]. Because electron micrographs have shown the presence of membrane-bound vesicles within papilla [76–78], this hints to unusual trafficking events and extrusion of vesicles in the paramural space, some of which might become trapped in the papillary cell wall scaffold. The directed movement of GFP-VAMP722 vesicles to pathogen portals and a *pen* phenocopy seen in *VAMP721*<sup>+/-</sup> *VAMP722*<sup>-/-</sup> mutant plants is consistent with the hypothesis that plasma membrane-resident PEN1 syntaxin acts through the formation of heterooligomeric SNARE complexes, enabling focal secretion of vesicle cargo at fungal entry sites [69]. Likewise, a distinct syntaxin family member, SYP132, has been shown to function in secretion-based antibacterial defense [79], suggesting that secretory processes are a general feature of plant immune responses against different pathogen classes but act through distinct pathways. A bacterial virulence factor, HopM1, was shown to interfere with the plant secretory system by degrading an adenosine diphosphate ribosylation factor guanine nucleotide exchange factor (ARF-GEF) [80].

### Killing cargo

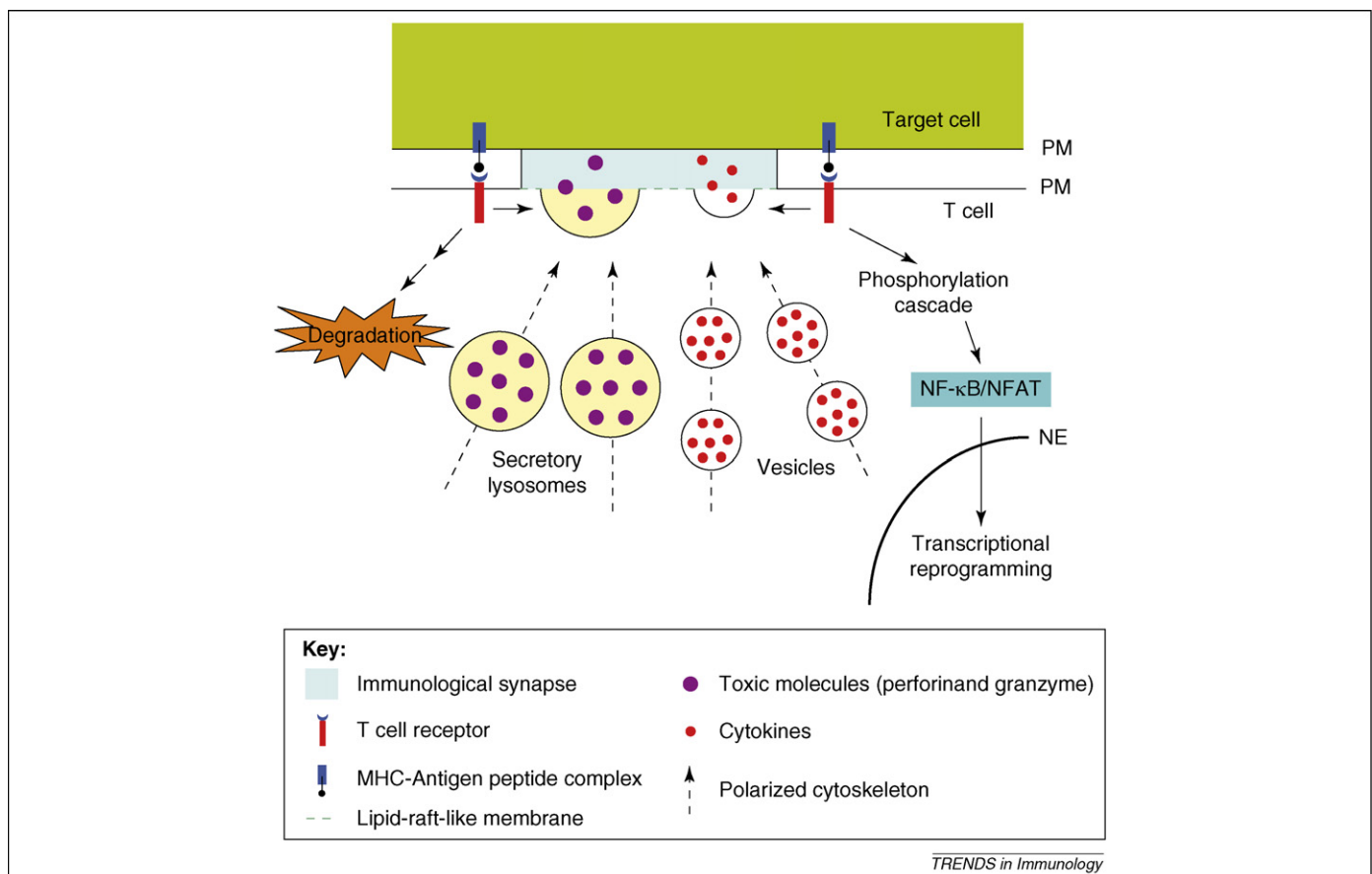
A delay in the timing of papilla formation both in *pen1* mutants [70] and in transgenic lines in which *VAMP721* and *VAMP722* are constitutively co-silenced suggests cell wall precursors and/or enzymes involved in papillary cell wall biosynthesis as potential vesicle cargo [69]. This and the genetic analysis of *pen* double mutants points to the combined action of SNARE-mediated discharge of VAMP721 and VAMP722 vesicle cargo and PEN3 ABC transporter-driven translocation of small antimicrobial compounds for a maximal immune response [67–69]. Like CTL-, NK- and NKT-dependent immune responses, plant cells executing PEN-dependent immune responses spare themselves from being killed. Possibly, formation of the papillary cell wall scaffold together with focal secretion enables containment of antimicrobials and contributes to a self-protection mechanism. However, a precise understanding of the toxic principle(s) underlying PEN-dependent termination of pathogenesis at the cell periphery await comprehensive compiling of VAMP721 and VAMP722 vesicle cargo and structure determination of molecules translocated by PEN3.

In animals, the lytic activity of CTLs and NK cells is known to be compartmentalized in specialized granules in their cytoplasm. Focal secretion of 'secretory lysosomes' at contact sites formed between these immune cells and their targets selectively induces target cell death [1,81,82]. This activity requires perforin, a soluble protein that is related to the pore-forming C9 component of complement, suggesting that secreted perforin acts through pore formation and disruption of the target plasma membrane [83–85]. Other components of lytic granules, such as the serine

proteases granzyme A and granzyme B, require perforin for their activity, possibly by facilitating the entry to target cells through a perforin pore either on the plasma membrane and/or endosomes to trigger apoptosis in target cells through caspase cleavage [83–85]. Increasingly more is becoming known about the molecular machinery that regulates the docking and fusion of this organelle with the plasma membrane. Recent insight comes from studies on inherited disorders in humans leading to defects in the granule-dependent cytotoxic function of lymphocytes [1,81,82]. Whereas mutations in *RAB27A* causing loss of function of the small GTPase Rab27a lead to defective granule docking [86,87], mutations in *UNC13D* causing loss of hMunc13–4 function impair the fusion of cytotoxic granules with target membrane that have docked at the immunological synapse [88]. A third component that has been genetically linked to granule-dependent dysfunction of NK cells is loss-of-function mutations in *STX11* encoding a syntaxin family member [89]. The involvement of Rab27a, hMunc13–4 and syntaxin 11 in vesicle fusion strongly suggests the engagement of SNAREs in cytotoxic granule exocytosis in cytolytic T cells. Interestingly, cytotoxic executor proteins such as perforin and granzyme B and fusion regulatory proteins Rab27a and hMunc13–4 localize on distinct vesicular structures [90]. Apparently only at the last step of the exocytic pathway do both types of vesicles fuse near the plasma membrane [90]. In this

context, it is tempting to speculate that the observed size variation of *Arabidopsis* GFP-VAMP722–tagged vesicles close to the plasma membrane (Figure 2f) reflects a similar mechanism of compound exocytosis in which vesicles fuse with each other before their fusion with the plasma membrane [91]. In addition, SNAREs seem to be engaged in TCR recycling and the secretion of cytokines in activated CD4<sup>+</sup> T cells, and it is likely that these are different family members from those involved in the secretion of cytotoxic granules [53,82].

**Co-stimulation is required for focal defense responses**  
Integrated functional and cell biological studies of plant PRRs have just begun. Thus, it is not yet known whether these receptors cluster at sites of attempted fungal ingress or beneath bacterial colonies. However, a GFP-tagged functional fusion protein of the plasma membrane-resident *Arabidopsis* FLS2 PRR, which detects bacterial flagellin, becomes within minutes endocytosed and degraded in a ligand-dependent manner [92]. Because these experiments involved treatment of plant cells with a single stimulus, the flg22 peptide, it should be interesting to study FLS2 localization underneath surface colony-forming pathogenic *Pseudomonas* cells. Direct evidence that MAMP treatment alone is insufficient to induce the characteristic cell polarization process seen in bacterial interactions or those with filamentous parasites was demonstrated by co-stimulation



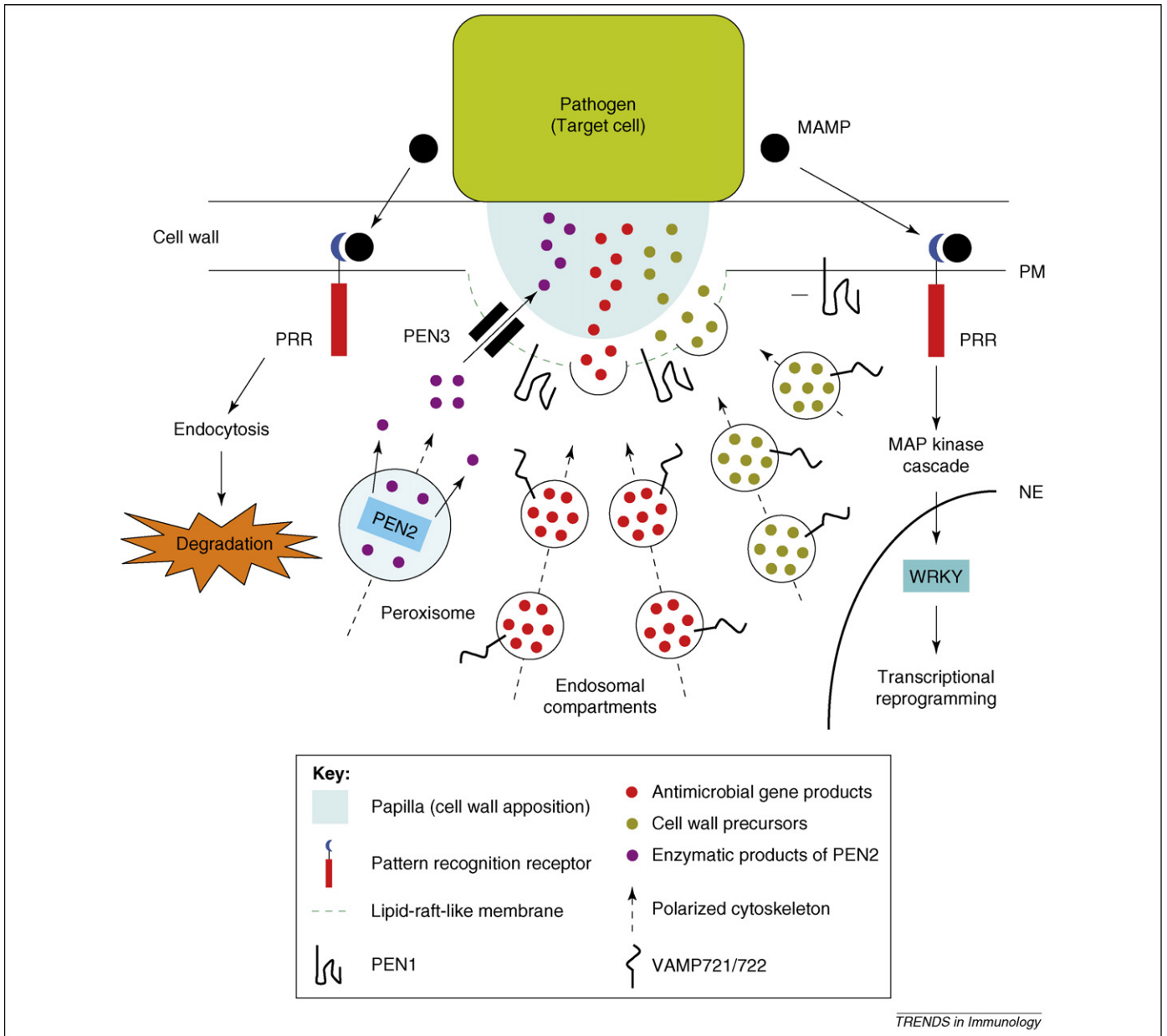
**Figure 3.** Schematic overview of the immunological synapse in cell-to-cell interactions in vertebrates and plants. A cartoon depicting key cellular processes for immunological synapse function in vertebrate T cells. The immunological synapse forms a tight interface between a T and a target cell and provides a scaffold for focal secretion of secretory lysosomes and vesicles releasing cytotoxic molecules (as in the case of cytotoxic T cells) or cytokines (as in the case of CD4<sup>+</sup> cells), respectively. The strength of signaling at the immunological synapse is controlled by continuous delivery and degradation of receptors.

experiments of individual plant cells with a MAMP from an oomycete pathogen (Pep-25) and mechanical stimulation using a needle of the same diameter as a fungal hypha [93]. Whereas mechanical stimulation alone induced polarized migration of intracellular organelles and the production of reactive oxygen intermediates (ROS), MAMP treatment induced ROS accumulation and the activation of a subset of defense-related genes [93]. However, co-stimulation triggered most of the early responses seen in interactions on fungal attack but was still insufficient to trigger papilla formation [93]. This finding supports the idea that execution of polarized resistance responses in individual plant cells demands integration of stimulatory signals in much the same way as co-stimulation is needed for the establishment of a stable immunological synapse. Because it is

likely that a host cell detects an array of pathogen-derived MAMPs, a single MAMP such as Pep-25 may trigger only a subset of the processes seen in the interaction between a host and pathogen cell.

**Conclusion**

Pattern recognition receptors in plants and animals are composed of similar structural protein modules [3–6]. This is, however, unlikely to reflect a common evolutionary origin but is likely a consequence of biochemical constraints to build non-self sensors from a limited number of eukaryotic protein modules [94]. Likewise, the similarities discussed above in cell-mediated immunity in plants and animals are likely to reflect shared biochemical and cellular constraints that demand integration of non-self sensor perception in



**Figure 4.** Schematic overview of the immunological synapse in cell-to-cell interactions in vertebrates and plants. Speculative scheme illustrating focal secretion at contact sites between plant and pathogen (target) cells. Although the clustering of plant pattern recognition receptors at pathogen contact sites remains to be tested, these sites might provide a platform for recognition of microbe-associated molecular patterns and for secretion of antimicrobials through vesicles and translocation of small toxic molecules produced by peroxisome-associated penetration 2 (PEN2). Genetic studies showed that the PEN1-dependent secretory pathway functions independently from the PEN2/PEN3 pathway [67,68]. PM, plasma membrane; NE, nuclear envelope.

single cell systems for controlled focal delivery of immune executors (Figures 3,4). In this context, we believe that future research on cell-mediated immunity in animals and plants will be mutually beneficial.

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