A STUDY OF THE IMMUNOLOGICAL SYNAPSES AND KINAPSES FORMED BY CD4 T CELL SUBSETS

By

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CERTIFICATE OF APPROVAL

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Abstract

The immunological synapse (IS) is a large-scale rearrangement of molecules at the interface between T cells and antigen presenting cells (APCs). The structure of the 'mature' IS is well defined, with a ring of adhesion molecules surrounding a centralized accumulation of T cell receptor (TCR)-peptide major histocompatibility complex (pMHC) interactions. However, there is evidence that the structure of the IS differs depending on experimental conditions and the differentiation state of the cells used. Given that IS are hypothesized to play a crucial role in the delivery of effector molecules, further studies are needed to determine the relationship between the phenotype of effector T cells and IS structure.

The aim of my thesis research was to test the hypothesis that CD4 T cell subsets possessing specialized effector functions form IS with distinct structures. To achieve this goal, I carried out experiments designed to examine the IS formed by Th1 and Th2 cells interacting with transfected fibroblast APCs and supported planar bilayers, and induced T regulatory cells (iTregs) interacting with bilayers and dendritic cells (DCs).

I provide evidence that Th1 and Th2 cells form morphologically distinct IS. Th1 cells form IS with adhesion molecules surrounding TCR-pMHC interactions, while Th2 cells form multifocal IS with adhesion molecules interspersed among multiple small accumulations of TCR-pMHC. There are also differences in the distribution of the phosphatase CD45 and phosphorylated signaling molecules in Th1 and Th2 IS. I hypothesize that these structural differences are related to differences in the effector molecules secreted by Th1 and Th2 cells, specifically

that the mature IS formed by Th1 cells act as gaskets for the directed secretion of cytotoxic molecules and CD40 ligand.

I also show that iTregs form either immunological kinapses or stable IS depending on the level of CD80 on the surface of APCs. Induced Tregs are capable of downmodulating the costimulatory ligand CD80 on the surface of DCs in an antigen specific manner. On the same timescale as CD80 downmodulation, iTregs transition from stable to motile contacts with DCs. Experiments utilizing supported planar bilayers containing pMHC, ICAM-1 and CD80 showed that iTregs form motile immunological kinapses with a highly polarized cell shape in the presence of low levels of CD80. However, iTregs arrest and form stable IS when interacting with bilayers containing high levels of CD80. Consistent with CD80 modulating the stop signals iTregs receive upon antigen recognition, intracellular calcium flux is more sustained in the presence of high levels of CD80. I hypothesize that mature IS formation is required for downmodulation of costimulatory molecules by iTregs.

Chapter 1

Introduction

Part A. CD4 T cell activation and function

The studies presented here concern immunological synapses (IS) formed between activated CD4 T cells and antigen presenting cells (APCs). Signaling proximal to the T cell receptor (TCR) precedes IS formation and is critical for IS maintenance. Section A-1 is an overview of the pathways involved in signal transduction through the TCR, and serves as an introduction to many of the molecules that will be discussed in the context of the IS. Section A-2 serves as an introduction to the differentiation and function of the cells that are used in the studies presented in Chapters 2 and 3.

A-1. T cell receptor proximal signaling

CD4 T cells recognize peptide antigens presented on major histocompatibility complex class II (MHC class II) molecules. The T cell receptor (TCR), first identified by several groups in the early 1980's, is the molecule responsible for the interaction with peptide-loaded MHC class II (pMHC) (1-4). Each T cell expresses a unique TCR, which is selected for in the thymus for its ability to bind to MHC molecules (5). When T cells recognize pMHC on the surface of APCs, a signal is transduced through the TCR, a process that is dependent on the TCR-associated CD3 complex (6). The molecules in the CD3 complex contain immunoreceptor tyrosine-based activation motifs (ITAMs), and phosphorylation of ITAMs is crucial for TCR signal transduction (7-10). The CD4 coreceptor is associated with the Src kinase Lck whose activity is necessary for ITAM phosphorylation (11-13). Upon TCR ligation, CD4 is brought within close proximity of the TCR-CD3 complex, resulting in the phosphorylation of ITAMs.

Dephosphorylation of an inhibitory tyrosine residue on Lck by the large phosphatase CD45 is also critical for this process (14).

Once phosphorylated, CD3 ITAMs recruit the Syk-family kinase ZAP-70 (15). ZAP-70 phosphorylates the adaptor molecules LAT and SLP-76, which form a trimolecular complex with Gads (16-18). This structure forms the core of the proximal TCR signaling complex, recruiting numerous proteins responsible for altering the cytoskeleton, and transducing signals to the nucleus. Of particular importance, phospholipase C (PLC)-γ is recruited to the LAT-SLP-76 complex and activated by the Tec kinase Itk (19, 20). PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of ER calcium stores, which results in activation of the phosphatase calcineurin. Calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT) leading to its translocation to the nucleus (18). DAG recruits protein kinase C (PKC)-θ to the plasma membrane, resulting in its activation (21). PKC-θ activates Ras GTPase via phosphorylation of the Ras guanine nucleotide exchange factor (GEF) RasGRP1, and active Ras initiates signal transduction through the MAP kinase pathway and gene transcription (22). PKC-θ is also required for phosphorylation of CARMA1, allowing formation of the CARMA1-BCL-10-MALT1 complex and activation of the NFκB pathway (23, 24). In addition to these roles, intracellular calcium flux and PKC-θ activation are also critical regulators of the dynamic T cell-APC interactions that ultimately result in IS formation, as discussed below.

The costimulatory molecule CD28 binds to CD80 and CD86 on the surface of APCs. This interaction is crucial, as signaling through the TCR without costimulation results in a state of anergy. Ligation of CD28 leads to its phosphorylation and the subsequent recruitment of phosphatidylinositol-3-OH kinase (PI3K) (25). PI3K phosphorylates PIP_2, producing phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 serves as a docking site for pleckstrin homology (PH)-domain containing proteins, many of which are important for T cell activation. In particular, 3-phosophoinositide-dependent protein kinase 1 (PDK1) and Akt are recruited to the plasma membrane via their PH-domains. PDK1 activates Akt, resulting in enhancement of the NFκB and NFAT pathways (18). Thus, signals through the TCR and CD28 converge on a similar set of transcription factors, resulting in complete T cell activation.

A-2. CD4 T cell subset differentiation and function

Upon receiving a productive signal via the TCR, naïve CD4 T cells have the ability to differentiate into at least four (and probably more) types of effector cells depending on the presence of cytokines and other factors. Th1, Th2 and induced T regulatory cells (iTregs) are utilized in the studies presented here.

Th1 cells

Th1 differentiation requires IL-12 produced by antigen presenting cells and interferon (IFN)-γ (perhaps produced by natural killer (NK) cells) (26). IFN-γ upregulates expression of the master transcription factor T-bet via a signal through signal transducer and activator of transcription 1 (STAT1). T-bet then induces the expression of IFN-γ and the IL-12 receptor β2. IL-12 (via STAT4) and

IFN-γ cooperate to maintain high levels of IFN-γ production, resulting in complete differentiation.

Th1 cells, which secrete IFN-γ, lymphotoxin (LT), tumor necrosis factor-α (TNF) and IL-2, are mediators of several facets of the immune response. IFN-γ secreted by Th1 cells is critical for the maturation of macrophages, increasing their ability to destroy phagocytosed microbes (26). IFN-γ is also important for isotype switching by B cells. In mice, the IFN-γ dependent isotypes are IgG2a and IgG3, and these isotypes are critical for the opsonization and subsequent phagocytosis of microbes (27). IL-2 produced early in an immune response by Th1 cells is important for the generation of memory $CD8^+$ cells capable of a secondary response (28), and IFN-γ also plays a role in stimulating CD8 T cells (27). In addition to secreting cytokines, Th1 cells express CD40 ligand (CD40L), which activates B cells, dendritic cells (DCs) and macrophages in a CD40-dependent manner. Th1 cells also have lytic granules (containing granzyme B and perforin) and are capable of killing APCs in an antigen specific manner (29).

Th2 cells

Th2 differentiation requires signaling through the interleukin (IL)-4 receptor (IL4R) (26, 30). IL-4 binding causes STAT6 mediated expression of the transcription factor GATA3, resulting in production of endogenous IL-4. Newly produced IL-4 then further upregulates GATA3 in a positive feedback loop. IL-2 signaling via STAT5 is also required for efficient IL-4 production and Th2 differentiation (26, 31).

Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and are critical regulators of the allergic response and immunity against extracellular parasites (26). IL-4

produced by Th2 cells helps B cells to secrete large quantities of neutralizing antibodies (predominantly IgG1) and IgE (32), and also acts in the positive feedback loop during the differentiation of Th2 cells (26). Mast cells and basophils bind IgE with high affinity, and cross-linking IgE on the surface of these cells by multivalent antigens results in degranulation and an allergic response (26, 27). Th2 derived IL-13 is essential for protective immunity to nematodes (33), and IL-5 mediates the maturation and activation of eosinophils (26, 27).

Induced T regulatory cells

Differentiation of naïve CD4 T cells to iTregs absolutely requires the presence of transforming growth factor (TGF)-β and IL-2 (26). Signals through the TCR, TGF-β receptor and the IL-2 receptor (acting via NFAT, Smad3 and STAT5 respectively) cooperate to induce expression of the transcription factor FoxP3. FoxP3 controls the expression of a multitude of genes that are required for iTreg function. All trans retinoic acid has been shown to enhance the TGF-β and IL-2 mediated conversion of naïve CD4 T cells to iTregs (34).

Induced Tregs function similarly to thymus-derived or natural Tregs (nTregs), although they tend to recognize foreign antigens instead of self-antigens and are critical for suppression of immune reactions to commensal gut bacteria (35). In vitro, Tregs suppress the proliferation of T cells in a contact dependent fashion (36). However, TGF-β, IL-10 and IL-35 are important for Treg function in vivo (37). Additionally, cytotoxic T-lymphocyte-associated protein (CTLA)-4 has recently been shown to be critical for Treg function in vivo (38).

Part B. The immunological synapse

The experiments presented in this thesis examine differences in the IS formed by CD4 T cell subsets. Therefore, a detailed introduction to IS formation, structure and function is important. This part of the introduction begins with section B-1, which describes T cell locomotion and the 'stop signal' and subsequent conjugate formation that follow recognition of antigen by the TCR. The transition from locomotion to stable conjugate formation is examined in detail for iTregs in Chapter 3. Sections B-2 through B-4 deal with the roles of the cytoskeleton and TCR proximal signaling in the formation and structure of the IS. The discussions in these sections are important for understanding the processes and signals involved in the formation of the structures described in Chapters 2 and 3. Actin dynamics are crucial for lamellapodium and uropod formation and the morphological changes associated with IS formation described in Chapter 3. The segregation of signaling and adhesion molecules seen in the IS described in Chapters 2 and 3 also requires actin remodeling. TCR proximal signaling is crucial for all of the processes described in this thesis. Chapter 2 examines the effect that reducing TCR signaling has on IS structure. Chapter 3 describes differences in the locomotion of iTregs in the presence and absence of signaling through the TCR. Section B-5 describes the role of costimulation in IS formation and structure. This section is an important introduction for Chapter 3, which describes a new role for CD80 in IS formation by iTregs. Section B-6 is a discussion of the function of the IS, which is germane to the discussion in Chapter 2, the model for iTreg function presented in Chapter 3 and the

discussion in Chapter 4. Section B-7 discusses the diversity of IS structures that have been observed, and can be viewed as a lead-in to the rest of the thesis.

B-1. T cell-APC interactions

T cell locomotion

Advances in in vivo imaging techniques have revealed that T cells migrate continuously on DCs within lymph nodes both in the absence of high-affinity cognate antigen, as well as 24 hr after initial activation (39-42). Migrating T cells can be divided into three zones: i. a protruding edge with filopodia generated by filamentous actin (F-actin) polymerization called the lamellapodium, ii. an F-actin and integrin rich zone at the center of the cell called the lamella, and iii. a myosin-II rich structure trailing the cell body that contains most of the cytoplasm called the uropod (43, 44). Polymerization of actin at the lamellapodium causes a retrograde flow of actin from the lamellapodium to the uropod, propelling the cell forward if the actin cytoskeleton is attached to the substrate via integrins (43, 45, 46). Maintenance of this polarized cell shape requires complexes containing proteins with PDZ domains, with the Scribble and Crumbs complexes primarily located in the uropod and the Par3 complex located in the cell body (47). These complexes of PDZ proteins maintain polarity by interacting with membrane proteins and the cytoskeleton, and are required for T cell motility (47). Members of the ezrin-radixin-moesin (ERM) family of proteins that connect cell surface molecules to the actin cytoskeleton are also indicated in cell polarity, with phosphorylated ezrin primarily found in the uropod (48). Overexpression of constitutively active forms of several members of the Rho family of GTPases, including RhoA, Rac1 and Cdc42, inhibit cell polarization and localization of the

ERM protein moesin to the uropod (49). T cell chemotaxis is inhibited in cells expressing both constitutively active and dominant negative Rac1 and Cdc42, indicating that cycles of Rho family GTPase activation and inactivation may be required for polarization and locomotion (49).

In T cells migrating on substrates coated with inter-cellular adhesion molecule (ICAM)-1, a large band of high-affinity lymphocyte function-associated antigen (LFA)-1 is found in the lamella (50). This LFA-1 rich 'focal zone' colocalizes with talin, and knocking down talin results in a drastic loss of motility, suggesting that it provides a crucial link between the substrate and the cytoskeleton via an interaction with LFA-1. Making forward progress requires migrating T cells to advance the lamellapodium and detach the uropod in concert. This process relies on two kinases that phosphorylate myosin light chains: myosin light chain kinase (MLCK) and Rho kinase (ROCK) (51). MLCK and its upstream activator calmodulin are required for extension of the lamellapodium and ROCK is necessary for both formation and detachment of the uropod (48, 51). The importance of the actomyosin cytoskeleton is further highlighted by the finding that the class II myosin heavy chain MyH9, which is located in the uropod of migrating T cells, is required for polarization and locomotion (52). Uropod detachment may also require the large glycoprotein CD43, which is located in the uropod of migrating T cells, and is hypothesized to play a role in LFA-1 de-adhesion (53).

Antigen recognition causes changes in T cell morphology

CD4 T cells recognize peptide antigens loaded on MHC class II molecules presented by DCs, B cells and other APCs. This arrangement necessitates a cell-

cell interaction, during which TCRs on the surface of T cells are triggered by pMHC on the surface of APCs. The morphological changes that occur during antigen recognition, and the subsequent formation of T cell-APC conjugates, were thoroughly described in studies of a human CD4 T cell clone interacting in an antigen-specific manner with a monolayer of MHC class II-transfected fibroblasts (54, 55). These studies showed that the morphological changes following antigen recognition can be broken down into three steps: i. CD4 T cells have a polarized shape and low levels of intracellular calcium while initially contacting the surface of APCs, ii. T cells exhibit a spike in calcium levels and become rounded up, and iii. T cells flatten against the APC while maintaining increased calcium levels. A similar flattening phenotype, with an associated increase in intracellular calcium is seen when naïve TCR transgenic CD4 T cells form conjugates with peptide-pulsed B cells (56). Actin polymerization and continuous signaling through the TCR are critical for maintenance of the morphological changes associated with antigen recognition, as treatment with cytochalasin D or blocking anti-MHC class II antibodies inhibits the flattening phenotype and associated calcium flux (57). Further, buffering intracellular calcium prevents T cells from forming stable interactions with APCs (58). The correlation between an increase in intracellular calcium and changes in T cell morphology is significant because calcium flux occurs only seconds after cross-linking the TCR (59).

The observation that T cells flatten against APCs upon antigen recognition was reminiscent of earlier studies examining the conjugates formed by cytotoxic T lymphocytes (CTL) and target cells, although this phenotype was not explicitly quantified (60, 61). These early studies did, however, find that the microtubule

organizing center (MTOC) and the Golgi apparatus in the CTL were polarized towards the CTL-target cell interface (60, 61). As a result of these observations, it was hypothesized that polarization of the MTOC served to direct cytotoxic molecules contained in secretory compartments towards the target cell (61). Further experiments with CD4 helper T cell clones and APCs showed that the MTOC and Golgi apparatus in helper T cells are polarized towards the APC as well (62). Significantly, experiments with a Th2 cell line showed that after several hours of incubation with antigen-loaded splenic B cells, IL-4 accumulated in the T cell is polarized towards the T cell-APC interface (63). Several molecules involved in antigen recognition and subsequent signaling events, including the TCR, CD4 and PKC–θ also accumulate at the interface of T cell-APC conjugates (64, 65). Together, these observations indicate that the purpose of prolonged interactions between T cells and APCs is two-fold: i. sustaining a signal through the T cell receptor, and ii. allowing for the efficient delivery of effector molecules towards the APC.

Adhesion molecules are critical for productive T cell-APC interactions

The adhesion molecule LFA-1 and the cytoskeleton-associated protein talin are also found at the T cell-APC interface (66, 67). The discovery that LFA-1 accumulates at the interface of T cell-APC conjugates, although not unexpected given its role in adhesion, is significant, because blocking assays inhibiting the binding of LFA-1 to its ligand prevent killing in cytotoxicity assays and proliferation in mixed lymphocyte reactions (68-70). LFA-1 binds to its ligand, ICAM-1, in a cation-dependent manner, and signaling through the TCR transiently increases the avidity of LFA-1 for ICAM-1 (71, 72). The coupling of

LFA-1 avidity to TCR signaling, known as inside-out signaling, is mediated by the scaffold proteins ADAP and SKAP55, which link SLP-76 in the TCR signaling complex to integrins (73, 74). The critical role that LFA-1-ICAM-1 interactions play in T cell-APC conjugate formation was further shown in experiments examining the interaction of CD4 T cells with planar lipid bilayers reconstituted with ICAM-1 and pMHC (75). In these experiments, T cells crawled around in the absence of pMHC, but immediately stopped when they reached a bilayer containing both ICAM-1 and pMHC. Remarkably, the T cells fluxed calcium and polarized the MTOC towards the planar bilayer in addition to stopping, revealing that TCR-pMHC and LFA-1-ICAM-1 interactions are sufficient to induce the morphological changes associated with antigen recognition noted earlier by Trautmann and colleagues (54).

When ligands for the adhesion molecule CD2 (CD48 in rodents and CD58 in humans) were incorporated into planar lipid bilayers along with ICAM-1, CD2-CD48/58 and LFA-1-ICAM-1 accumulated in distinct domains at the T cell-lipid bilayer interface (76, 77). Specifically, CD2-CD48/58 interactions accumulated at the center of the interface, surrounded by a ring of LFA-1-ICAM-1. This segregation only happened if the T cells were pre-treated with the phorbol ester PMA and ionomycin or if pMHC was also present in the planar bilayers, suggesting that large-scale segregation of receptor-ligand interactions occur at the T cell-APC interface during antigen recognition. This hypothesis was shown to be correct in two seminal papers describing what would come to be known as the immunological synapse (78, 79).

B-2. Supramolecular activation clusters

Monks et al. examined antigen-specific conjugates formed between a cloned T cell line and the CH12 B cell lymphoma line (79). In this study, stacks of images were taken of CD4 T cell-APC conjugates that had been fixed and stained after a 30 min incubation, and the cell-cell interfaces in these conjugates were reconstructed three-dimensionally. Staining for the TCR complex, MHC class II and PKC–θ showed a central location at the cell-cell interface, and this collection of proteins was coined the central supramolecular activation cluster (cSMAC). Surrounding these accumulations of TCR-pMHC and PKC–θ, in a region termed the peripheral SMAC (pSMAC), were the adhesion molecule LFA-1 and the cytoskeletal-linker protein talin. Also, the Src-kinases Lck and Fyn were found to rapidly accumulate in the cSMAC. Based on these results, it was hypothesized that the function of the cSMAC might be to cluster the TCR complex and associated signaling molecules, thus potentiating the signal through the TCR. The additional finding that recognition of agonist, but not weak agonist/antagonist altered peptide ligands (APLs), resulted in cSMAC and pSMAC formation bolstered this hypothesis. Further work by the Kupfer group showed that CD8 T cells form IS that are morphologically very similar to those formed by CD4 T cells (80).

Grakoui et al. utilized the minimalist supported planar bilayer system described above (75) to examine the T cell-APC interface immediately following antigen recognition (78). Fluorescently labeled glycophosphatidylinositol (GPI)-linked MHC and ICAM-1 were incorporated into liposomes that were then spread out on clean glass coverslips in a flow chamber and incubated with peptide

overnight. The GPI-linked proteins are able to diffuse freely in this system, and naïve T cells flux calcium, proliferate and produce IL-2 when incubated with peptide-loaded bilayers. Activated T cell blasts were introduced to the system and the organization of MHC and ICAM-1 at the T cell-APC interface was examined. The 'mature' SMACs that Monks et al. observed, with a ring of LFA-1-ICAM-1 surrounding TCR-pMHC interactions, could be clearly seen after ten minutes in this simplified system. Importantly, time-lapse microscopy revealed that the initial TCR-pMHC interactions occurred in the periphery of the contact region and gradually moved to the cSMAC over the next ten minutes. These large-scale rearrangements were dependent on actin polymerization, as treatment with cytochalasin D prevented the movement of TCR-pMHC into the cSMAC. Experiments with APLs showed that the formation of a cSMAC and the density of TCR-pMHC interactions depend on a strong signal through the TCR. Together, Monks et al. and Grakoui et al. convincingly demonstrated that the IS is composed of distinct SMACs containing signaling and adhesion molecules, and that the formation of the IS is a dynamic process correlated with T cell activation.

B-3. The actin cytoskeleton at the immunological synapse

Polarity proteins reorganize during immunological synapse formation

The flattening phenotype observed when T cells conjugate with APCs necessitates a deformation of the plasma membrane and the underlying cortical actin. The ERM family of proteins that link membrane proteins to the actin cytoskeleton are crucial for this process (81). During the process of IS formation, CD43 is relocated from the uropod to the rear of the T cell, where it colocalizes with ERM proteins in a structure called the distal pole complex (81-84). A signal

through the TCR results in dephosphorylation of ERM proteins (via the GEF VAV1 and the small GTPases Rac1 and Cdc42) (85). Dephosphorylation of ERM proteins causes the dislocation of the cortical actin cytoskeleton from the plasma membrane, increasing the deformability of the membrane and enhancing T cell-APC conjugate formation. It is thought that the initial dephosphorylation of ERM proteins decouples CD43 from the cytoskeleton, and rephosphorylation results in the reattachment of ERM proteins to CD43 and the cytoskeleton at the distal pole (86).

During conjugation with an APC, the PDZ-containing polarity proteins Disks large (Dlg) and Scribble are transiently located at the nascent IS, while the Crumbs3 and Par3 complexes colocalize with CD3 at the T cell-APC interface at later time points after Dlg and Scribble have moved away (47). T cells lacking Scribble fail to recruit the TCR and PKC–θ to the interface, suggesting that rearrangement of polarity proteins is required for the transition from migration to IS formation. While at the IS, the Dlg isoform Dlgh1 forms a complex with Lck, Zap-70 and Wiskott-Aldrich Syndrome protein (WASp), indicating that reorientation of polarity proteins may be linked to initiation of actin polymerization at the IS (87).

Actin polymerization at the immunological synapse

As mentioned above, actin polymerization is crucial for IS formation (78). The necessity for actin remodeling during IS formation was further demonstrated in experiments examining the effects of inhibition of the protein cofilin. Cofilin induces F-actin depolymerization and cleavage, and inhibition resulted in impaired T cell-APC conjugation and IS formation (88). Actin remodeling during

the first minutes after antigen recognition, results in an actin rich ring just inside the peripheral lamellapodial extensions at the T cell-APC interface (89). The formation of this actin ring is Lat dependent, and WASp and the adaptor protein Nck are localized to the ring in a Lat and Slp-76 dependent manner (89, 90). The finding that WASp is located at the site of actin polymerization is significant because activated WASp binds and activates the Arp2/3 complex resulting in the nucleation of F-actin branches (91, 92). Activation of WASp requires a conformational change induced by the GTPase Cdc42, which allows an interaction between WASp and Arp2/3 (93). Significantly, WASp and Cdc42-GTP are found at the IS along with the GEF Vav1 (94, 95). The TCR proximal kinase Itk is necessary for recruitment of Vav1 to the IS, resulting in the activation of Cdc42 and actin polymerization at the T cell-APC interface (95, 96). WASp is recruited to the IS via an interaction with the adaptor protein Nck, which in turn interacts with phosphorylated Slp-76. Vav1 and Cdc42 are also recruited to phosphorylated Slp-76, bringing all of the proteins required for actin polymerization together in a complex proximal to the TCR (97).

WASp deficient T cells have defects in TCR signaling and exhibit reduced proliferation and IL-2 production after activation (98). It has been reported that accumulation of F-actin at the IS is impaired in T cells lacking WASp function (99). Also, T cells defective in WASp-interacting protein (WIP), which is known to stabilize WASp, have defects in APC conjugation and IS formation (100, 101).

WASp was recently shown to play a critical role in maintaining IS integrity. Experiments examining naïve CD4 T cells interacting with supported planar bilayers containing pMHC, ICAM-1 and CD80 revealed that naïve cells

continuously break IS symmetry, migrate and then reform IS (102). While PKC–θ is necessary to break IS symmetry, WASp deficient T cells were unable to maintain symmetry and had a predominantly migratory phenotype while forming only short-lived IS.

Although WASp clearly plays an important role in IS dynamics, other experiments have shown that actin and PKC–θ accumulate normally at the T cell-APC interface in WASp deficient T cells when high concentrations of antigen are used, with only a slight defect in talin and PKC–θ accumulation at low antigen doses (103). Given that actin polymerization and IS formation occur in WASp deficient T cells, other actin regulatory proteins must necessarily be involved in these processes. Indeed, the Abi/WAVE complex has also been shown to control actin polymerization at the IS (104, 105). The three WAVE isoforms are in the WASp family, but unlike WASp they are not activated directly by Rho GTPases. Instead, WAVE proteins are stably associated with Abi-1/2 in a protein complex including Hem family adaptors and Sra1. Rac1-GTP binds Sra1, recruiting the WAVE complex to the plasma membrane where it interacts with and activates Arp2/3 (106). All of the members of the WAVE complex are present in T cells, and multiple WAVE isoforms are recruited to the IS. Importantly, actin polymerization at the IS is significantly impaired when WAVE proteins are knocked down, as are proliferation and IL-2 production (104, 105). Surprisingly, talin can be found in a complex with WAVE and Arp2/3 (107). This arrangement most likely functions to enhance the recruitment of integrins to the nascent IS, a site of active actin polymerization. In addition to the WAVE complex, the actin regulatory protein HS1 is also recruited to the IS where it promotes actin

polymerization via the Arp2/3 complex (108, 109). HS1 is phosphorylated in an Lck and Zap-70 dependent manner, and is recruited to the IS via interactions with SH2 domain containing proteins in the TCR signaling complex where it stabilizes Vav1 localization, probably in concert with Slp-76 and Itk (96, 97, 108).

B-4. TCR proximal signaling at the immunological synapse

Dynamic localization of CD45 and Lck during immunological synapse formation

Given that IS formation is a dynamic process involving localization of the TCR that occurs simultaneously with signaling cascades initiated at the TCR, experiments examining the role of the IS in T cell signaling are of interest. The initial studies of the IS identified the Src-kinases Lck and Fyn as well as the serine/threonine kinase PKC–θ as examples of signaling molecules that reside within the SMACs at the T cell-APC interface (65, 79). Further analysis revealed that three minutes after conjugation Lck and CD45 were both in the cSMAC while talin and molecules containing phosphorylated tyrosine residues (pTyr) resided in the pSMAC. However, by seven minutes post conjugation, CD45 was absent from the interface, pTyr and Lck were randomly distributed and phospho-Zap-70 was localized in the cSMAC (110). These findings corroborated and extended on the earlier finding that CD45 is excluded from the T cell-B cell interface five minutes after conjugate formation (111). However, other experiments have shown that small amounts of CD45 remain at the T cell-APC interface at later time points where it is hypothesized to play a role in keeping Lck in a state of continual activation during IS formation (112). Significantly, the colocalization of CD45 and Lck soon after antigen recognition provides a spatiotemporal

explanation for the findings that the phosphatase activity of CD45 is necessary to dephosphorylate an inhibitory tyrosine residue in Lck (113-115). Furthermore, the eventual exclusion of most CD45 from the T-cell-APC interface is likely necessary to prevent the undesirable dephosphorylation of crucial signaling molecules proximal to the TCR (116).

The Pseudodimer Model: Recruitment of CD4 to the immunological synapse is crucial when antigen is limiting

The cytoplasmic tail of the CD4 (and CD8) co-receptor associates with Lck, implying that CD4 functions by recruiting Src-kinases to the site of antigen recognition (117). Moreover, fluorescence resonance energy transfer (FRET) experiments showed that agonist, but not antagonist, peptide ligands induce a close association between CD4 and the TCR at the T-cell-APC interface, suggesting that the inability to interact closely with CD4 is at least partially responsible for the defective TCR-signaling observed for low-affinity, short half-life TCR-pMHC interactions (118). Not surprisingly, given its importance in signaling, CD4 is critical for IS formation. CD4 knockout T cells are able to form conjugates with peptide-loaded splenocytes, but fail to cluster TCR and PKC–θ at the T cell-APC interface (119).

In a series of elegant experiments, Mark Davis and colleagues examined the activation of T cells with small numbers of agonist pMHC complexes and the function of the CD4 co-receptor in this process (120-122). Using fluorescently labeled peptides, Irvine et al. showed that only one pMHC complex was needed to cause CD4 T cells to arrest on the surface of an APC and flux calcium. However, a large excess of MHC class II molecules containing endogenous peptides were

simultaneously recruited to the T-cell-APC interface, in agreement with experiments showing the inclusion of null pMHC complexes, along with agonist pMHC, at the IS (120, 123). Blocking CD4 with antibodies increased to 25 the number of pMHC complexes required to initiate a calcium flux (120). Further, Lck is only recruited to the T-cell-APC interface in response to a small number of agonist pMHC complexes when CD4 is available (122). These data led the authors to suggest a pseudodimer model wherein a CD4 molecule associated with a TCR interacting with its cognate pMHC recruits a nearby MHC molecule containing an irrelevant peptide. The recruited MHC then interacts with a second TCR, and this dimerization of TCR-MHC interactions leads to productive signaling by CD4-associated Lck. This model is supported by the fact that when CD4 interacts with an MHC class II molecule, it juts out at almost a 90-degree angle, making it unlikely that CD4 interacts with both the TCR and MHC in a single TCR-MHC complex (124). The pseudodimer model has been confirmed with experiments with soluble heterodimers containing linked MHC molecules loaded with one agonist and one endogenous peptide. These heterodimers, but not agonist pMHC monomers, stimulate T cells to flux calcium and make IL-2 (121, 125). Clustering large numbers of TCRs, in a CD4-dependent manner, at the T cell-APC interface even in the presence of very small numbers of agonist pMHC complexes is certainly an important consequence of the molecular segregation that occurs during synapse formation. However, experiments from a number of laboratories have definitively shown that the large-scale segregation resulting in the formation of a cSMAC and a pSMAC is a consequence of, rather than a requirement for, signaling through the TCR.

TCR signaling precedes the formation of a mature immunological synapse

Soon after the discovery of SMACs at the T cell-APC interface, Krummel et al. observed that calcium flux was coincident with the formation of small, unstable clusters of the CD3 ζ-chain and CD4. CD3, but not CD4, eventually coalesced in a cSMAC, but not until 5-10 minutes after the onset of calcium signaling (126). Much like CD4, Lck is excluded from the TCR-enriched cSMAC in mature IS (127). These data suggested that signaling through the TCR (as measured by an increase in intracellular calcium) precedes the formation of a cSMAC, and this hypothesis was further supported by data published by Andrey Shaw and colleagues (128). Using naïve T cells and a B cell lymphoma line as APCs, Lee et al. showed that a mature IS containing a cSMAC and pSMAC did not form until approximately thirty minutes after conjugation, while phospho-Lck and phospho-Zap-70 were predominantly found at the periphery of the T cell-APC interface at much earlier time points (128). It is important to note that although TCR-signaling clearly precedes the formation of a 'mature' IS, the two processes are not independent of one another. Signaling through the TCR is necessary for stable conjugate formation and, therefore, IS formation, and is also required for the maintenance of the synapse. Experiments where T cell-B cell conjugates were treated with blocking antibodies to MHC class II showed that within minutes of the abrogation of signaling through the TCR, the large scale molecular segregation of LFA-1-ICAM-1 interactions to the pSMAC is no longer seen, resulting in a diffuse, random distribution throughout the interface (129).

Antigen recognition occurs in TCR microclusters

Imaging experiments examining Jurkat cells interacting with anti-TCR coated coverslips revealed small, dynamic TCR clusters distributed throughout the interface (130). The appearance of theses clusters coincided with calcium flux and the clusters colocalized with ZAP-70, pTyr and the adaptor proteins LAT, Grb2, Gads and SLP-76, indicating that they represented small but functional platforms for TCR proximal signaling. Although these studies clearly showed that TCR signaling can occur in small microdomains, the use of plate-bound, and presumably immobile, antibodies against the TCR complex to induce T cell signaling and activation made interpretation difficult. This obstacle was overcome in total internal reflection fluorescence microscopy (TIRFM) experiments, performed by Saito and colleagues, with supported planar bilayers containing freely mobile pMHC and ICAM-1 (131). When CD4 T cells transduced with green fluorescent protein (GFP)-tagged CD3 ζ-chain were placed on bilayers, small microclusters containing 40-150 TCR molecules each were formed throughout the T cell-bilayer contact region. These microclusters began appearing 5 sec after the initial contact and simultaneous to calcium flux. After the T cell was completely flattened against the lipid bilayer, the TCR microclusters began moving centripetally, eventually coalescing and forming a cSMAC after approximately 5 min. These microclusters were associated with pTyr, phospho-ZAP-70 and SLP-76 and excluded the large phosphatase CD45 (131, 132). However, experiments with T cells transduced with GFP-tagged ZAP-70 or SLP-76 showed that although these molecules did move centripetally in TCR microclusters, they disassociated from the microclusters prior to entry into

the cSMAC. TCR signaling did not cease after the formation of a cSMAC as newly formed TCR microclusters associated with signaling molecules continued to arise in the periphery and move centripetally towards the cSMAC after the appearance of a mature IS (131).

The results described above expanded on the extant hypothesis that the majority of TCR signaling happens in the periphery of the T cell-APC contact, by showing that TCR signaling occurs in continuously formed, peripherally located TCR microclusters (133, 134). Indeed, after the formation of a cSMAC, 95% of the pLck and pZAP-70 in the IS is located in the periphery, associated with new TCR microclusters (135). Experiments with supported planar bilayers layered on coverslips fabricated with a 1 μm square grid of 5 nm high, 100 nm wide chromium lines further demonstrated the importance of spatial positioning in TCR signaling (136). Due to the barriers, these IS did not have cSMACs, instead TCR clusters accumulated in each square of the grid. TCR clusters that were trapped at the periphery contained abundant pTyr, while those in the center of the cell did not. Furthermore, the peripherally trapped TCR clusters had more pTyr than peripheral clusters in IS formed on substrates lacking grids, and this increase in pTyr was associated with an enhanced calcium flux. TCR microclusters have subsequently been shown to be high avidity structures, given that treatment with blocking antibodies to MHC class II prevents the formation of new microclusters but does not disrupt existing ones (132). The formation of TCR microclusters does not depend on signal transduction through the TCR, as treatment with the Src-kinase inhibitor PP2 does not prevent the formation of

microclusters (although microclusters formed under these conditions are necessarily devoid of active TCR proximal signaling molecules) (135).

TCR and LFA-1 microclusters are transiently associated with the actin cytoskeleton

Treatment with latrunculin A showed that the formation and centripetal movement of TCR microclusters is dependent on actin polymerization (132, 135). Interestingly, latrunculin A treatment does not destabilize preexisting TCR microclusters or the cSMAC, but does destabilize the pSMAC (132, 135). This observation was expanded upon by Vale and colleagues in imaging experiments of Jurkat cells interacting with supported planar bilayers (45). These experiments demonstrated that LFA-1 microdomains segregate from TCR domains prior to IS formation and move centripetally, but unlike TCR microclusters, fail to enter the cSMAC. This result was explained with the observation that LFA-1 microdomains are destabilized by latrunculin B treatment and thus cannot enter the cSMAC due to the relative lack of actin polymerization at this site. Although the centripetal movement of TCR and LFA-1 clusters is dependent on retrograde flow, fluorescent speckle microscopy of cells transfected with actin-GFP showed that actin-GFP speckles move approximately 2-fold faster than TCR or LFA-1 clusters, suggesting that the coupling of TCR and LFA-1 microclusters to actin is transient (45). This hypothesis was tested by imaging T cells interacting with supported planar bilayers that were layered onto coverslips fabricated with chromium obstacles (137). TCR microclusters could not go through these barriers but were able to go around them, confirming that the linkage between microclusters and actin fibers is transient. The strong evidence

that microclusters are linked to the actin cytoskeleton provide an explanation for the earlier observation that the velocity of TCR clusters during IS formation is greater than the rate expected for unrestricted diffusion, and cast into serious doubt the theory that IS formation is a spontaneous process depending in part on the differing lengths of TCR-pMHC and LFA-1-ICAM-1 interactions (138, 139).

Myosin motors are required for some aspects of immunological synapse formation

Myosin motor proteins are known to be important for large-scale rearrangement of the cortical actin cytoskeleton during antigen recognition (140). The role of myosin motors in IS formation is less clear, with one paper showing that the reorientation of actin towards the T cell-APC interface and the formation of SMACs still occur when the ATPase activity of class II myosin motor proteins is blocked with the chemical inhibitor blebbistatin (52). T cell crawling is dependent on the activity of the class II heavy chain myosin MyH9, indicating that distinct modes of actin rearrangement occur during locomotion and IS assembly. However, a recent report showed that knocking down MyH9 with siRNA or inhibiting its activity with blebbistatin prevented the centripetal movement and fusion of TCR microclusters in the cSMAC (141). The accumulation of pZAP-70 and pLAT, calcium flux, cSMAC formation and IS stability are defective when class II myosin activity is absent, but T cell-APC conjugates still show accumulation of TCR and F-actin at the interface. Thus, actomyosin-dependent centripetal movement of TCR microclusters is critical for complete T cell activation even though large accumulations of receptor-ligand pairs appear at the T cell-APC interface in the absence of class II myosin activity.

B-5. Costimulation at the immunological synapse

Immunological synapse formation does not require costimulation

Although costimulatory signals are critical for complete T cell activation and differentiation, their role in the formation of the IS is controversial. A careful examination of the IS formed by CD4 T cells introduced to supported planar bilayers containing pMHC and ICAM-1 with or without GPI-linked CD80 revealed no significant differences in gross morphology (142). CD28-CD80 interactions colocalized to the cSMAC with pMHC-TCR, but the structure of the IS and the number of pMHC and ICAM-1 molecules accumulated in the cSMAC and pSMAC were unchanged relative to IS formed with bilayers lacking CD80. Although IS morphology was unchanged, the authors did note that inclusion of CD80 in the bilayers increased T cell proliferation and IL-2 secretion, as expected (142). The efficient recruitment of CD28 to the cSMAC of the IS requires the phosphorylation of Tyr 188 in its cytoplasmic tail, most likely by Src family kinases proximal to the TCR (143). Interestingly, the phosphorylation of Tyr 188 is also crucial for the costimulatory function of CD28, as measured by IL-2 production, suggesting that CD28 might need to localize to the IS to function (144). Time-lapse data of T cells co-transduced with fluorescently tagged CD3 and CD28 interacting with B cells showed that accumulation of CD3 and CD28 at the T cell-APC interface coincides both spatially and temporally. The absence of CD28 renders T cells incapable of maintaining increased calcium levels only minutes after antigen recognition, suggesting that CD28 signaling, like signaling through the TCR, begins prior to the formation of a mature IS (145).

Costimulation may alter the fine structure of immunological synapses

The idea that costimulation via CD28 signaling has no effect on IS morphology is controversial because experiments examining the IS formed between CD4 T cell blasts and $CD80^+$ fibroblasts transfected with pMHC and ICAM-1 showed that costimulation blockade with CTLA-4-Ig results in smaller and more diffuse accumulations of TCR-pMHC interactions (146). These data agreed with the observation that treatment with blocking antibodies to CD80 and CD86 results in a decrease in the number of IS displaying a compact accumulation of pMHC in the cSMAC (123). Also, the sustained accumulation of actin at the T cell-APC interface is inhibited by a blockade of costimulation (147). Upon TCR activation, the TCR and associated signaling complexes are found in detergent-insoluble membrane fractions, termed lipid rafts (148). Several groups have published data showing that the recruitment of lipid rafts enriched with Lck and other signaling molecules to the IS depends on signaling through CD28 (149-153). However, other experiments have convincingly shown that CD28 is not required for the recruitment of lipid rafts (154). Furthermore, the importance of lipid rafts in T cell signaling is highly controversial (155). Together, these data indicate that, while IS formation certainly occurs in the absence of costimulation, signaling through CD28 most likely results in differences in the fine structure of the mature IS.

PKC–θ focusing requires costimulation

While the function of costimulation in IS formation is somewhat unclear, the presence of CD28 in the IS clearly has an effect on the activation and localization of signaling molecules downstream of the TCR. Experiments with an

antibody specific for an auto-phosphorylation site on Lck showed that while CD4 is critical for the initial recruitment and phosphorylation of Lck, the presence of CD28 potentiates the auto-phosphorylation of Lck at later time points, most likely via an interaction between the SH3 domain of Lck and proline residues in the cytoplasmic tail of CD28 (156). In addition to modulating signaling molecules directly proximal to the TCR, CD28 signaling also effects the localization of PKC–θ at the IS. Specifically, T cells lacking CD28 fail to focus PKC–θ at the cSMAC (157). TIRFM studies with supported planar bilayers showed that CD28 microclusters initially colocalize with the TCR in the periphery, but form a dynamic annular ring surrounding TCR-pMHC interactions in the cSMAC (158). CD28 colocalizes with PKC–θ both in microclusters and in the annular ring in mature IS. Mutating a residue in a YMNM domain in the cytoplasmic tail of CD28 known to be critical for the recruitment of PI3K results in a loss of PKC–θ focusing at the cSMAC of the IS and deficient NFκB activity downstream of PKC–θ (159). Also, treatment with chemical inhibitors of PI3K prevents localization of PKC–θ to the cSMAC (160). These results suggest that a critical function of CD28 in the cSMAC is the recruitment of PI3K, resulting in the localized production of PIP_3 and the subsequent accumulation of proteins containing PH domains (161). PI3K and its product PIP_3 accumulate at the T cell-APC interface immediately after antigen recognition (before a detectable increase in intracellular calcium) and persist at the IS for hours (162-164). The PH-domain containing protein PDK1 is recruited to the T cell-APC interface soon after antigen recognition and has recently been shown to interact with, and activate, PKC–θ (165). PKC–θ itself is recruited to the membrane via an

interaction with DAG produced by PLC–γ, and the activation of PLC–γ depends on a signal through the TCR (18). A DAG gradient is set up soon after conjugation, with the highest concentration at the center of the T cell-APC interface (166). Thus, the efficient activation of PKC–θ requires the integration of signals through the TCR and CD28. The hypothesis that the IS functions as a platform for this signal integration was elegantly tested by stimulating CD4 T cells with CD80/86-deficient APCs and then providing the costimulatory signal in *trans* (i.e. with anti-CD28 coated beads or class II$^-$, CD80$^+$ cells) (167). These experiments showed that when costimulation is provided in *trans*, IL-2 production is greater than the no-costimulation controls, but the increase in IL-2 secretion occurs in a PKC-θ-independent manner. Consistent with these results, when costimulation is provided in *trans*, PKC–θ accumulates at the T cell-APC interface but fails to focus to the cSMAC, localization of NFκB to the nucleus is defective and IL-2 transcription is not upregulated (167).

CTLA-4 is rapidly recruited to the immunological synapse

Given the importance of CD28 at the IS, the role that its homolog CTLA-4 plays at the IS is of interest. Although the bulk of CTLA-4 is located in an intracellular compartment within the uropod of migrating T cells, it is recruited to the plasma membrane at the T cell-APC interface within three minutes of antigen recognition, only slightly slower than CD28 (168). Interestingly, the accumulation of CTLA-4 (in contrast to CD28) at the IS is highly dependent on the strength of signal through the TCR, with very little accumulation seen when T cells are stimulated with weak agonist peptides. These results indicate that the recruitment of CTLA-4 to the IS in response to strong signals through the TCR

might act as a negative feedback mechanism (168). It is known that CD28 and CTLA-4 bind CD80 and CD86 with different affinities. CTLA-4 has a higher affinity for both CD80 and CD86 than CD28, but the preferred binding partner for CTLA-4 is CD80, while the preferred binding partner for CD28 is CD86 (169). Using CD80 and CD86 knockouts, Allison and colleagues showed that CD80 and CD86 preferentially recruit CTLA-4 and CD28 to the IS respectively (170). Thus, the strength of the TCR signal and the ratio of CD80/CD86 on the APC both affect the recruitment of CD28 and CTLA-4 to the IS, and presumably the degree of T cell activation.

B-6. Immunological synapse function

The cSMAC is the site of TCR downmodulation

T cell signaling is critical for IS formation, and signaling continues in the periphery of mature IS, with newly formed TCR microclusters continuously migrating to the cSMAC. Given that IS are formed in minutes, while full activation of T cells takes considerably longer (129), the IS clearly plays a role in T cell signaling and activation. Consistent with this idea, it has been shown that the cSMAC is critical for TCR downmodulation and degradation (171). Using CD4 T cells from CD2AP-deficient mice that are defective in cSMAC formation (77) and TCR downmodulation, Shaw and colleagues used in vitro and in silico methods to demonstrate that TCR downmodulation at the cSMAC is critical for the attenuation of strong signals (171). In agreement with these results, lipobisphosphatidic acid (LBPA), a lipid present at sites where membrane proteins are degraded, is found at the cSMAC (132). Also, recycling endosomes, known to be crucial for TCR internalization, can be found at the center of the T

cell-APC interface (172, 173). The idea that the cSMAC is a site for TCR downmodulation fits nicely with the observation that weak agonist and antagonist APLs fail to induce the formation of a mature IS (78), leading to the hypothesis that the cSMAC might act as a negative feedback mechanism to control the signaling induced by strong agonists. This hypothesis was tested in experiments with AND TCR transgenic T cells that paradoxically proliferate to a greater extent in response to the APL K99A than the agonist MCC peptide. When cSMAC formation was coerced by incubating T cells transduced with NKG2D and DAP10 with APCs transduced with Rae-1ε, the enhanced proliferative response to K99A was ablated (174). Recent experiments have shown that the cSMAC can also function to enhance signaling in response to weak stimuli. When IS are formed in response to weak agonist peptides or low doses of agonist peptides, the signaling molecules phospho-ZAP-70 and PI3K can be found in the cSMAC at late (1 hr), but not early (0-20 min), time points (175).

The immunological synapse plays a role in Th cell differentiation

A study examining the IS formed by naïve CD4 T cells showed that the IFN–γ receptor (IFNGR) colocalizes with the TCR, and this colocalization is inhibited when IS are formed in the presence of IL-4 (176). There is evidence that Th1 differentiation is the default pathway during activation of naïve T cells in the absence of polarizing cytokines, and recruitment of the IFNGR and the downstream transcription factor STAT1 to the IS allows for differentiation to a Th1 phenotype in the absence of IFN–γ (177). Signaling through the IL4R prevents polarization of the IFNGR to the IS by an unknown mechanism, preventing Th1 polarization in the presence of IL-4. The IL4R is only recruited to

the IS in T cells that are defective in signaling through the IFNGR (e.g. *stat1*$^{-/-}$ or *ifngr1*$^{-/-}$), indicating that Th2 differentiation does not require colocalization of the TCR and IL4R under normal circumstances (177).

The pSMAC is important for CTL-mediated killing

Early imaging studies conducted by Kupfer and others noted that in T cell-APC conjugates, the MTOC and associated Golgi apparatus in the T cell were polarized towards the APC or other source of stimulus (60-63, 178). The obvious implication of these observations is that T cells reorient themselves so as to secrete cytokines or cytotoxic molecules towards the APC they are interacting with. While the data support a role for the cSMAC in TCR downmodulation, there is compelling evidence that the ring of adhesion molecules that make up the pSMAC functions as a gasket (179-182).

In a seminal paper, Gillian Griffiths's group showed that CTL interacting with target cells polarize lytic granules towards the APC where they accumulate in the cSMAC (183). The lytic granules congregate in a sub-domain of the cSMAC distinct from accumulations of Lck. Electron micrographs showed that the lytic granules are released into a cleft formed by an indentation in the target cell membrane (183). Interestingly, portions of the target cell membrane appeared to be transferred to the CTL after the cells detached, a phenomenon that has also been noted in CD4 T cells interacting with APC (184, 185). Further experiments demonstrated that lytic granules move along microtubules in a minus-end direction towards the centrosome, which is in contact with the plasma membrane during killing (186). Loops of microtubules originating from the centrosome and ending at the plasma membrane have been observed passing close to the CTL-

target cell interface (187). The scaffold protein ADAP is associated with the actin cytoskeleton (188), and is thought to link microtubules with cortical actin via an association with the minus-end motor protein dynein (189). Given that polarization of the MTOC towards the target cell fails to occur in the absence of ADAP, it is possible that association between dynein and the actin cytoskeleton functions to pull the MTOC to the T cell-APC interface (189), explaining why cytochalasin D inhibits CTL degranulation even though lytic granules travel along microtubules (190). Consistent with this hypothesis, it has recently been shown that localization of dynein to the site of antigen recognition precedes recruitment of the MTOC (191).

The importance of the pSMAC was highlighted in experiments designed to examine the role of LFA-1 in CTL-mediated killing (192). Disruption of the pSMAC by treatment with anti-LFA-1 resulted in a reduction in specific lysis but not granule release. $CD8^+$ CTL periodically form LFA-1-ICAM-1 rings, or presynapses, on lipid bilayers lacking pMHC (193). These presynapses might enhance the efficiency of CTL by bypassing the time consuming steps involved in mature IS formation, allowing the delivery of cytolytic granules as soon as antigen recognition occurs. The pSMAC seems to be generally important in killing, as NK cells form 'lytic' IS with a similar structure to those formed by CTL, albeit with activating receptors and their ligands concentrated in the cSMAC instead of TCR-pMHC clusters (194).

Although lytic granules are released within the ring of adhesion molecules when a pSMAC is present, this observation does not prove that IS formation is required for CTL killing. To the contrary, several published reports bring into

question the necessity of a mature IS for killing. As discussed above, signaling through CD28 promotes the focusing of PKC–θ to the cSMAC (157), a phenotype that is often considered synonymous with mature IS formation. However, PKC–θ focusing is not a requirement for polarization of lytic granules to the CTL-target cell interface, and the presence of CD80 on the target cell increases PKC–θ focusing but not specific killing (195). Also, killing still occurs at low antigen concentrations when CD2 and pTyr accumulation aren't detectable at the interface (196). Live cell imaging experiments have shown that single CTL can simultaneously interact with two targets pulsed with 10,000-fold different concentrations of peptide. In these three-cell conjugates, the TCR-CD3 complex is polarized towards the cell with the higher antigen concentration, but lytic granules are polarized towards both targets, which are both subsequently killed (197). Furthermore, experiments with fluorescently labeled peptides demonstrated that only 3 pMHC complexes are needed at the CTL-target cell interface to induce killing, but 10 pMHC complexes are required for pSMAC formation (198).

The nature of the target cells used in the studies described above could account for the disparity in results regarding the need for a pSMAC during killing by CTL. Immortalized cell lines (e.g. P815 and JY cells) were used as targets, and the fact that these cells are killed only minutes after contact with a CTL demonstrates their sensitivity to the contents of lytic granules (197). The formation of a pSMAC, while certainly occurring in some conjugates, might be dispensable for the killing of these sensitive targets. Physiologically relevant targets, such as virus-infected fibroblasts, are far less sensitive to CTL-mediated

lysis, and the formation of a pSMAC might be crucial for the killing of such targets (Ann Hill, personal communication).

The immunological synapse and the delivery of cytokines

Given the importance of the IS in the delivery of cytotoxic molecules to target cells, the hypothesis that the IS also functions to direct effector molecules such as cytokines towards the APC is appealing. Janeway first tested this hypothesis in experiments where CD4 T cells were spun down on a filter and stimulated on either their top or bottom sides with antibodies against the TCR (178). IL-4 tended to be preferentially secreted towards the side of stimulation, although cytokine could be found in both the top and bottom halves of the chamber regardless of the site of stimulation. Further experiments by the Kupfer group showed that the cytokines IL-2, IL-4 and IFN–γ were polarized towards the T cell-APC interface in 1-2 day old conjugates (63, 199). Although these experiments only measured the location of cytokines within the cell (presumably in the Golgi apparatus associated with the MTOC), the continued polarization within long-term conjugates suggested that cytokines might generally be secreted in the direction of the APC. A recent paper showed that IL-2 and IFN–γ, but not TNF and IL-4, were predominantly located at the T cell-APC interface two hours after conjugation (200). Importantly, experiments where CD4 T cells were stimulated with glass coverslips coated with pMHC, CD80 and capture antibodies to cytokines showed that IFN–γ and IL-10, but not TNF and IL-4 were secreted in a focused spot smaller than the average contact area between the cell and coverslip (200). This focused secretion was dependent on microtubules, as treatment with nocodazole resulted in a diffuse secretion of IFN–γ. These

experiments demonstrated that both Th1 and Th2 cytokines can be secreted synaptically or diffusely and presumably are located in different intracellular compartments. The conflicting results regarding directed IL-4 secretion between early experiments using the D10 cell line and later experiments using Th2-polarized blasts indicate that differences in polarization conditions or activation state could affect whether or not a particular cytokine is secreted synaptically (178, 200).

In addition to secreted cytokines, two members of the TNF superfamily, CD40L and Fas ligand (FasL), are recruited to the IS. FasL is located within secretory lysosomes in both $CD4^+$ cells and $CD8^+$ cells, and is polarized to the center of the CTL-target cell interface (201). CD40L is also found in secretory lysosomes in CD4 T cells and is rapidly mobilized to the cell surface after activation, suggesting that CD40L, like FasL, is delivered synaptically (202). Indeed, CD40 is centrally localized at the IS within one minute of antigen recognition, and recruitment of CD40 requires both CD40L and LFA-1-ICAM-1 interactions (203). Directly staining T cell-B cell conjugates for CD40L confirmed that it is centrally located at the interface (204).

B-7. Immunological synapse diversity

Most of the experiments examining IS formation described above used either transformed B cells, transfected fibroblasts or supported planar bilayers as APCs. Given the central role played by DCs in T cell activation, studies examining the IS formed by T cells and DCs are of crucial importance. One of the first reports describing the T cell-DC IS showed that CD3, talin, CD4 or CD8, PKC–θ, pTyr and the MTOC are all polarized towards the cell-cell interface in the

absence of cognate antigen (205). This large-scale rearrangement of cell surface proteins was associated with an increase in intracellular calcium. Surprisingly, IS formation was seen at the same frequency when MHC class II deficient DCs were used as APC (205). This result indicates that DCs are an active partner in receptor clustering under these conditions, as IS formation in the supported planar bilayer system is strictly antigen dependent (78). Consistent with this hypothesis, the DC cytoskeleton is polarized towards the IS, and treatment of DCs with cytochalasin D results in decreased T cell-DC conjugate formation and T cell proliferation (206). The crosslinking of MHC class II is required for polarization of the DC cytoskeleton towards the IS, suggesting that IS formed in the absence of antigen do not depend on the DC cytoskeleton (207).

In a careful study of T cell-DC IS, Trautmann and colleagues reported that these IS lack clearly defined SMACs (208). Instead, T cell-DC IS are multifocal, with large dynamic TCR clusters spread throughout the interface. LFA-1, talin and PKC–θ also show a multifocal distribution. These immunofluorescence experiments were confirmed by electron microscopy, which revealed multiple regions of close apposition between T cell and APC membranes separated by clefts (208). Microvilli on the DC surface may be responsible for these multiple contact regions (209). Further experiments by another group showed that CD28-CD80 interactions occur in discrete clusters that are separate from pMHC-TCR accumulations. These CD28-CD80 clusters colocalized with PKC–θ, confirming the importance of CD28 ligation in the recruitment of PKC–θ to the IS (210). Although T cell-DC IS are predominantly multifocal in nature, the activation state of DCs plays a role in IS formation, as DCs matured overnight with LPS form IS

with clearly defined SMACs at a higher frequency than immature DCs (211). Together, these experiments show that the DC is an active participant in IS formation, and this participation causes T cell-DC IS to have a multifocal phenotype.

The T cell differentiation state has also been shown to play a role in IS formation and structure. Experiments examining double positive (DP) thymocytes interacting with thymic stromal cells in vitro showed that IS formed by these thymocytes failed to cluster CD3 centrally under conditions that encourage negative selection (212). These results were confirmed with experiments using supported planar bilayers that showed double negative (DN), DP and single positive (SP) thymocytes all form multifocal IS with LFA-1-ICAM-1 interactions interspersed between clusters of TCR-pMHC (213). The majority of IS formed by naïve CD4 T cells are also multifocal, suggesting that the ability to form a mature IS with a cSMAC and pSMAC might be unique to activated T cells (214). Anergic T cells also form non-canonical IS, with decreased levels of Lck and CD45 recruited to the T cell-APC interface (215). Additionally, Th2 cells have been reported to have a defect in the accumulation of the TCR and CD4 at the IS when compared to Th1 cells (216). Recently, it has been shown that this defect in TCR accumulation at the Th2 IS only occurs when naïve B cells are used as APC and is due to the high levels of CTLA-4 on the surface of Th2 cells (217).

Given the range of IS structures described above, and working under the hypothesis that IS structure is related to effector T cell function, I set out to examine the IS formed by in vitro polarized CD4 T cell subsets.

Chapter 2

Th1 and Th2 cells form morphologically distinct immunological synapses

This work was published in the Journal of Immunology, and is presented here as published, with only minor stylistic alterations.

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Summary

The arrangement of molecules at the interface between T cells and APC is known as the IS. I conducted experiments with supported planar bilayers and transfected fibroblast APC to examine the IS formed by polarized Th1 and Th2 cells. Th1 cells formed classic 'bullseye' IS with a ring of adhesion molecules surrounding TCR-pMHC interactions at all antigen concentrations tested, while Th2 cells formed multifocal IS at high concentrations of antigen. At low antigen concentrations, the majority of Th2 cells formed IS with a compact, central accumulation of TCR-pMHC, but ICAM-1 was not excluded from the center of the IS. Additionally, CD45 was excluded from the center of the interface between Th1 cells and APC, while CD45 was found throughout the Th2-APC interface. Finally, phosphorylated signaling molecules colocalized with TCR-pMHC to a greater extent in Th2 IS. Together, our results indicate that the IS formed by Th1 and Th2 cells are distinct in structure, with Th2 cells failing to form bullseye IS. I hypothesize that the differences in IS structure observed for Th1 and Th2 cells have important implications in the delivery of effector molecules by these cells.

Introduction

Immunological synapses are stable cell-cell junctions formed between APCs and T cells during antigen recognition. Mature IS are characterized by a ring of LFA-1-ICAM-1 interactions, known as the pSMAC, surrounding a cSMAC composed of TCR-pMHC clusters (77-79, 218). Maintenance of radial symmetry is important for IS stability (102). A number of other molecules, including CD28 and PKC-θ are known to concentrate within the cSMAC (79, 142), while CD45 (110, 112) and some phosphorylated signaling proteins, including Lck and Zap-70 (110, 128, 171), reside mainly outside of the cSMAC of the mature IS.

The IS has been implicated in a number of functions, yet these have been difficult to prove due to the complexity of manipulating this phenomenon specifically. Although IS formation correlates with activation, the cSMAC is not required for the initiation of TCR signaling, since signaling is initiated before the cSMAC is formed (128). Experiments with T cells from $CD2AP^{-/-}$ mice, which are incapable of forming mature IS, indicated that the function of the cSMAC may be to enhance signaling while at the same time promoting TCR downmodulation (171). Additionally, it has been shown that the lipid LBPA, which is known to exist at sites where membrane proteins are being sorted for degradation, accumulates in the cSMAC (132). The ability to signal without forming a cSMAC is consistent with recent data showing that TCR signaling is initiated in small TCR microclusters that form rapidly after contact and sustain signaling through continual formation in the periphery of the IS (131, 132, 135).

Another possible function of the IS is the delivery of effector function. IS formed by $CD8^+$ T cells have separate domains within the cSMAC containing

signaling molecules and lytic granules (183). These data suggested that the peripheral ring of adhesion molecules functions to prevent lytic granules from harming bystander cells (192, 219). However, it has been shown that $CD8^+$ cells can kill sensitive targets without forming mature IS (195, 196, 198). Recently, it has been determined that the cytokines IL-2, IFN-γ and IL-10 are directly secreted into the IS, whereas chemokines, TNF and IL-4 are directed away from the IS (200). Thus, both Th1 and Th2 cytokines can be released into the IS or away from the IS.

Upon antigen recognition, $CD4^+$ T cells proliferate and differentiate into effector cells. Depending upon the strength of stimulation and cytokines present at the time of stimulation, $CD4^+$ cells can differentiate into either Th1 or Th2 cells (30, 220, 221). Th1 cells secrete IFN-γ and lymphotoxin, which serve to activate macrophages and induce inflammation by recruiting other leukocytes. Th1 cells also express FasL and can kill target cells. Th2 cells secrete IL-4, IL-5 and IL-13 and promote allergic reactions in epithelial tissues. A recent study has shown that Th2 cells, but not Th1 cells, fail to cluster TCR at the cell-cell interface when forming conjugates with primary B cells due to increased expression of CTLA-4 (217). Although it has been shown that co-clustering of TCR with CD4 and lipid rafts is much more common in Th1 than Th2 cells (216), the organization of the IS formed by polarized Th1 and Th2 cells has not been thoroughly studied.

In this study, we used a supported planar bilayer system, and transfected fibroblast APC, to study IS formed by polarized Th1 and Th2 cells. We report that Th1 cells form synapses with a compact accumulation of TCR-pMHC surrounded by a ring of adhesion molecules. Unlike Th1 cells, Th2 cells predominantly form

multifocal synapses at high antigen concentrations with multiple small accumulations of TCR-pMHC and LFA-1-ICAM-1 interspersed throughout the interface. At low concentrations of antigen, the majority of Th2 cells form IS with a central, compact accumulation of TCR-pMHC, but LFA-1-ICAM-1 is not excluded to the pSMAC, as in Th1 cells. We propose that the morphological differences in IS between Th1 cells and Th2 cells, specifically the formation of a 'gasket' structure in Th1 cells, correlates with differences in the delivery of effector functions by these distinct types of cells.

Materials and Methods

Animals

Heterozygous AD10 TCR transgenic mice on a B10.BR background that are specific for pigeon cytochrome *c* 88-104 and reactive against moth cytochrome *c* 88-103 (MCC), were provided by S. Hedrick (University of California at San Diego, La Jolla, CA) by way of P. Marrack (National Jewish Medical Center, Denver, CO). Mice were housed in specific-pathogen free conditions at Oregon Health & Science University according to institutional standards.

Antibodies

The antibodies used for immunofluorescence were as follows: anti-LFA-1 (I21/7; Southern Biotech), anti-CD45 (30-F11; eBioscience), biotinylated anti-phosphotyrosine (4G10; Upstate), Texas Red-conjugated goat anti-rat IgG, Cy-5-conjugated goat anti-rat IgG and streptavidin-conjugated Cy5 (Jackson Immunoresearch).

Antigen Presenting Cells

MCC:GFP fibroblasts expressing CD80, ICAM-1, wild type I-E^k α-chain and β-chain along with GFP-labeled I-E^k β-chain covalently attached to MCC have been described previously (146).

In vitro T cell polarization

Th1 conditions: AD10 splenocytes were used after removal of red blood cells by hypotonic lysis. Splenocytes were cultured in RPMI 1640, supplemented as previously described (146), with 2.5 μM moth cytochrome *c* (MCC) 88-103

(KAERADLIAYLKQATK) in the presence of 5 ng/ml IL-12 (Cell Sciences) and 20 μg/ml anti-IL-4 (11B11).

Th2 conditions: After removal of red blood cells by hypotonic lysis, $CD3^+$ cells were purified by negative selection with Mouse T Cell Enrichment Columns (R&D Systems). $CD3^+$ cells were incubated at 10^6/ml with irradiated B10.BR splenocytes (5:1) in the presence of 2.5 μM MCC 88-103, 100 ng/ml IL-4 (as a transfected plasmacytoma culture supernatant) and 50 μg/ml anti-IFN-γ (XMG 1.2). 80 U/ml IL-2 was added on day 2 of culture. After 6-7 days in culture, Th2 cells were restimulated with irradiated B10.BR splenocytes and peptide. Th2 lines were maintained for up to five weeks. Intracellular cytokine staining of acutely activated Th1 and Th2 cells for IFN-γ and IL-4 confirmed polarization.

Bilayers

GPI-anchored forms of Oregon Green 488 labeled $I\text{-}E^k$ (200 molecules/μm^2) and Cy5-labeled ICAM-1 (300 molecules/μm^2) were incorporated into dioleoylphosphatidylcholine bilayers as described (78). These bilayers were supported on a coverslip in a Bioptechs flow cell, and were loaded with various concentrations of peptide in a PBS/citrate buffer for 24 hr at 37°C (78).

Live Cell Microscopy

10^7 Th1 or Th2 cells in 1 ml HBS buffer with 1% human serum albumin were injected onto bilayers at 37°C. Imaging was performed with a 40X or 60X objective using two Applied Precision DeltaVision systems . These systems included an Applied Precision chassis with a motorized XYZ stage, a Nikon TE200 or Olympus IX71 inverted fluorescent microscope, halogen illumination, a

CH350L or CoolSnap HQ^2 camera and the DeltaVision SoftWorx software package.

Fixed Cell Microscopy

2.5 x 10^4 MCC:GFP APC were seeded onto a LabTek II eight-chambered #1.5 (0.17 mm) coverglass in complete DMEM and incubated overnight at 37°C. After removal of DMEM, 5 x 10^5 Th1 or Th2 cells in RPMI 1640 were added per chamber. After a brief centrifugation, the chambers were incubated at 37°C for 10 or 30 min. The cells were fixed and stained as previously described (146). Conjugates to be imaged were chosen based on the presence of a GFP signal at the T cell-APC interface. Stacks of fluorescent images spaced 0.2 μm apart were obtained with a 1.4 NA 60x oil immersion lens on the DeltaVision system and deconvolved with an iterative, constrained algorithm. Deconvolution and 3D reconstructions were performed with the Applied Precision SoftWorx software. Colocalization studies were conducted with Imaris software from Bitplane.

Results

TCR-pMHC clusters do not coalesce to form a cSMAC in Th2 IS

Previous studies using the supported planar bilayer system demonstrated the early stages of IS formation (78). Upon antigen recognition, TCR-pMHC interactions are initiated in the periphery and immediately start moving towards the center of the T-cell-APC interface (78, 131, 132, 135). After 4-5 minutes, most of the TCR-pMHC interactions are centrally located, and this structure with a centrally located cluster of TCR-pMHC surrounded by a ring of adhesion molecules is stable for at least an hour (78).

I conducted experiments with supported planar bilayers to observe IS formation by Th1 and Th2 cells. Initially upon antigen recognition, both Th1 and Th2 cells spread out and showed broad accumulation of ICAM-1 across the T cell-bilayer interface (Fig. 2-1). Small accumulations of TCR-pMHC were randomly distributed across the interface very rapidly after the initiation of synapse formation. After 1 minute, both Th1 and Th2 cells showed clusters of TCR-pMHC across the interface. Between 2 and 3 minutes, TCR-pMHC clusters moved to the center of synapses formed by Th1 cells (Fig. 2-1 A). By 5 minutes, most Th1 synapses had a compact central accumulation of TCR-pMHC surrounded by a ring of ICAM-1, and this pattern was stable past 10 min. TCR-pMHC clusters in most Th2 IS did not coalesce into a central accumulation (Fig. 2-1 B). Unlike Th1 cells, the IS formed by Th2 cells had multiple distinct clusters of TCR-pMHC that were maintained for at least ten minutes. These data indicate that Th2 cells do not form mature IS, with a single, central accumulation of TCR-pMHC surrounded by a ring of adhesion molecules.

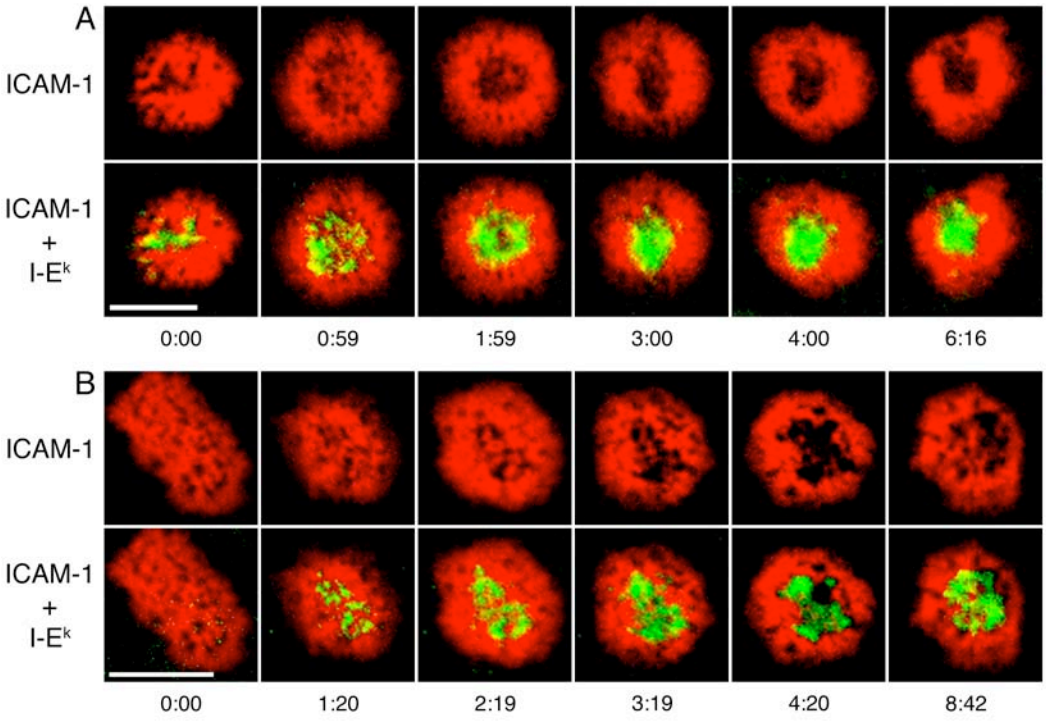

Figure 2-1: Dynamics of IS formation in Th1 and Th2 cells. Th1 (A) and Th2 (B) cells were injected onto supported planar bilayers containing ICAM-1-Cy5 (red) and peptide-loaded I-Ek-488 (green). Wide-field fluorescence microscopy was used to image T-cell-bilayer contacts. One set of ICAM-1-Cy5/I-E^k-488 images was obtained every minute. The time indicated is relative to the initial detection of T cell-bilayer contact. TCR-pMHC clusters did not coalesce into a cSMAC in most Th2 IS. Data are from one representative experiment of two with n=14 for Th1 cells and n=83 for Th2 cells. Scale bars represent 5 μm.

Th2 cells form multifocal IS

To confirm that Th2 cells do not form mature IS with a central accumulation of TCR-pMHC, I observed Th1 and Th2 synapses that were 20 to 35 minutes old. As in Figure 2-1, I allowed Th1 or Th2 cells to settle onto supported lipid bilayers loaded with 100 μM MCC, and let the cells interact with the bilayers for 20 minutes at 37°C before commencing imaging. Th1 cells consistently formed tight, compact IS with an ICAM-1 ring surrounding a central

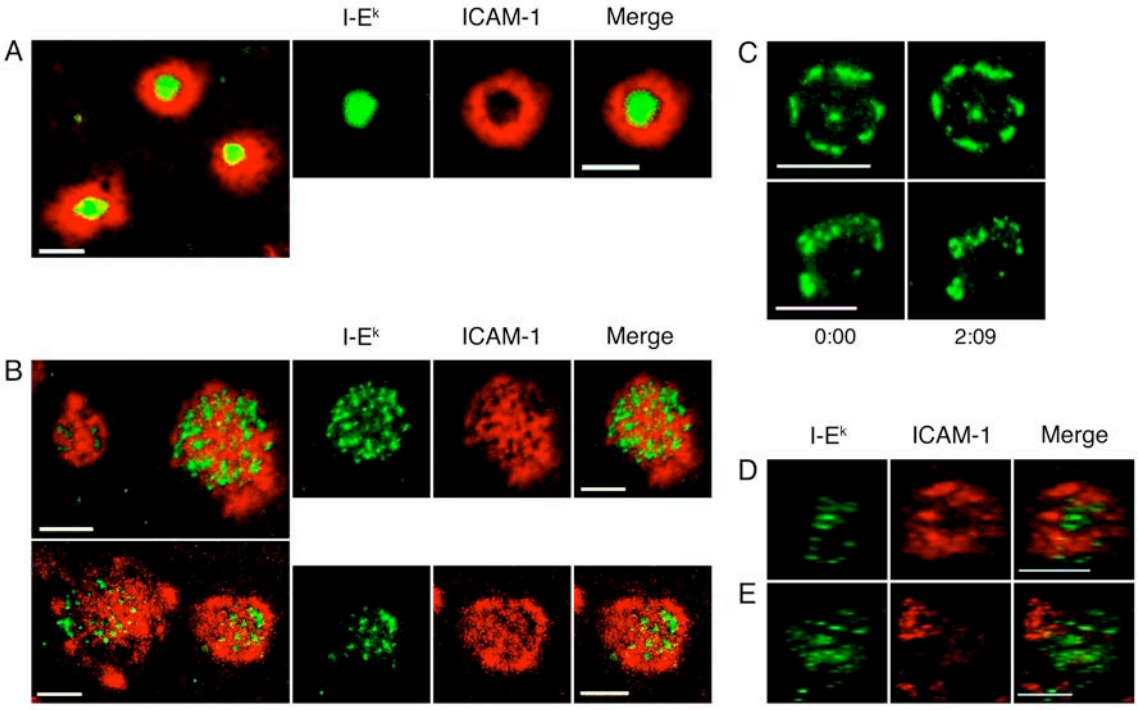

Figure 2-2: Th2 cells form multifocal IS.
Th1 and Th2 cells were incubated on supported planar bilayers containing ICAM-1-Cy5 (red) and peptide-loaded I-E^k-488 (green) for 20 minutes prior to imaging. Images were then captured between 20 and 35 minutes. (A) Th1 cells consistently formed IS with a compact, monofocal accumulation of TCR-pMHC. (B) The majority of Th2 cells formed multifocal IS. (C) Th2 cells were imaged over time, 20 min after IS formation. Th1 (D) and Th2 (E) cells were allowed to interact with MCC:GFP fibroblast APC for 30 minutes. Conjugates were fixed, permeabilized and stained with an antibody to LFA-1 followed by a Cy-5-conjugated secondary antibody. Following deconvolution, 3-D reconstructions of T-cell-APC interfaces were made. En face views from the 3D reconstructions of T cell-APC interfaces are shown. Data are representative of two experiments with n=20 for Th1 cells and n=19 for Th2 cells. Scale bars represent 5 μm.

accumulation of TCR-pMHC (Fig. 2-2 A). This structure agrees well with IS observed in a variety of systems using both CD4$^+$ T cell blasts and CD8$^+$ CTLs (78-80). Only cells with TCR-pMHC accumulations were scored. The pattern of TCR-pMHC was only scored as compact if there was a single cluster of TCR-pMHC. Multiple TCR-pMHC accumulations were scored as multifocal even if all of the clusters were centrally located. Although a compact accumulation of TCR-

pMHC was the dominant phenotype for Th1 IS, a multifocal structure was seen in 23% of the cells (Table 2-1).

Table 2-1: *Quantitation of Th1 and Th2 IS formed on supported planar bilayers*[a]

		Pattern of TCR-pMHC in IS		Pattern of ICAM-1 in IS	
Antigen Conc.	n	% Compact	% Multifocal	% Ring	% Diffuse
Th1 100 μM	407	77	23	65	35
10 μM	284	76	24	52	48
1 μM	518	75	25	56	44
Th2 100 μM	805	31	69	14	86
10 μM	483	55	45	21	79
1 μM	238	54	46	19	81

[a]Quantitation of data from Figs. 1 and 2 (100 μM) and Fig. 5 (10 and 1 μM).

Th2 cells formed synapses with the same frequency as Th1 cells, but with a markedly different structure. Instead of containing a single, compact accumulation of TCR-pMHC, Th2 IS were distinctly multifocal in nature (Fig. 2-2 B). The majority of IS formed by Th2 cells had multiple small accumulations of TCR-pMHC, with ICAM-1 interspersed throughout the T-cell-APC interface. A quantitative assessment showed that nearly 70% of the IS formed by Th2 cells had a diffuse multifocal distribution of TCR-pMHC (Table 2-1). The number of TCR-pMHC clusters varied from cell to cell with some having as few as 5 and others more than 20. I also examined the distribution of ICAM-1 in Th1 and Th2 IS. Two-thirds of the IS formed by Th1 cells have the classic 'bullseye' pattern with ICAM-1 excluded from the cSMAC, resulting in a ring-like structure. Th2 cells, on the other hand, only formed ring structures in 14% of the IS examined

(Table 2-1). The vast majority of Th2 IS had a diffuse distribution of ICAM-1 throughout the interface with the planar bilayer. I also imaged Th2 IS over time, 20 min after introduction to the bilayers, to determine if the location of TCR-pMHC clusters was dynamic after the initial steps of IS formation. As shown in Figure 2-2 C, the pattern of TCR-pMHC clusters in Th2 IS was stable.

To confirm that Th1 and Th2 cells formed distinctly different IS, I conducted experiments with viable APC instead of supported planar bilayers. For these experiments I used $CD80^+$ fibroblasts transfected with ICAM-1 and GFp-tagged pMHC. These fibroblasts have been shown to form IS with AD10 T cell blasts and to induce T cell proliferation, but it should be noted that IS formed with these fibroblasts have a patchier distribution of TCR-pMHC and LFA-1-ICAM-1 than IS formed on the highly simplified planar bilayers (146). T cell-APC conjugates were stained with an antibody to LFA-1. Some of the IS formed by Th1 cells and fibroblast APCs were characterized by a ring of LFA-1 surrounding the largest accumulations of TCR-pMHC, as shown in Figure 2-2 D. On the other hand, all of the Th2 cells examined formed multifocal IS with LFA-1 distributed homogenously throughout the T cell-APC interface (Fig 2-2 E).

CD45 is not excluded from the T-cell-APC interface in Th2 cells

The large tyrosine phosphatase CD45 is involved in both the positive and negative regulation of TCR signaling (116, 222). The role of CD45 in TCR signaling makes its spatial and temporal regulation extremely important. Initially upon antigen recognition and TCR clustering, CD45 is located centrally, along with TCR-pMHC (110), where it may positively regulate TCR signaling by

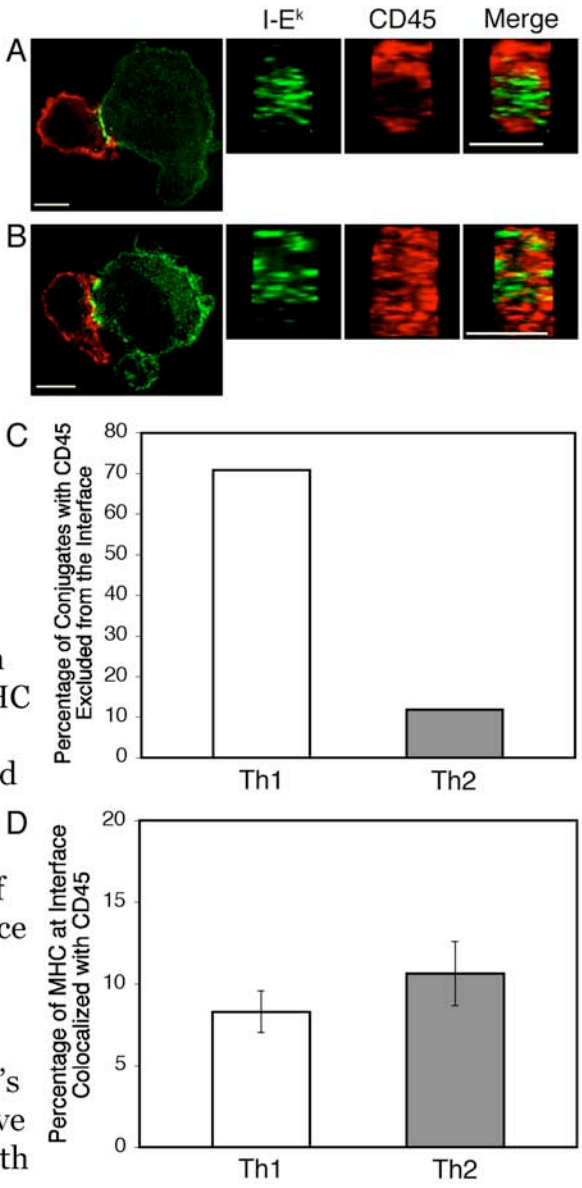

Figure 2-3: CD45 is not excluded from Th2 IS. Th1 (A) and Th2 (B) cells were incubated with MCC:GFP fibroblast APC for 10 minutes, fixed, permeabilized and stained with an antibody to CD45 followed by a Texas Red-conjugated secondary antibody (red). 3-D reconstructions of T-cell-APC interfaces were made after deconvolution. (C) Conjugates were scored for the exclusion of CD45 from the center of the T cell-APC interface. (D) Although CD45 is not excluded from the center of the T cell-APC interface in Th2 cells, there is not a significant difference in the colocalization between TCR-pMHC and CD45 in Th1 and Th2 cells. Colocalization analysis was conducted with Imaris software from Bitplane using the thresholding technique. The percentage of the total volume of TCR-pMHC at the T cell-APC interface that was colocalized with CD45 is shown. The difference between Th1 and Th2 cells was not statistically significant (P=0.3; unpaired Student's *t* Test). Data shown are representative of three independent experiments with n=32 for Th1 cells and n=33 for Th2 cells. Scale bars represent 5 μm.

dephosphorylating the negative regulatory tyrosine phosphorylation sites on the C-terminus of Lck (14, 223). However, by 5 to 7 min CD45 is completely excluded from the T-cell-APC interface (110, 111). Recent experiments with LFA-1 deficient T cells have shown that this adhesion molecule is critical for the exclusion of CD45 from the cSMAC (224). Importantly, it has been reported that after

stimulation through the TCR, CD45 is located in lipid rafts in Th1 but not Th2 cells (216). This led me to conduct experiments to examine the location of CD45 in IS formed by Th1 and Th2 cells.

By ten minutes after conjugation, CD45 was excluded from the central region of the interface in the majority of the Th1-APC conjugates examined (Fig. 2-3 A and C). With viable fibroblast APC, TCR-pMHC clusters at the interface did not completely coalesce into a cSMAC in many of the conjugates. Nevertheless, TCR-pMHC clusters rarely overlapped the ring of CD45 at the periphery of the Th1-APC interface. In Th2-APC conjugates, TCR-pMHC clusters were diffusely spread throughout the interface. Strikingly, CD45 was not excluded from the interface between Th2 cells and APC (Fig. 2-3 B and C). Instead, CD45 was interspersed among TCR-pMHC clusters in the majority of conjugates examined. Although CD45 was not excluded from the central region of the interface in Th2 cells, minimal colocalization with TCR-pMHC clusters was seen (Fig. 2-3 D). This is consistent with recent studies that have shown that CD45 is excluded from TCR microclusters (132).

Phosphotyrosine colocalizes with TCR-pMHC clusters to a greater extent in Th2 IS

Recent publications have shown that signaling continued after the formation of a mature IS, as measured by the presence of phosphorylated proteins, is largely excluded from the cSMAC (110, 128). Total internal reflection fluorescent microscopy of T cells interacting with a supported lipid bilayer demonstrated that TCR microclusters in the periphery, but not the center of an IS, colocalized with phospho-Lck, phospho-Zap-70 and phospho-Lat (131, 135).

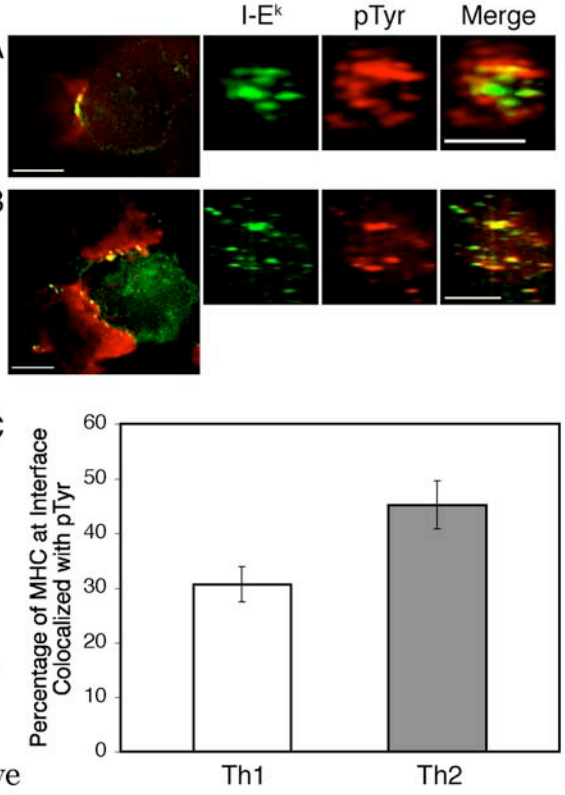

Figure 2-4: Phosphotyrosine localization is distinct in Th1 and Th2 IS.
Th1 (A) and Th2 (B) cells were incubated with MCC:GFP fibroblasts for 30 minutes. The conjugates were then fixed, permeabilized and stained with a biotinylated antibody to phosphotyrosine followed by Cy-5-conjugated streptavidin (red). Following deconvolution, 3-D reconstructions of the T cell:APC interfaces were made. (C) A greater percentage of the TCR-pMHC at the T cell-APC interface is colocalized with pTyr in Th2 cells. Colocalization analysis was performed as in Figure 3. The difference between Th1 and Th2 cells is significant (P = 0.01; unpaired Student's *t* test) Data are representative of three independent experiments with n=32 for Th1 cells and n=28 for Th2 cells. Scale bars represent 5 μm.

I conducted experiments with MCC:GFP fibroblasts to observe the pattern of pTyr staining in mature IS formed by Th1 and Th2 cells. I chose to look at 30 min conjugates to assure that the majority of IS I observed were mature. Phosphotyrosine and TCR-pMHC generally occupied distinct domains in the IS formed by Th1 cells (Fig. 2-4 A). However, there was significant colocalization at the boundary between the domains containing pTyr and TCR-pMHC. I performed a quantitative colocalization analysis to determine the degree of overlap between pTyr and TCR-pMHC at the T cell-APC interface. On average, approximately 30% of the TCR-pMHC at the interface was colocalized with TCR-pMHC in Th1 IS (Fig. 2-4 C). In Th2 IS, the TCR-pMHC and pTyr domains were not as distinct as for Th1 cells (Fig. 2-4 B). Colocalization analysis showed that

for Th2 cells, a significantly greater percentage of the TCR-pMHC at the interface was colocalized with pTyr (Fig. 2-4 C).

A greater percentage of Th2 cells form compact IS at low antigen concentrations, but ICAM-1 does not form a ring structure

T cell blasts from AND TCR-transgenic mice on a B10.BR background, which skew towards a Th1 phenotype (225), continue to form mature IS with a central accumulation of TCR-pMHC surrounded by a ring of ICAM-1 even when the number of available pMHC molecules is reduced several fold (78, 132). I conducted experiments with bilayers loaded with 10- and 100-fold less peptide to determine if the IS formed by Th1 and Th2 cells were still morphologically distinct when less antigen was available. These bilayers contained 200 I-E^k molecules/μm^2 as in the preceding experiments. Th1 cells continued to form compact IS at a similar frequency to experiments with planar bilayers loaded with 100 μM peptide. Interestingly, when the bilayers were loaded with 10- or 100-fold less antigen, a greater percentage of the IS formed by Th2 cells had a compact accumulation of TCR-pMHC, although still significantly less than for Th1 cells (Figure 2-5 and Table 2-1). However, unlike Th1 IS, Th2 IS formed on bilayers loaded with 10 or 1 μM MCC did not segregate ICAM-1 into a pSMAC. Instead, ICAM-1 was distributed diffusely across the IS in approximately 80% of the IS examined regardless of the antigen concentration (Figure 2-5 and Table 2-1).

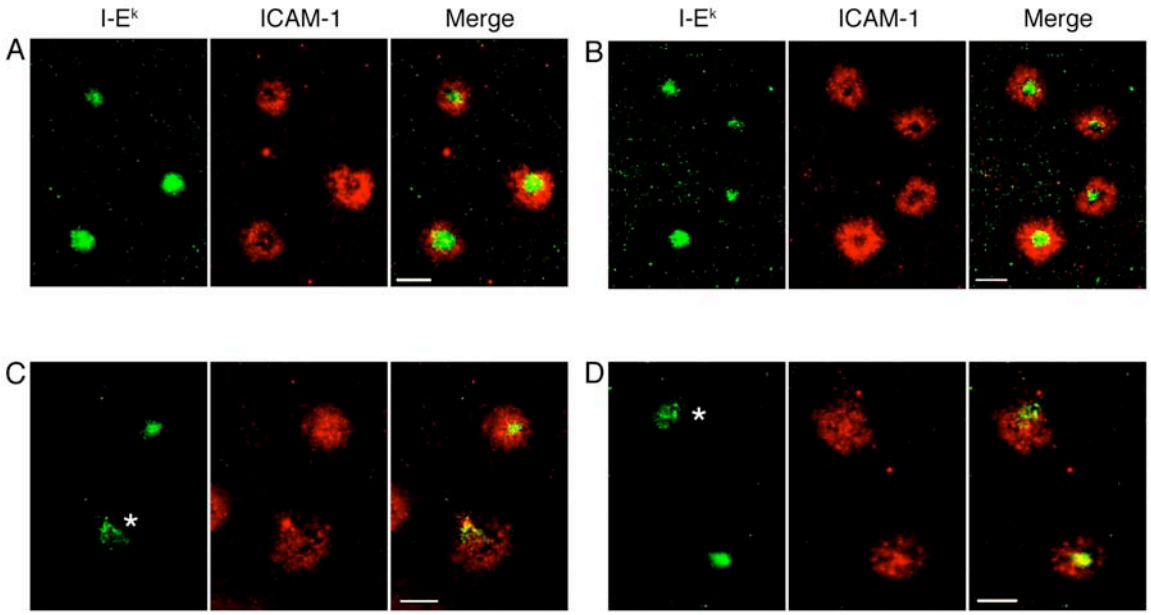

Figure 2-5: Th2 cells form compact IS at low antigen concentration, but ICAM-1 is not excluded from the cSMAC.
For these experiments, 10 or 1 μM MCC was used during the peptide-loading step instead of 100 μM MCC, as in the preceding figures. Th1 (A and B) or Th2 (C and D) cells were incubated on supported planar bilayers containing ICAM-1-Cy5 (red) and I-E^k-488 (green) loaded with 10 μM MCC (A and C) or 1 μM MCC (B and D) for 15 minutes prior to imaging. Images were then captured between 15 and 35 minutes. Cells marked with an asterisk were scored as multifocal for TCR-pMHC. Data are from one representative experiment of three. Scale bars represent 5 μm.

Discussion

In this report, I have shown that polarized Th1 and Th2 cells form morphologically distinct IS. Th1 cells form IS with a compact central cluster of TCR-pMHC surrounded by a ring of adhesion molecules, that closely resemble the IS described in the literature for T cell blasts. This resemblance is not surprising, considering that most of the published experiments designed to study IS formation used T cell blasts from B6 or B10 mice. Blasts from these mice tend to skew towards an IFN-γ secreting Th1 phenotype (225). Th1 IS structure was not markedly different, even when planar bilayers were loaded with 100-fold less peptide antigen. In contrast, I found that at high antigen concentrations, Th2 cells formed multifocal IS, characterized by multiple TCR-pMHC accumulations. When the amount of antigen used to load I-E^k in the bilayers was reduced by 10 to 100-fold, slightly more than half of Th2 cells formed compact IS. However, even when Th2 cells formed compact IS, ICAM-1 did not form a ring. This apparent co-localization of TCR-pMHC and LFA-1-ICAM-1 interactions in Th2 cells forming compact IS may reflect the close packing of many submicron TCR and LFA-1 microclusters in this region - a continuation of the multifocal paradigm to a shorter length scale. Further study will be needed to determine if this change in organization alters some of the proposed functions of the cSMAC, such as signal termination. Inactivation of signaling in the cSMAC may allow Th1 cells to maintain relatively constant signaling intensity over a wide range of antigen densities. Perhaps the biology of Th2 cells requires that signal strength is more proportional to antigen density and this might be achieved by delaying or eliminating functional cSMAC formation.

Multifocal IS have been described in several reports. Immunofluorescence and transmission electron microscopy were used to show that IS formed between naive T cells and DCs are multifocal (208). IS with a multifocal structure were also seen in conjugates between naïve $CD4^+$ T cells and APC in the absence of CD80 (214). Finally, DP thymocytes have been shown to form multifocal IS, with pTyr stably associated with TCR-pMHC foci (213). IS formed by these immature thymocytes are remarkably similar in structure to the multifocal IS formed by Th2 cells at high antigen doses. Strikingly, pTyr continues to be associated with TCR-pMHC clusters for at least 30 min in both double positive thymocytes and Th2 cells. Hence, it is possible that continuous tyrosine kinase signaling at the site of TCR-pMHC accumulation is a general property of multifocal synapses. Th2 cells are thought to make lengthy contacts with B cells during the delivery of T cell help (63, 226), and continuous signaling at the IS may be important for this function.

It is known that the phosphorylation of signaling proteins, including Zap-70, is less complete in Th2 compared to Th1 cells upon activation through the TCR (227, 228). A recent report has shown that Th2 cells have twofold less surface CD4 than Th1 cell (229), and I have confirmed this result with polarized AD10 cells (data not shown). When CD4 levels of Th2 cells were elevated via retroviral transduction, stimulated Th2 cells had increased levels of ζ-chain and Zap-70 phosphorylation, comparable to Th1 cells (229). Additionally, CD4 is excluded from lipid rafts in Th2-APC conjugates (216), and CD4 is necessary for T cells to arrest and form mature IS (78, 119). Therefore, the inability of Th2 cells to form mature IS when there are large quantities of pMHC available, as

demonstrated in this report, could be due to the lower levels of CD4 compared to Th1 cells.

Th1 cells form mature IS with a 'bullseye' structure and have cytotoxic functions. Th2 cells are generally incapable of killing (230-232), and I have shown that a significant percentage of the IS formed by Th2 cells are multifocal, especially at high concentrations of antigen. Importantly, even at much lower antigen concentrations, where the majority of IS displayed compact accumulations of TCR-pMHC, Th2 cells failed to form IS with ring-like distribution of ICAM-1. It has been hypothesized that the delivery of cytotoxic effector molecules inside the ring of adhesion molecules in IS formed between CTL and target cells could prevent damage to bystander cells (43, 182, 233). In this model, the ring structure formed by LFA-1-ICAM-1 interactions acts as a 'gasket'. This gasket may not be a crucial component of the IS formed by naïve $CD4^+$ T cells, Th2 cells and thymocytes, as these cell types do not have cytotoxic function. Th1 cells contain a pool of preformed CD40L that is rapidly mobilized to the cell surface upon stimulation (202), and it is possible that the LFA-1-ICAM-1 gasket could serve to direct secretion of molecules involved in T cell help directly towards the source of antigen, thereby preventing activation of bystander cells. Further experimentation will be required to determine if the primary function of the bullseye-like IS formed by Th1 cells is to facilitate the delivery of cytotoxic agents and/or molecules involved in T cell help to APCs.

Chapter 3

An immunological synapse/kinapse transition in induced T regulatory cells is regulated by CD80 density

Summary

T regulatory cells are capable of modulating the surface phenotype of DCs and altering naïve CD4-DC interactions. In this chapter, I show that iTregs decrease surface expression of the costimulatory molecule CD80 on DCs. Induced Tregs form stable conjugates with DCs before, but not after, alteration of the DC surface phenotype. Decreases in CD80 are important because I also show that this costimulatory molecule plays a crucial role in determining the phenotype of the IS formed between iTregs and DC. Using supported planar bilayer technology, I show that in the absence of costimulation, iTregs maintain a highly polarized and motile phenotype after recognizing antigen. These motile cells are characterized by distinct accumulations of ICAM-1 in the lamella and TCR-pMHC in the uropod, a description that is consistent with a motile immunological synapse or 'kinapse'. However, in the presence of high concentrations of CD80, iTreg form stationary, mature IS at the same frequency as Th1 cells. Intriguingly, iTregs fail to form stable IS in the presence of 5 or 10 fold lower levels of CD80. Together, these results indicate that iTregs are tuned to form mature IS only in the presence of high levels of costimulation. I speculate that the transition from kinapse to synapse may be crucial for downmodulation of costimulatory molecules on the surface of DCs.

Introduction

$CD4^+$ Tregs are critical for controlling immune responses and promoting tolerance to autoantigens and commensal gut microflora (37, 234). Tregs were first identified as a subset of anergic $CD4^+CD25^+$ cells critical for maintaining tolerance to self and preventing autoimmunity (235, 236). Subsequent work revealed that Tregs express the transcription factor FoxP3, which is necessary for their development and function (237-239). FoxP3 is a master transcription factor that controls the expression or repression of hundreds of genes, including many that are crucial for Treg function such as *Ctla4* and *Cd25* (37, 240, 241). Thymocytes with a somewhat higher degree of self-reactivity than conventional CD4 T cells are selected and mature into $Foxp3^+$ T cells in the thymus before they leave for the periphery, where they account for approximately 10% of CD4 T cells (242, 243).

In addition to thymus derived or nTregs, $FoxP3^+$ Tregs can be induced from peripheral CD4 T cells both in vitro and in vivo (35). Differentiation of naïve CD4 T cells into iTregs requires the presence of TGF-β and IL-2, and retinoic acid enhances iTreg generation (244-248). Two recent reports have shown that DCs in both the lamina propria of the small intestine and the mesenteric lymph nodes efficiently convert naïve CD4 T cells to iTregs in vivo (249, 250). Conversion depends on TGF-β and retinoic acid, confirming the in vitro results. Naïve B cells are also adept at converting CD4 T cells to iTregs in the presence of polarizing cytokines (246, 251). Induced Tregs are thought to play a crucial role in promoting tolerance to foreign antigens, especially antigens derived from the gut microflora (234, 252). Significantly, oral tolerance can be

achieved in the absence of nTregs via induction of Tregs in a TGF-β dependent manner (253).

Tregs use a variety of processes to suppress immune reactions both in vitro and in vivo (252, 254). Early experiments demonstrated that ex vivo derived $CD4^+CD25^+$ Tregs suppress T cell proliferation in a contact dependent, cytokine independent manner in vitro (36). Tregs must be stimulated through the TCR to mediate suppression, but once activated they can suppress T cells of different specificities (255). This suppressive effect has been hypothesized to involve multiple mechanisms, including presentation of membrane bound TGF-β and delivery of cAMP to target cells via gap junctions (256, 257). However, in vivo experiments have suggested that IL-10 produced by Tregs is critical for control and prevention of inflammatory bowel disease and allergic inflammation (258, 259). TGF-β and IL-35 produced by Tregs have also been implicated in suppression in vivo (260, 261)

Tregs are also capable of controlling T cell activation by modulating DCs. Tregs express high levels of CTLA-4, and interactions between CTLA-4 and CD80/86 molecules on DCs upregulate expression of indoleamine 2,3-dioxygenase (IDO) by DCs (262). IDO catabolizes tryptophan, resulting in localized tryptophan deficiency and the production of pro-apoptotic catabolites. Through these mechanisms, IDO production by DCs discourages robust T cell proliferation.

Several reports have also shown that nTregs can modulate expression of CD80 and CD86 on DCs (38, 263-266). Downmodulation of CD80/86 on DCs requires expression of CTLA-4 by nTregs, and occurs in an antigen and LFA-1-

ICAM-1 dependent manner (265, 266). This effect was seen even when nTregs and DCs were cocultured in the presence of LPS, IFN-γ and other potent maturing stimuli, indicating that CTLA-4-mediated regulation of DCs by nTregs is a powerful mechanism for preventing DC maturation (266). In vivo imaging experiments have demonstrated that in the presence of antigen, nTregs prevent sustained naïve T cell-DC interactions (267, 268). Although Tregs were not observed directly interacting with naïve T cells, the mechanism responsible for suppression of naïve T cell-DC contacts was not established in these experiments (268). In experiments with DO11.10 TCR transgenic nTregs and naïve CD4 T cells cocultured with peptide-pulsed splenic DCs, Tregs were highly motile, swarmed around antigen loaded DCs and out-competed the naïve cells for space around the DCs (266). This swarming phenotype, possibly in conjunction with CTLA-4 mediated downmodulation of CD80/86, could explain the in vivo imaging results described above. Treg-DC IS have been described in one report examining the effect of homotypic Neuropilin-1 (Nrp-1) interactions on the length of Treg-DC interactions (269). This report indicated that although enhanced expression of Nrp-1 by nTregs increased the length of interactions with immature DCs compared to naïve T cells, the IS formed between nTregs and DCs was unaffected by Nrp-1 and were grossly similar to the IS formed by CD4 Th cells.

Given the importance of Tregs in modulating the surface phenotype of DCs and the lack of studies focusing on IS formed by Tregs, the factors governing Treg-DC interactions are of interest. In this chapter, the hypothesis that costimulatory molecules on the APC surface are responsible for determining Treg IS structure was tested. Given that Tregs are capable of downmodulating

CD80/86 molecules on the surface of DCs, I examined the role that CD80 plays in Treg-DC IS formation. I show here that in vitro generated iTregs, like nTregs, are capable of downmodulating CD80 on the surface of DCs, and that alteration of the DC surface phenotype affects subsequent iTreg-DC interactions. Using supported planar bilayers as artificial APCs, I show that iTregs are highly motile when they recognize antigen in the absence of costimulation. However, the presence of high, but not low, levels of CD80 is sufficient to induce the formation of classical, mature IS by iTregs. Differences in calcium flux upon antigen recognition in the presence or absence of CD80 could provide a mechanism for the enhanced IS formation seen in the presence of costimulation.

Materials and Methods

Animals

Heterozygous AD10 TCR transgenic mice on a B10.BR background, specific for pigeon cytochrome *c* 88-104 and reactive against moth cytochrome *c* 88-103 (MCC) (270), were provided by S. Hedrick (University of California at San Diego) by way of P. Marrack (National Jewish Medical Center). Mice were housed in specific-pathogen free conditions at Oregon Health & Science University according to institutional standards.

Antibodies

The antibodies used for FACS experiments were as follows: anti-CD4 Alexa Fluor 488 (GK1.5; eBioscience), anti-CD4 PerCP (RM4-5; BioLegend), anti-CD152 PE (UC10-4B9; BioLegend), anti-FoxP3 Alexa Fluor 647 (150D; BioLegend), anti-CD11c (HL3; Bioscience) and anti-CD80 FITC (16-10A1; BD Bioscience).

Dendritic cell culture

Bone marrow derived dendritic cells (BMDCs) were cultured as described (271). The femora and tibiae of B10.BR mice were harvested, cleaned and sterilized in 70% ethanol. The ends of the bones were cut and the marrow was flushed out with PBS loaded in a syringe. Cells were cultured in 100 mm bacteriological Petri dishes at 4 x 10^5/mL in 10 mL complete RPMI 1640 media supplemented with GM-CSF supernatant (final conc.: 20 ng/mL). On day 3, 10 mL of media supplemented with 20 ng/mL GM-CSF was added to the plates. On days 6 and 8, half of the supernatant was collected from the plates and spun down. The cell pellet was resuspended in 10 mL of media with 20 ng/mL GM-

CSF and added back to the original plates. On day 9, immature DCs were removed from the plates by gentle scraping, washed, resuspended in fresh media with 20 ng/mL GM-CSF and 1 μg/mL LPS and plated in LabTek II eight-well chambers (#1.5) (Nunc) or 6 well plates.

In vitro T cell polarization

Th1 conditions: AD10 splenocytes were used after removal of red blood cells by hypotonic lysis. Splenocytes were cultured at 10^7 cells/well in 6 well plates in 2 mL complete RPMI 1640, supplemented as previously described (146). Cultures contained 2.5 μM moth cytochrome *c* (MCC) 88-103 (KAERADLIAYLKQATK) and 5 ng/ml rmIL-12 (PeproTech). Cells were used on day 4.

Induced Treg conditions: $CD4^+$ cells and B cells were purified from AD10 and B10.BR spleen cell suspensions respectively using EasySep immunomagnetic negative selection (STEMCELL technologies). 5 x 10^6 B cells and 2.5 x 10^6 $CD4^+$ cells were cultured in 1 mL complete RPMI 1640 media in 6 well plates with 2.5 μm MCC peptide, 20 ng/mL TGF-β, 100 U/mL IL-2 and 10 nM all trans retinoic acid. On days 2 and 3, 1 mL of media supplemented with 100 U/mL IL-2 was added to the cultures. To confirm that T cells were polarized to an iTreg phenotype, cells were fixed, permeabilized and stained for CD4, CTLA-4 and FoxP3. Fixation and permeabilization reagents were from BioLegend. Cells were used on day 4.

CD80 downmodulation assay

1.6 x 10^5 day 9 BMDCs were seeded onto 12 well plates and treated LPS as described above. One day later, 8 x 10^5 or 8 x 10^4 day 4 iTregs were added per

well in the presence or absence of 2.5 μm MCC. As a control, iTregs were not added to some wells. 24 hrs later, the cells were harvested and stained for 30 min on ice for CD4, CD11c and CD80. All antibodies were used at 1:200. EDTA (1 mM) was included in the FACS buffer to discourage continued interactions. Samples were collected on a BD FACSCalibur with CellQuest software and analyzed with FlowJo (Tree Star, Inc.)

Imaging iTreg-DC interactions

3×10^4 day 9 BMDCs were seeded onto coverslips in 8 well chambers and treated with LPS as described above. One day later, 1.5×10^5 CFSE-loaded, day 4 iTregs were added to the wells. For CFSE loading, cells were incubated in 0.1% BSA in PBS with 5 μM CFSE for 10 min at 37º Celsius, and then washed in complete RPMI 1640. Imaging commenced as soon as iTregs were added to the wells. DIC and fluorescent images were obtained every minute for 2 hrs with a 20x objective. For some experiments, 1.5×10^5 unlabeled iTregs were added to the wells containing LPS matured BMDCs. 24 hrs later 1.5×10^5 fresh CFSE labeled iTregs were added per well. After the addition of labeled cells, imaging experiments lasting two hours were conducted. For some experiments, 10 μg/mL anti-CD80 (1G10; BD Bioscience) and anti-CD86 (GL1; BD Bioscience) were added to the wells 10 min before addition of iTregs. Cells were scored as motile if they adopted a polarized phenotype and moved more than one cell diameter over the course of the experiment. In some cases, the DCs became highly motile during the course of the experiment. In this situation, the conjugate was only scored if the T cell was clearly interacting with the same spot on the DC during the entire experiment. Imaging was performed with an Applied Precision

DeltaVision system. This system included an Applied Precision chassis with a motorized XYZ stage, Weatherstation environmental chamber, Olympus IX71 inverted fluorescent microscope, Xenon lamp and CoolSnap HQ^2 camera. The DeltaVision SoftWorx software package was used for image acquisition.

Supported planar bilayer experiments

GPI-linked forms of Oregon Green 488 labeled I-E^k (200 molecules/μm^2) and Cy5-labeled ICAM-1 (300 molecules/μm^2) were incorporated into dioleoylphosphatidylcholine bilayers exactly as described (78). For some experiments, GPI-linked CD80 was incorporated into bilayers at 20, 40 or 200 μm^2. These bilayers were supported on a coverslip in a Bioptechs flow cell, and were loaded with 100 μm MCC or Hb peptide (GKKVITAFNEGLK) in a PBS/citrate buffer (pH 4.5) for 24 hr at 37°C (78). 10^7 iTreg or Th1 cells in 1 ml HBS buffer with 1% human serum albumin were injected onto bilayers at 37°C. Images were acquired every minute for 1 hour using a 60x objective. Imaris 6.3 (Bitplane) was used to track cells interacting with the bilayer. To ensure that only cells productively interacting with the bilayer were analyzed, Cy5 fluorescence (ICAM-1 accumulation) was tracked automatically with the Spots tool in Surpass mode. In situations where the ICAM-1 signal was ambiguous, DIC images were examined to determine if the cell was flattened against the bilayer. DIC images were used to track cells interacting with bilayers loaded with irrelevant peptide, as there was only sparse ICAM-1 accumulation under these conditions.

Scoring T cell-bilayer interactions

Cells were scored as non-motile if they moved one cell diameter or less during the entire imaging session. Cells were considered to have reformed an IS

if they stopped forward progress, lost their uropod and ICAM-1 arc and remained non-motile for at least 10 min. Cells were considered to have broken symmetry if they were non-motile for 10 min or more before gaining motility and moving more than one cell diameter. The vast majority of cells were symmetrical when first contacting the bilayer, but this state was transient (much less than 10 min) and cells that subsequently became motile were not considered to have broken symmetry. Cells were scored as having a uropod anchor if the uropod of a motile cell was attached to the same place for at least 10 min, causing the cell to pivot.

Imaging calcium flux

Induced Tregs were loaded with the calcium indicator Fluo-4 (1 μm) for 30 min at room temperature in complete RPMI 1640, washed and introduced to supported planar bilayers containing I-E^k and ICAM-1, with or without 200 molecules/μm^2 GPI-CD80, and loaded with 100 μM MCC as above. DIC and fluorescent images were obtained every 2 sec for 10 min. Fluorescent intensity at each time point was measured using Imaris from Bitplane. Cells were only included in the analysis if they flattened against the bilayer.

Results

Induced Tregs downmodulate CD80 on the surface of BMDCs

Natural Tregs have been shown to downmodulate costimulatory molecules on the surface of DCs in an antigen specific manner (265, 266). To extend these findings to iTregs, I generated Tregs in vitro using a protocol established by Noelle and colleagues (246). Naïve AD10 $CD4^+$ splenocytes were cultured with naïve B cells in the presence of peptide antigen (MCC), TGF-β, IL-2 and all trans retinoic acid. Cells analyzed for CTLA-4 and FoxP3 at day 4 showed almost complete polarization to a Treg phenotype (Fig. 3-1 A). All of the experiments described in this chapter used these day 4 in vitro generated iTregs.

Bone marrow derived dendritic cells were cultured as described (271), transferred to 6 well plates and matured with LPS. Twenty-four hours later, day 4 iTregs were added to the mature BMDCs with or without peptide. As shown in Fig. 3-1 B, after a 24 hr co-culture with iTregs at a 5:1 iTreg:DC ratio, CD80 levels on BMDCs were reduced approximately 10 fold. This reduction was not seen when the cells were cocultured together without peptide, showing that this effect is antigen specific, even when using activated iTregs. Interestingly, when the number of iTregs in the co-cultures was reduced 10 fold, there was a bimodal distribution of CD80 expression on BMDCs, indicating that each Treg may downmodulate CD80 on one DC, implying that the iTreg-DC interactions are stable. If the conjugates formed in the co-cultures were unstable, it would be expected that each DC would receive roughly the same amount of contact time with iTregs, even if DCs outnumbered iTregs.

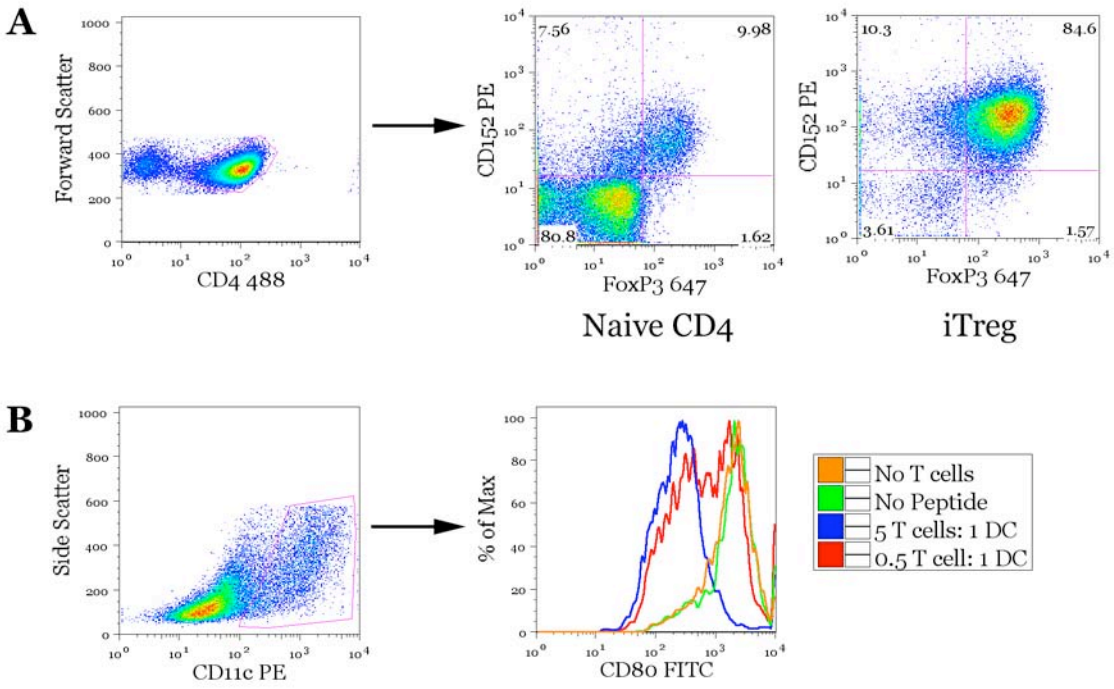

Figure 3-1: Induced Tregs downmodulate CD80 on the surface of DCs. (A) Day 4 in vitro generated Tregs were assayed for the expression of CTLA-4 and FoxP3 and compared to naive CD4 T cells. The leftmost panel was gated on live cells. (B) Induced Tregs were cocultered with BMDCs for 24 hrs at the indicated ratios. $CD11c^+$ live cells were analyzed for expression of CD80 and compared to BMDCs cultured without iTregs or with iTregs but without peptide. Results are representative of three experiments.

Modulation of DCs by iTregs results in decreased stability of iTreg:DC conjugates

Sakaguchi recently showed that nTregs swarm around peptide loaded splenic DCs, and hypothesized that this highly motile behavior could prevent naïve T cells from interacting with DCs (266). Additionally, nTregs have been observed swarming around DCs in an antigen-specific manner in lymph nodes (268). However, neither of these studies reported whether Treg-DC interactions changed over time. This question is of interest given the ability of Tregs to alter the surface phenotype of DCs.

Table 3-1: *Classification of iTreg-BMDC contacts*[a]

Time	n	Non-Motile (%)	Motile (%)	Contact time less than 30 min (%)
0	43	84[b,c]	16	9
24 hr	77	43[b]	57	16
0 (anti-CD80/86)	97	47[c]	53	N.D.[d]

[a]Quantitation of data shown in Figure 3-2.
[b]P<0.0001, Fisher's exact test.
[c]P<0.0001, Fisher's exact test.
[d]N.D.=not determined

I conducted imaging experiments to examine the initial interactions between iTregs and mature BMDCS, as well as interactions 1 day later, when costimulatory molecules on the DCs are downmodulated. The number of DCs/mm^2 was the same as for the CD80 downmodulation assay shown in Figure 3-1 B, and the ratio of iTregs:DCs was 5:1. As shown in Figure 3-2 A and quantified in Table 3-1, the initial interactions between mature BMDCs and day 4 iTregs were quite stable. Over 80% of cells did not move more than one cell diameter over the course of the experiment. Consistent with these results, less than 10% of cells had short contact times, defined as less than 30 min (Table 3-1). One day later, the iTreg-DC conjugates were no longer stable. Instead the iTregs had a polarized shape and actively crawled around DCs. To determine if the change in iTreg behavior after 24 hrs was due to changes in the DCs or the iTregs, fresh CFSE labeled iTregs were added to the co-cultures prior to imaging. Figure 3-2 B shows that both labeled and unlabeled iTregs were in the swarms surrounding DCs. At this time point, some of the DCs were detached from the coverslip while others remained attached. I quantified the interactions between

labeled iTregs and attached DCs. Compared to the initial iTreg-DC interactions, interactions involving DCs that had been incubated with iTregs for 24 hrs were

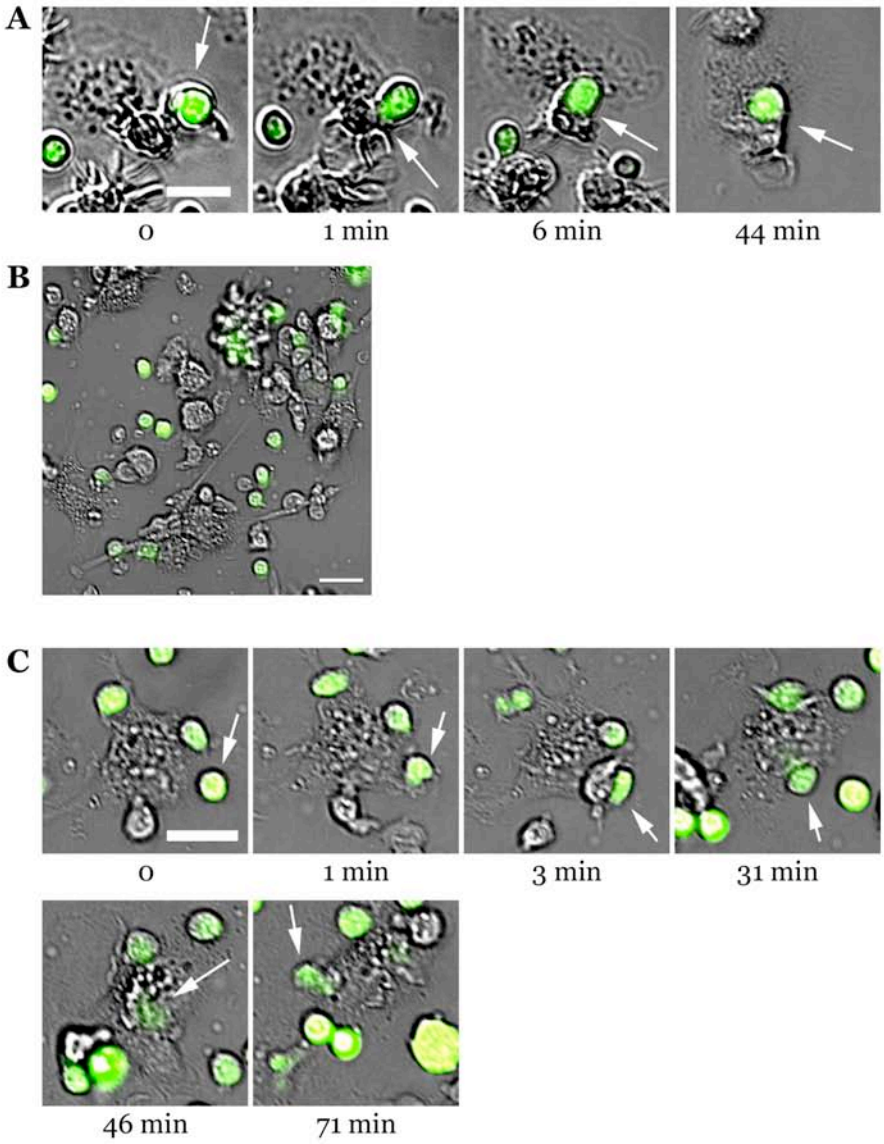

Figure 3-2: The stability of new iTreg-DC interactions are altered after DCs are incubated with iTregs for 24 hrs.
(A) CFSE labeled iTregs were added to peptide-pulsed mature BMDCs. Most of the iTreg-DC interactions were extremely stable over the length of the experiment. In this example, the cell of interest flattens against the DC at the 1 min time point. (B) and (C) Unlabeled iTregs were incubated with BMDCs for 24 hrs followed by the addition of fresh CFSE labeled iTregs. (B) Images were acquired one hour after addition of labeled iTregs. Both labeled and unlabeled iTregs were in large clusters surrounding DCs. (C) Fresh (labeled) iTregs showed increaed motility on 24 hr DCs. In this example the iTreg flattens against the BMDC at 1 min. The iTreg maintains a polarized phenotype throughout the experiment, and between 31 and 71 min it travels underneath the DC. Scale bars represent 10 μm.

much less stable. An example of one such interaction is shown in Figure 3-2 C. At 24 hrs, approximately 60% of the freshly added iTregs were motile, although the number of short-lived conjugates was not significantly different than at T=0 (Table 3-1). This increase in motility was due to decreased costimulatory molecules, as roughly half of the iTregs examined were motile at T=0 when the DCs were preincubated with antibodies to CD80 and CD86. The motility observed at the 24 hr time point is generally consistent with previously reported results for nTregs interacting with immature BMDCs (266). However, the fraction of motile nTregs was not reported by Onishi et al., making a direct comparison difficult. The results reported here support the hypothesis that interactions between iTregs and DCs are altered by iTreg-mediated downmodulation of costimulatory molecules on the surface of DCs.

iTregs form kinapses on supported planar bilayers

To more closely examine the interaction between iTregs and APCs, I introduced iTregs onto supported planar bilayers containing GPI-linked, fluorescently labeled MHC class II (I-E^k) and ICAM-1 and loaded with MCC peptide. Under these conditions, iTregs failed to form mature IS with a clearly defined cSMAC and pSMAC. Instead, the vast majority of iTregs were motile (Table 3-2). These motile iTregs had a very polarized phenotype with well-defined lamellapodia and uropods. An arc of ICAM-1 defined the lamella and pMHC-TCR interactions accumulated in the uropod (Fig. 3-3 A and Table 3-3). This description is consistent with the definition of an 'immunological kinapse' recently proposed by Dustin (44). However, the accumulations of pMHC-TCR interactions in the uropod have not, to my knowledge, been previously described.

Retrograde actin flow initiated in the lamellapodium is known to transport TCR clusters centripetally to the cSMAC (45), and it is likely that TCR microclusters initiated at the leading edge of these iTreg kinapses are transported via actin flow

Table 3-2: *Classification of T cell-supported planar bilayer contacts*[a,b]

			Motile	
Cell Type	CD80 (molec./mm^2)	Non-Motile (%)	Continuous (%)	Reform or Break (%)
iTreg	None	6[c]	84[d]	10[e]
iTreg	20	2	82	16
iTreg	40	6	87	7
iTreg	200	20[c]	50[d]	30[e]
Th1	None	25	48	27

[a]Data from at least two experiments with a total of n=73-129 for each condition.
[b]Representative examples of motile cells are shown in Figures 3-3 and 3-4.
[c]P=0.0013, Fisher's exact test.
[d]P<0.0001, Fisher's exact test.
[e]P<0.0001, Fisher's exact test.

to the uropod where they become detectable. When iTregs were injected onto bilayers loaded with an irrelevant peptide antigen, they flattened against the substrate, adopted a polarized phenotype and migrated rapidly. Unlike the kinapses formed when iTregs interacted with bilayers loaded with MCC, non-cognate iTreg-bilayer interactions were characterized by only occasional and diffuse accumulations of ICAM-1 and no pMHC-TCR interactions (Figure 3-3 B). Timelapse images of iTregs interacting with bilayers loaded with MCC were

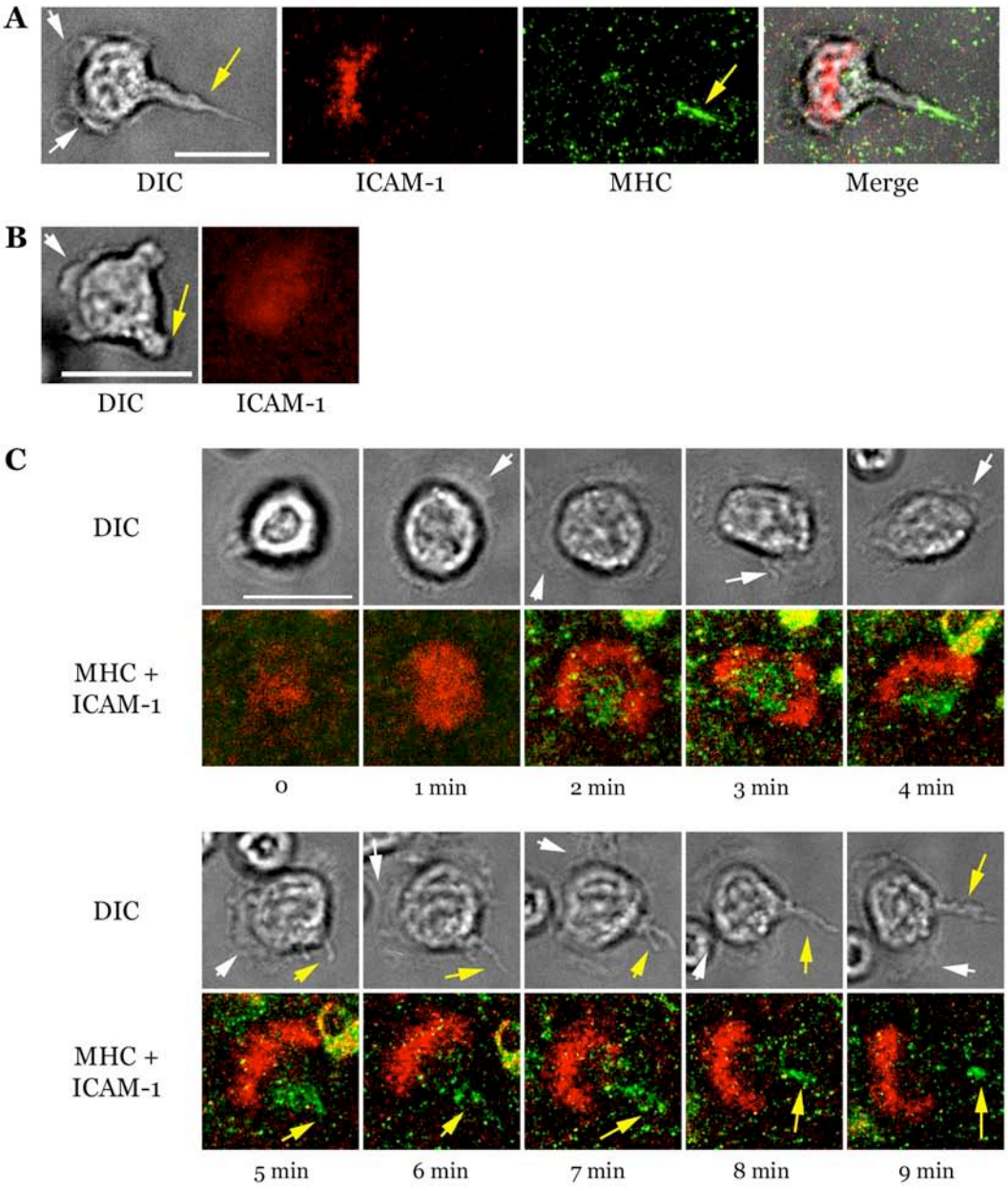

Figure 3-3: iTregs form kinapses on supported planar bilayers.
iTregs were injected onto supported planar bilayers containing 200 molecules/μm² I-E^k-Oregon Green 488 and 300 molecules/μm² ICAM-1-Cy5. (A) iTregs adopted a highly polarized phenotype with clearly defined uropods and lamellapodia when interacting with bilayers loaded with 100 μm MCC. An ICAM-1 arc ran across the center of the cell and pMHC accumulated in the uropod. (B) iTregs interacting with bilayers loaded with an irrelevant peptide (Hb 64-76) also adopted a polarized shape, but had only diffuse ICAM-1 accumulation and no pMHC accumulation. (C) Time lapse images of the initial iTreg-bilayer contact. This cell initially had a symmetrical footprint (1 min), but lost symmetry by 2 min and formed a kinapse with a clearly defined uropod and ICAM-1 arc by 5 min. White arrows indicate lamellapodia and yellow arrows indicate uropods. Scale bars are 10 μm.

Table 3-3: *Classification of kinapses*[a,b]

Cell Type	CD80 (molec./mm^2)	Uropod + ICAM-1 Arc (%)	Uropod Anchor (%)
iTreg	None	78[c]	6[e,f]
iTreg	20	88	12
iTreg	40	74	19[e]
iTreg	200	60[c,d]	29[f,g]
Th1	None	27[d]	4[g]

[a]Data from at least two experiments with n=73-129 for each condition.
[b]Representative examples of these phenotypes are shown in Figures 3-3 and 3-4.
[c]P=0.0043, Fisher's exact test.
[d]P<0.0001, Fisher's exact test.
[e]P=0.012, Fisher's exact test.
[f]P<0.0001, Fisher's exact test.
[g]P<0.0001, Fisher's exact test.

acquired to define the initial steps in kinapse formation. The iTregs were symmetrical when first contacting the bilayer, but lost symmetry immediately (Fig. 3-3 C). In the example shown, MHC accumulated within a partial ICAM-1 ring within 2 min of the initial contact. However, an ICAM-1 arc was formed, and centrally located accumulations of MHC were relocated to the nascent uropod within 5 min. Between 5 and 10 min, the uropod became more clearly defined and the cell began migrating.

Although 84% of iTregs were motile over the entire length of the experiment, 6% of the cells were non motile for the whole experiment (Table 3-2). An additional 10% of the cells either formed stable, symmetrical IS and then

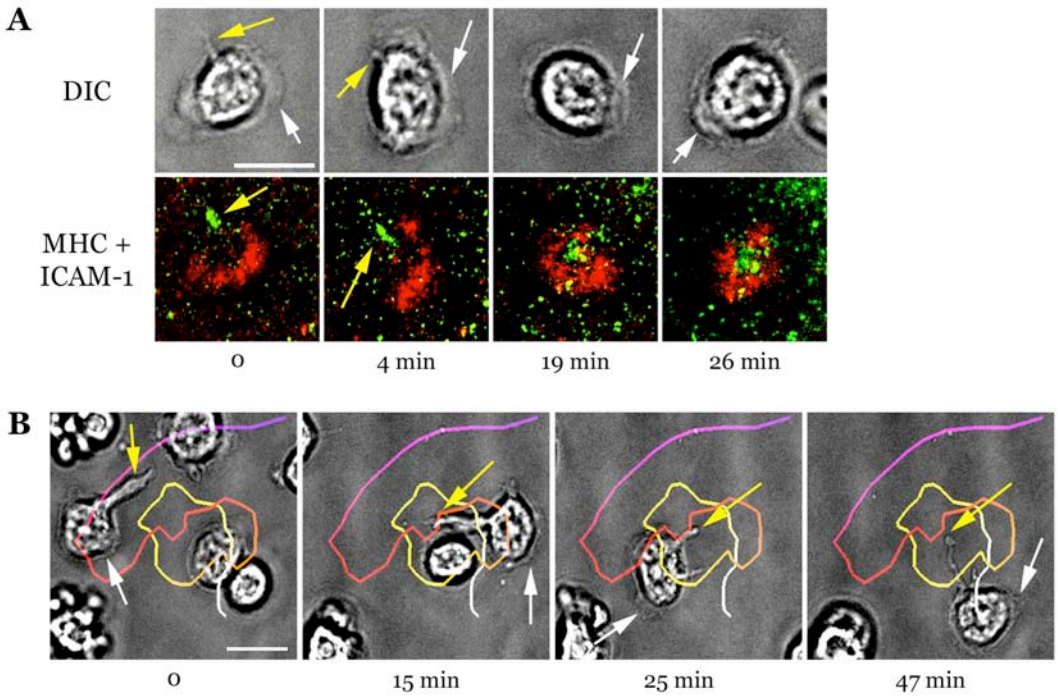

Figure 3-4: Examples of phenotypes displayed by iTregs on supported planar bilayers.
iTregs were injected onto supported planar bilayers as in Figure 3-3. (A) iTregs occasionally regain symmetry. In this example, a motile iTreg with an ICAM-1 arc and a clearly discernible uropod (1 min) begins to lose its uropod (4 min) and forms a symmetrical IS with an ICAM-1 ring surrounding accumulations of pMHC. (B) Motile iTregs occasionally became anchored to the bilayer by their uropod. In this example the uropod becomes anchored at the 15 min timepoint and pivots around this anchor until the end of the experiment. The track overlaid on the DIC images shows the path the the cell took over the course of the experiment. The track is color coded from blue to white to indicate the progresion of time from the beginning to the end of the experiment. White arrows point to lamellapodia and yellow arrows indicate uropods. Scale bars represent 10 μm.

broke symmetry or transitioned from a kinapse to a symmetrical IS (Table 3-2). Figure 3-4 A shows an example of an iTreg transitioning from a kinapse to a stable IS. Occasionally, a motile kinapse would become anchored to the substrate by its uropod. When this occurred, the cell maintained a polarized phenotype but pivoted around the site of uropod attachment. This phenotype was observed in 6% of the motile iTregs (Table 3-3). Figure 3-4 B shows a cell that is anchored in this manner for over 30 min.

CD80 changes the balance between kinapses and stable synapses for iTregs

Induced Treg-DC interactions become less stable on the same time frame that iTregs downmodulate costimulatory molecules on the surface of DCs.

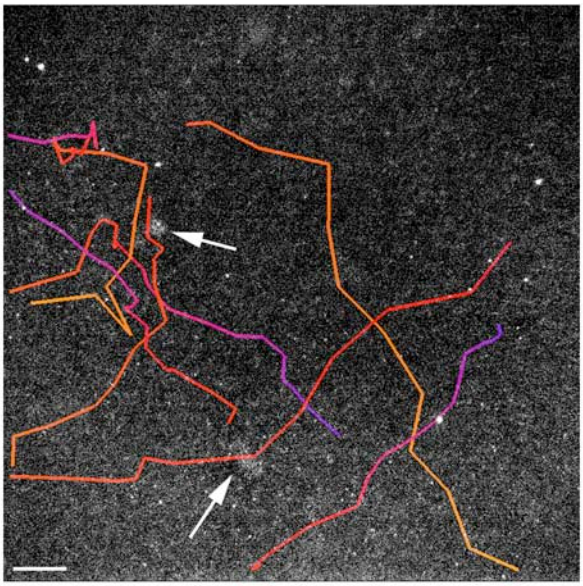

Figure 3-5: iTregs rapidly migrate on bilayers loaded with irrelevant peptide. iTregs were injected onto planar bilayers loaded with Hb peptide as in Figure 3-3 B. Under these conditions, iTregs flattened against the bilayers and were highly motile. No pMHC accumulation occured. ICAM-1 accumulations were only occasionally visible. This figure shows cell tracks overlaid on an ICAM-1 image taken approximately halfway (30 min) through the experiment. The white arrows point to faint ICAM-1 accumulations that were visible for some of the cells. The tracks are color coded blue to white to indicate the progression of time from the beginning to the end of the experiment. All of the tracks depicted here concluded before the end of the experiment. Scale bar represents 10 μm.

Therefore, I examined interactions between iTregs and bilayers containing increasing levels of CD80. As a control, iTregs were observed interacting with bilayers lacking antigen. Figure 3-5 shows tracks tracing the path of iTregs on bilayers loaded with irrelevant peptide antigen. Under these conditions, the iTregs were highly motile and accumulated very little ICAM-1 (see also Fig. 3-3 B). When iTregs were injected onto bilayers loaded with MCC, they maintained a high degree of motility and only rarely formed stable IS, as discussed above. However, iTregs displaced 3-fold less distance per unit time and their tracks were significantly less straight on bilayers loaded with their cognate antigen (Fig. 3-6 A and Fig. 3-7 A and B). Next, iTregs were introduced to bilayers containing 20, 40

or 200 molecules/μm^2 CD80. Induced Tregs placed on bilayers containing the two low concentrations of CD80 behaved similarly to iTregs interacting with bilayers containing no CD80 (Fig. 3-6 B and C). Induced Tregs formed similar

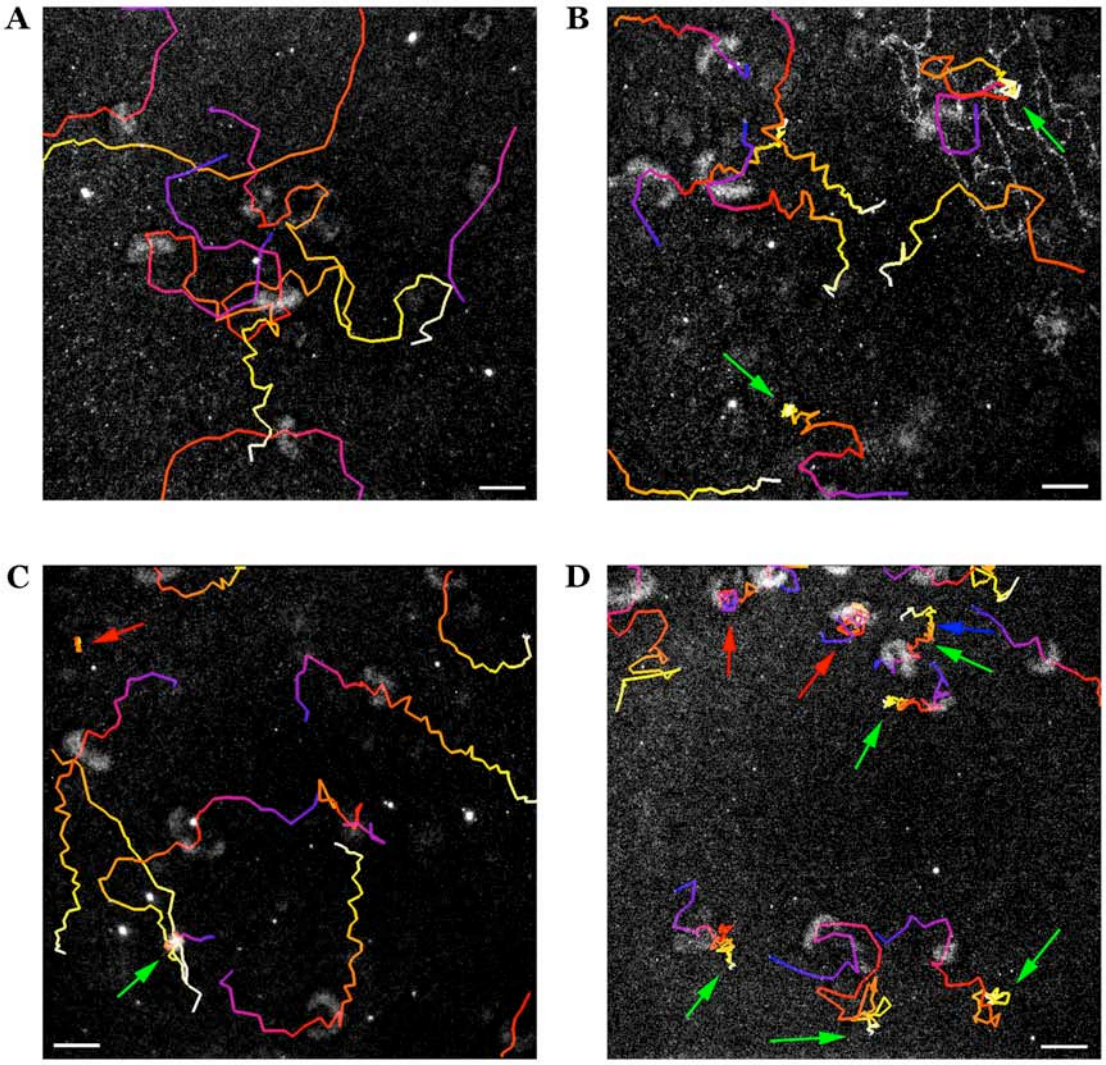

Figure 3-6: High levels of CD80 reduces iTreg motility.
iTregs were injected onto supported planar bilayers containing pMHC, ICAM-1 and either zero (A), 20 (B), 40 (C) or 200 (D) molecules/µm2 CD80. These images show tracks overlaid on images of ICAM-1 fluorescence and color coded from blue to white to indicate the progression of time from the beginning to the end of the experiment (1 hr total). Green arrows point to cells that regain symmetrical IS, red arrows point to non motile cells and blue arrows point to cells that break symmetry. Note the greater proportion of non motile cells and cells that regain symmetry in (D). Tracks in (D) are also significantly less straight. Scale bar represents 10 µm.

numbers of non-motile IS and regained or lost symmetry at a similar frequency at 0, 20 and 40 molecules/μm2 CD80 (Table 3-2). However, more cells became anchored by the uropod at 40 molecules/μm2 (Table 3-3).

Strikingly, a much greater percentage of iTregs interacting with bilayers containing 200 molecules/μm2 CD80 were non-motile for the entire length of an hour-long experiment. In fact, 50% of the cells were non-motile for at least part of the experiment, compared to approximately 15% at the lower concentrations of CD80 (Table 3-2). The kinapses formed on bilayers with high levels of CD80 were also significantly different. A lower percentage of cells adopted a highly

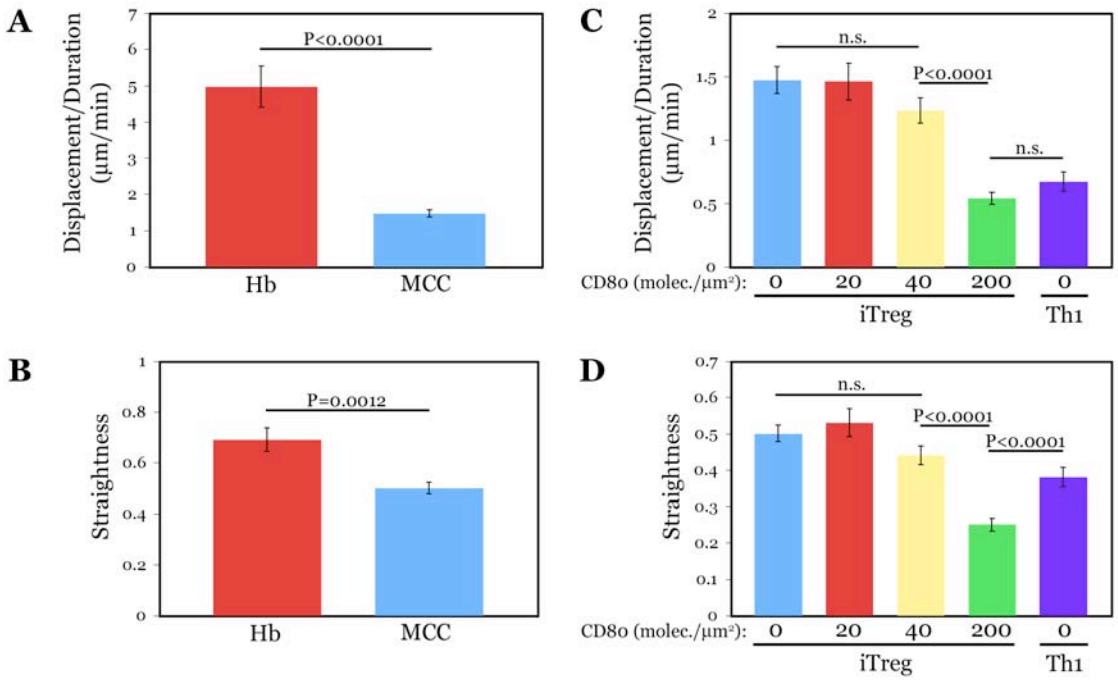

Figure 3-7: Quantitaton of tracks formed by iTregs and Th1 cells interacting with supported planar bilayers.
Tracks from iTregs interacting with bilayers containing I-E^k and ICAM-1 and loaded with Hb or MCC peptide were analyzed for displacement/duration (μm/min) (A) or straightness (track displacement/length) (B). The same measurements were made for iTregs and Th1 cells interacting with bilayers containing I-E^k, ICAM-1 and varying amounts of CD80 and loaded with MCC (C and D). Two tailed Student's *t* tests were conducted to determine P values. Each bar represents data from at least two experiments with n=73-129.

polarized phenotype with a clearly discernible uropod and ICAM-1 arc. Also, a much higher percentage (approximately 30%) of the cells were anchored to the substrate by their uropod (Table 3-3). These uropod anchors are reminiscent of structures formed by T cells when the myosin light chain kinase ROCK is inhibited (51). ROCK is required for detachment of the uropod during migration, while another light chain kinase, MLCK is required for detachment of the leading edge. The increased number of cells attached by their uropods in the presence of high levels of CD80 indicates that the cycling between MLCK and ROCK activation required for effective migration breaks down under these conditions (51).

Measurements of the tracks made by iTregs on bilayers with high levels of CD80 revealed that the paths taken by cells under this condition were significantly less straight than at low concentrations of CD80. Induced Tregs only displaced one-third as much distance per minute at the higher CD80 levels (Fig. 3-7 C and D). This result is most likely the result of a combination of reduced straightness and reduced speed.

Most of the studies of IS structure published over the past decade have examined IS formed by T cell blasts. These T cell blasts have generally been generated in vitro from splenocytes harvested from TCR Tg mice on a B6 or B10 background, and blasting cells from these mice tend to differentiate towards an IFN-γ secreting, Th1 phenotype (225). The IS formed by these cells have been thoroughly described on the planar bilayer system (43). Therefore, I decided to examine the synapses and kinapses formed by Th1 cells on supported planar

bilayers. As shown in Figure 3-8, Th1 cells formed a similar distribution of synapses and kinapses in the absence of CD80 as iTregs did in the presence of high levels of CD80. Th1 cells had a similar displacement/duration but traveled significantly straighter than iTregs on bilayers with high CD80 (Fig. 3-7 C and D), indicating that Th1 cells migrated at a lower speed than iTregs under these conditions. Although Th1 cells formed a similar number of completely non-motile IS and reformed or broke symmetry as often as iTregs did in the presence of high levels of CD80 (Table 3-2), the kinapses formed by Th1 cells were less polarized, and very few of these cells became anchored to the substrate by their uropods (Table 3-3). The lack of uropod anchors could explain the relative straightness of the Th1 tracks.

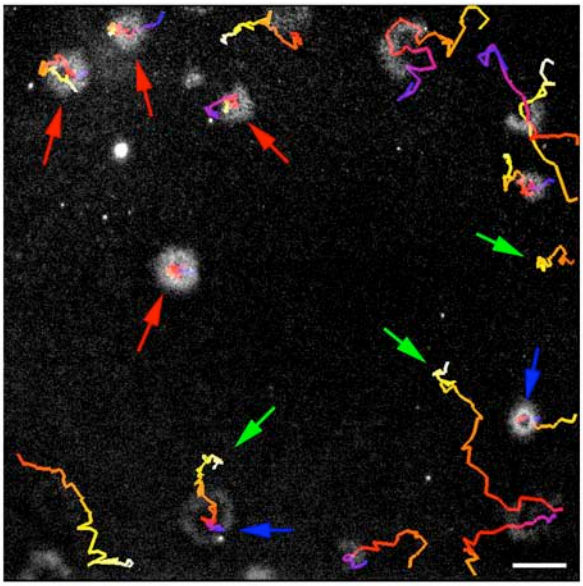

Figure 3-8: Th1 cells form both stable IS and kinapses. Th1 cells were injected onto supported planar bilayers containing pMHC and ICAM-1, as in Figure 3-3. Cell tracks are color coded as in Figures 3-5 and 3-6 and overlaid on an image of ICAM-1 fluorescence. Green arrows point to cells that regain symmetry, red arrows point to non motile cells and blue arrows point to cells that lose symmetry. Note the similarity between the tracks in this figure and those in Figure 3-6 D. Scale bar represents 10 μm.

CD80 alters the calcium flux of iTregs

A sustained calcium flux is associated with the 'stop signal' given to migrating T cells upon antigen recognition, resulting in stable T-cell-APC conjugates and IS formation (75, 272). Furthermore, costimulation is necessary

for sustained calcium flux when TCR signals are suboptimal (145). Induced Tregs clearly were not receiving a stop signal in the absence of high levels of CD80, so I decided to measure the calcium flux in iTregs interacting with bilayers with or without CD80. For these experiments, the cells were loaded with the calcium indicator Fluo-4 and injected onto bilayers containing 0 or 200 molecules/μm^2 CD80. As

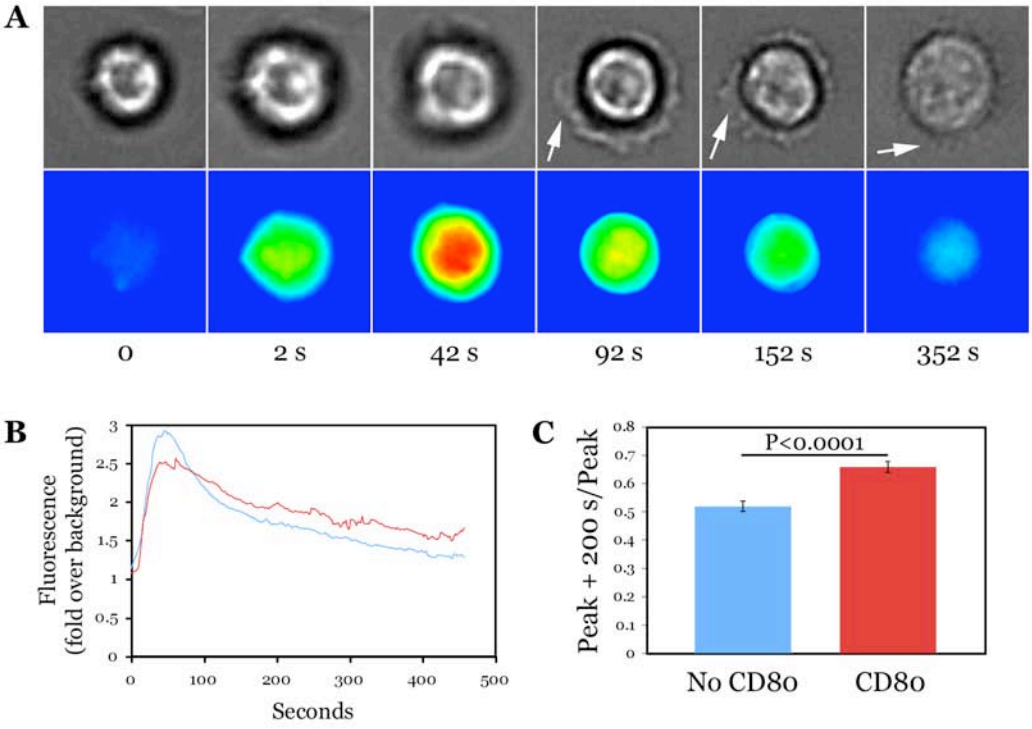

Figure 3-9: Calcium flux in iTregs is more sustained in the presence of high levels of CD80.
iTregs loaded with Fluo-4 were injected onto bilayers containing pMHC, ICAM-1 and 0 or 200 molecules/μm^2 CD80. Images were acquired every 2 sec. (A) Calcium flux precedes flattening. The bottom row has pseudocolored images of the calcium flux, where blue is the background fluorescence and red is the peak (3.2-fold above background in this example). (B) After background subtraction, the peak fluorescence from each cell (n=19 and n=12 for 0 and 200 molecules/µm2 CD80 respectively) was aligned and averages for each condition were determined. The blue line is 0 and the red line is 200 molecules/μm^2 CD80. Values were not significantly different at any time point. (C) iTreg calcium flux is more sustained in the presence of CD80. The ratio of fluorescence at 200 sec post peak to peak fluorescence was determined for each cell. This ratio was significantly higher in the presence of CD80. The P value was determined with a two tailed Student's *t* test.

shown in Figure 3-9 A, calcium flux preceded complete flattening of the cell against the bilayer and lamellapodium formation. Although the peak calcium flux appeared to be higher on average without CD80 and calcium levels at later time points appeared to be higher with CD80, these differences did not rise to the level of statistical significance (Fig. 3-9 B). This was due to high cell to cell variability, most likely caused by inconsistent loading of the cells with Fluo-4. To control for this variable, I calculated the ratio between the fluorescence at 200 sec after the peak (during the plateau phase) and the peak fluorescence. As shown in Figure 3-9 C, this ratio was significantly higher in the presence of CD80, demonstrating that intracellular calcium increases are more sustained when iTregs interact with bilayers containing high levels of CD80. The ratio of fluorescence at 100 sec post peak: peak fluorescence was also significantly higher with CD80 (P=0.0013; two tailed Student's *t* test). These data indicate that iTregs may be tuned to flux calcium at the sustained levels necessary to deliver a stop signal only when high levels of costimulation are available.

Discussion

In this chapter, I showed that iTregs, like nTregs, are capable of downmodulating costimulatory molecules in an antigen-specific fashion. After a 24 hr incubation there was a roughly 10-fold reduction in surface CD80 on LPS-matured BMDC. Over the same time scale, iTregs altered the nature of their interactions with DCs. When iTregs were initially introduced to mature DCs, they formed extremely stable conjugates, with most iTregs failing to move more than one cell diameter over the entire length of the imaging experiment. After a 24 hr incubation, newly added iTregs formed less stable conjugates, characterized by the T cells crawling along the surface of DCs. This phenotype is consistent with the dynamic kinapses described for low avidity interactions between naïve T cells and DC in vivo (40). Taken together with the results from the bilayer experiments, the simplest explanation for the change in behavior at 24 hrs is that the motility of iTregs, or lack thereof, is governed by the levels of costimulatory molecules on the surface of the DCs. This interpretation is supported by the experiments examining the effects of blocking antibodies to CD80 and CD86. These results suggest a model, described in Figure 3-10, wherein iTreg-DC interactions are modulated by the relative levels of costimulatory receptors on the surface of APCs. In the absence of antigen recognition, iTregs rapidly migrate over the surface of the APC, scanning for their cognate antigen. Upon antigen recognition, iTregs form either relatively slow moving kinapses or non-motile IS depending on the levels of costimulatory molecules. In the presence of high levels of CD80/86, symmetrical IS are favored, and this structure may allow the Treg to exert its regulatory function on the DC, including downregulation of

CD80 and CD86. After CD80/86 levels are reduced, kinapse formation is favored and the iTreg starts migrating. By alternating between kinapses and synapses, iTregs could contact and modulate CD80/86 levels on multiple DCs.

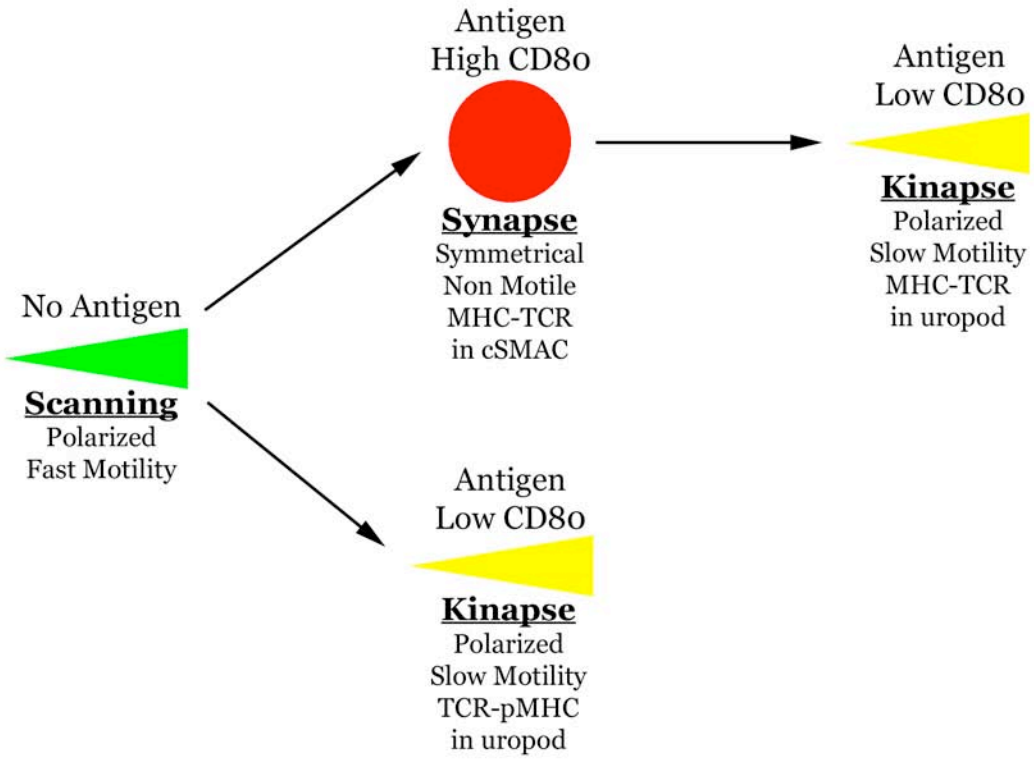

Figure 3-10: Model for Treg-DC interactions.
In this model, Tregs rapidly survey their environment, crawling on DCs in an ICAM-1-dependent manner. Upon antigen recognition, Tregs flux calcium and decide whether to form a stable synapse or kinapse. If the DC has an immature phenotype with low levels of costimulatory molecules, the Treg forms a kinapse characterized by relatively slow motility and accumulation of TCR-pMHC interactions in the uropod. If the Treg encounters high levels of costimulatory molecules, it forms a stable, symmetrical synapse. This structure could be required for CTLA-4-mediated downmodulation of CD80 and CD86. After downmodulation of CD80/86 the Treg continues to recognize antigen, but loses symmetry and forms a kinapse.

When I conducted experiments adding blocking antibodies for CD80 and CD86 to the DCs before the addition of iTregs, only half of the cells displayed a motile phenotype. This result was in stark contrast to the planar bilayer experiments where the cells were almost uniformly motile in the absence of CD80. Given the complexity of T-DC interactions, it is likely that other molecules on the of DCs also modulate interactions with iTregs, resulting in a close balance between synapse and kinapse formation when CD80 and CD86 are blocked. For example, the CD28 family costimulatory molecule ICOS is expressed on nTregs and iTregs, and shares an ability to modulate the PI3K pathway with CD28 and CTLA-4 (161, 273). It is possible that iTregs downmodulate ICOS ligand (a B-7 family member) or other molecules on the surface of DCs via a similar mechanism to CTLA-4 mediated downmodulation of CD80 and CD86. However, only 57% of newly introduced iTregs were motile on DCs that had interacted with iTregs for 24 hrs, leaving open the possibility that this close balance between synapse and kinapse formation is the default state for iTreg-DC interactions. Further experiments examining iTreg-DC interactions at later time points would be useful in determining if continued modulation of the DC surface phenotype results in a greater percentage of iTregs forming kinapses.

In an attempt to determine which receptor for CD80 was responsible for providing the stop signal for iTregs, I conducted experiments where I coated iTregs with anti-CD28 or CTLA-4 and then added an anti-isotype crosslinking antibody before introducing the cells to planar bilayers that lacked CD80. Cells that had these receptors crosslinked still almost exclusively formed kinapses. Given this result, it is likely that CD28 or CTLA-4 ligation at the IS is required.

This is consistent with experiments showing that IS formation, and downstream signals, are altered when costimulation is provided in trans (via beads coated with anti-CD28) (167). Further experiments using blocking Fab antibodies to CD28 and CTLA-4 will be needed to determine which of these receptors is required for delivery of a stop signal in the presence of high levels of CD80.

Experiments from the Rudd laboratory have shown that ligation of CTLA-4 actually increases motility of T cells (274, 275). Therefore, it is likely that CD28 is necessary for the stop signal. If this is indeed the case, it suggests a model where high levels of CD80/86 are needed to deliver a stop signal via CD28 because CTLA-4 has a much higher affinity for CD80 and CD86 (169). At low levels of CD80/86, CTLA-4 out-competes CD28. When CD80/86 levels are high, CD28 signaling occurs and mature IS formation is favored, and CD80/86 are downmodulated in a CTLA-4-dependent manner. When CD80/86 levels are reduced below a certain threshold, CD28 is no longer able to compete with CTLA-4 for its ligands, dampening the stop signal and resulting in a transition from a mature IS to a kinapse.

Mice deficient for the actin regulatory protein WASp develop chronic colitis, and Tregs from these mice are poor suppressors both in vitro and in vivo (276-278). Also, it has recently been shown that T cells require WASp to maintain IS symmetry (102). Although defective migration to lymphoid tissue likely plays a role in the phenotype described for WASp deficient Tregs, it is tempting to speculate that defective IS formation could also play a role. Tregs incapable of IS formation would also be deficient in CD80/86 downmodulation if the model proposed in Figure 3-10 is correct. Thus, constant surveillance and

modulation of DCs (via IS dependent downmodulation of costimulatory molecules) may be required to prevent autoimmune disease. Consistent with this idea, Tang et al. have demonstrated that nTregs from BDC2.5 TCR Tg mice (which have TCRs specific for an antigen associated with pancreatic β-cells) are motile in the pancreatic LN when transferred into NOD mice. However, when the same nTregs were transferred into NOD.CD28$^{-/-}$ mice that lack Tregs, they were non-motile (268). Given the results presented here, it is likely that the DCs in the Treg-deficient animals had relatively high levels of CD80/86 compared to animals containing endogenous Tregs, thereby altering the behavior of the transferred Tregs. Higher levels of costimulatory molecules on DCs in the pancreatic LN of these already diabetes-prone animals could partially explain why NOD mice lacking Tregs develop diabetes more quickly than wt mice (279).

Chapter 4

Discussion

Summary of findings

The basic finding of this thesis is that CD4 T cell subsets form a diverse array of IS structures. Th1 cells form IS with clearly defined SMACs over a range of antigen concentrations. These structures were identical to the classical IS described by Kupfer (79) and Dustin (78). Th2 cells, on the other hand, form multifocal IS without segregation of adhesion molecules to the pSMAC. Differences in the distribution of signaling molecules are also noted for Th1 and Th2 IS. I hypothesize that IS structure is related to the delivery of effector functions, with the secretion of inflammatory and cytotoxic molecules requiring formation of a pSMAC that acts as a gasket.

Induced Tregs predominantly form kinapses when low levels of costimulatory molecules are available. However, in the presence of APCs displaying high levels of CD80, iTregs transition to stable IS, and then start moving again; this transition to a motile phenotype occurs in the same time frame as CD80 downmodulation by iTregs. These findings suggest a model wherein iTregs constantly survey DCs for antigen, but are tuned to only form stable IS when costimulation is in abundance. I hypothesize that formation of a mature IS may be required for CTLA-4-mediated downmodulation of costimulatory molecules.

Multifocal immunological synapses

The results presented in Chapter 2 show that Th2 cells form predominantly multifocal IS with both fibroblast APC and supported planar bilayers containing pMHC and ICAM-1. These results are somewhat at odds with reports from the Bottomly laboratory on IS formation in Th2 cells (216, 217).

Those reports showed that Th2 cells, generated in vitro from TCR Tg mice on a B10.BR background, failed to cluster TCR at the T cell-APC interface when resting splenic B cells were used as APC, but were able to cluster TCR when DCs or LPS activated B cells were used. These results were explained with the observation that Th2 cells express high levels of CTLA-4 (217). The higher levels of costimulatory molecules on activated B cells and DCs were hypothesized to be necessary to overcome inhibition by CTLA-4, allowing clustering of the TCR. The experiments with $CD80^+$ $CD86^-$ fibroblasts shown in Chapter 2 argue against this theory. CTLA-4 has a much higher affinity for CD80 than CD28 (CD28 and CTLA-4 compete for CD86 on a more equal footing). Therefore, Th2 cells should not be expected to cluster TCR at the interface of conjugates with these fibroblasts due to the CTLA-4-favoring environment. However, TCR was clustered at IS formed with these fibroblasts, suggesting that CTLA-4 does not inhibit IS formation in Th2 cells. An explanation for this apparent contradiction could be that multifocal IS lacking a strong centralized accumulation of TCR-pMHC interactions were scored as lacking TCR clustering in the above-mentioned studies. Thus, a lack of centralized clustering of TCR-pMHC may be a general feature of Th2 IS.

An exception to this generality might be situations where very little antigen is available. I found that when planar bilayers were loaded with low concentrations of peptide antigen, significantly fewer Th2 IS had a distinctly multifocal phenotype. One interpretation for this finding is that the number of foci is directly related to the amount of antigen. If this is the case, one would predict that monofocal IS would be formed below a certain antigen

concentration. Indeed, the large IS with 10 or more large foci spread throughout the interface, as seen in Figure 2-2, were never seen at low concentrations of antigen. Significantly, Th2 cells failed to form IS with clearly defined pSMACs and cSMACs at all antigen concentrations. This result indicates that the forces responsible for segregating LFA-1-ICAM-1 interactions at the T cell-APC interface are altered in Th2 IS. Clusters of LFA-1-ICAM-1 move centripetally in an actin dependent manner (45), and it is thought that the border between the pSMAC and the cSMAC is really a transition from a relatively actin-rich zone to an actin-poor zone in the center of the contact (280). My results show that LFA-1-ICAM-1 interactions occur throughout the interface. This observation suggests that the center of the Th2-APC interface is richer in filamentous actin than the Th1-APC interface. Further experimentation will be required to test this hypothesis.

Are multifocal IS formed by a unique process? Kupfer described a complex series of events during IS formation, with phosphorylated signaling molecules and CD45 moving in and out of the nascent cSMAC (110). More recently, it has been demonstrated that TCR microclusters form in the periphery and move towards the center of the interface where they form the cSMAC (131). However, TCR microclusters are also known to coalesce before reaching the center of the interface (132). Thus the TCR-pMHC foci seen in multifocal IS could be formed by microcluster coalescence just as the cSMAC of mature IS are. Cytoskeletal differences (such as the lack of an F-actin gradient as described above) might prevent the eventual deposition of these foci at the center of the interface. In turn these cytoskeletal differences could be explained by differences in TCR proximal signaling, as discussed in Chapter 2. TIRFM imaging of Th2

cells forming IS with supported planar bilayers could be used to test the hypothesis that the foci in multifocal IS are formed from coalescing microclusters.

Are bullseye IS the exception rather than the rule for CD4 cells?

In addition to Th2 cells, multifocal IS are formed by DP and CD4 SP thymocytes and naïve CD4 T cells (212-214). Multifocal IS have also been described for T-DC interactions (208). Significantly, multifocal IS have been observed in conjugates between T cell blasts and DCs, indicating that T cells capable of forming bullseye IS on supported planar bilayers or with B cells do form multifocal IS in some situations (210).

T cells also form kinapses following antigen recognition in many circumstances. In vivo imaging experiments have shown that CD4 T cell priming by DCs in lymph nodes is characterized by stages of varying T cell motility (39, 42, 281). Upon antigen recognition and before beginning to proliferate, CD4 T cells form short interactions followed by a stage of relatively stable T-DC contacts and finally motile, swarming interactions. The T cells retain a degree of motility throughout this entire process. A recent report made progress in explaining these results by showing that CD4 T cells alternate between motile and non-motile phases (with a periodicity of approximately 30 min) when undergoing activation (102). The results presented in Chapter 3 show that iTregs form kinapses unless costimulatory requirements are met. These observations are consistent with in vitro and in vivo results showing that nTregs are highly motile when interacting with dendritic cells (266, 268). Furthermore, I show in Chapter 3 that Th1 cells transition between stable IS and kinapses when interacting with

supported planar bilayers containing pMHC and ICAM-1 (Fig. 3-7). To my knowledge, this is the first report of activated Th cells forming kinapses. As shown in Chapter 2, the stable IS formed by Th1 cells tend to have well defined SMACs. Additional experiments are needed to determine if CD80 encourages increased formation of stable IS by activated Th cells.

The structure of the mature IS was determined using $CD4^+$ cells, and most studies examining the structure and function of the IS continue to use activated CD4 T cells in fixed conjugates with B cells. I posit that the particular cells and systems used in these studies, while providing key insights into the formation and structure of the mature IS, have overstated its prevalence. Instead, the studies described in the preceding paragraphs combined with the data presented in this thesis suggest that the mature IS is a specialized structure. There is solid evidence indicating that T cell selection in the thymus and activation of naïve CD4 T cells by DCs both occur without the formation of mature IS. It has also been shown that mature IS with a clearly defined cSMAC are not formed during activation of naïve CD8 T cells (282). Thus, mature IS formation does not appear to be necessary for the initiation of an immune response.

What types of cells do reliably form mature IS? The literature points to both $CD8^+$ CTL and NK cells predominantly forming mature IS (180, 194). I show here that a large majority of Th1 cells interacting with either supported planar bilayers or fibroblast APC form IS with clearly defined SMACs. Additionally, one report has showed that Tregs form bullseye IS (269), in agreement with data shown here in Chapter 3. Therefore, a disparate, but select group of effector cells form mature IS.

What is the function of the mature IS?

There is convincing data showing that the cSMAC is the site of TCR downmodulation (132, 171). Although the presence of LBPA at the cSMAC indicates that TCR internalization occurs at this location, it does not prove the necessity of the cSMAC for these processes. Additionally, it has been proposed that the cSMAC allows for similar responses to a range of antigen densities by balancing TCR signaling and degradation (175). Although I suggest in Chapter 2 that the absence of a cSMAC in Th2 IS could allow Th2 cells to make responses in proportion to antigen density, the large accumulations of TCR-pMHC that make up the foci in multifocal IS could also serve the functions described above. If TCR microclusters coalesce to form the foci in multifocal IS, which then serve as sites of TCR downmodulation, the collection of foci could be considered functionally equivalent to a cSMAC.

The idea that one of the functions of the mature IS is directed secretion of effector molecules towards target cells is well established (181). All of the cells listed above that predominantly form mature IS, including Th1 cells and Tregs (in the presence of high CD80/86) contain granzymes and are capable of killing target cells (29, 283-285). A recent paper showed that preventing pSMAC formation by anti-LFA-1 treatment results in a significant reduction in maximal specific lysis by $CD4^+$ and $CD8^+$ CTL (233). The pSMACs formed by $CD4^+$ CTL are less stable than for $CD8^+$ CTL, and $CD4^+$ CTL are less efficient killers (233). When mature IS formation was enhanced in CD4+ CTL via treatment with a PKC-θ inhibitor, they became more efficient killers (233). Thus pSMAC formation is critical for efficient killing of target cells. In these circumstances, the

pSMAC may act as a gasket, with adhesion molecules attached to the T cell cytoskeleton forming a seal between the two cells (43).

In addition to delivery of cytolytic granules, I propose that the pSMAC is also crucial for the targeted delivery of other effector molecules, including FasL, CD40L and CTLA-4. FasL is stored intracellularly in secretory lysosomes in CD4 and CD8 T cells and is delivered to the cell surface in response to a signal through the TCR (201). Preformed CD40L has recently been shown to reside in a similar compartment and is rapidly mobilized to the cell surface upon antigen recognition (202). CTLA-4 is also located in an intracellular compartment and is polarized towards the APC during IS formation (168). Targeted delivery of these molecules to cells displaying antigen may be of critical importance for preventing bystander death and activation. Indiscriminate killing with granzymes and perforin or FasL could result in excess tissue damage, while CD40L-mediated activation of B cells or DCs in an antigen non-specific manner could result in autoimmunity. Targeted delivery of CTLA-4 towards DCs by Tregs might be important for preventing downmodulation of costimulatory molecules on bystander cells presenting foreign antigen during an immune response. The formation of mature IS by Tregs in the face of high CD80/86 levels may be important for this function.

Further experiments are needed to visualize the delivery of effector molecules towards APCs, and to measure the role of mature IS formation in the avoidance of bystander effects. Experiments utilizing CD4 T cells transduced with CTLA-4, FasL and CD40L-GFP fusion proteins will be useful for determining whether intracellular stores of these proteins are secreted through

the pSMAC after IS formation. TCR transgenic CD4 T cells deficient for WASp or PKC-θ, which are defective for immunological synapse and kinapse formation respectively, could be utilized to examine the importance of the formation of a symmetrical IS in the delivery of effector functions without bystander effects.

References

1. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J Exp Med* 157:1149-1169.
2. Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* 308:149-153.
3. Malissen, M., K. Minard, S. Mjolsness, M. Kronenberg, J. Goverman, T. Hunkapiller, M. B. Prystowsky, Y. Yoshikai, F. Fitch, T. W. Mak, and et al. 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell* 37:1101-1110.
4. Yanagi, Y., Y. Yoshikai, K. Leggett, S. P. Clark, I. Aleksander, and T. W. Mak. 1984. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature* 308:145-149.
5. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21:139-176.
6. Weiss, A., and J. D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med* 160:1284-1299.
7. Irving, B. A., and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell* 64:891-901.

8. June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc Natl Acad Sci U S A* 87:7722-7726.

9. Reth, M. 1989. Antigen receptor tail clue. *Nature* 338:383-384.

10. Wegener, A. M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell* 68:83-95.

11. Barber, E. K., J. D. Dasgupta, S. F. Schlossman, J. M. Trevillyan, and C. E. Rudd. 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc Natl Acad Sci U S A* 86:3277-3281.

12. Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc Natl Acad Sci U S A* 85:5190-5194.

13. Straus, D. B., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* 70:585-593.

14. Sieh, M., J. B. Bolen, and A. Weiss. 1993. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. *Embo J* 12:315-321.

15. Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71:649-662.

16. Jackman, J. K., D. G. Motto, Q. Sun, M. Tanemoto, C. W. Turck, G. A. Peltz, G. A. Koretzky, and P. R. Findell. 1995. Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J Biol Chem* 270:7029-7032.

17. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Trible, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92:83-92.

18. Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T Cell Activation. *Annu Rev Immunol* 27:591-619.

19. Schaeffer, E. M., J. Debnath, G. Yap, D. McVicar, X. C. Liao, D. R. Littman, A. Sher, H. E. Varmus, M. J. Lenardo, and P. L. Schwartzberg. 1999. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. *Science* 284:638-641.

20. Qi, Q., and A. August. 2007. Keeping the (kinase) party going: SLP-76 and ITK dance to the beat. *Sci STKE* 2007:pe39.

21. Hayashi, K., and A. Altman. 2007. Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacol Res* 55:537-544.

22. Roose, J. P., M. Mollenauer, V. A. Gupta, J. Stone, and A. Weiss. 2005. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol Cell Biol* 25:4426-4441.

23. Matsumoto, R., D. Wang, M. Blonska, H. Li, M. Kobayashi, B. Pappu, Y. Chen, and X. Lin. 2005. Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation. *Immunity* 23:575-585.

24. Thome, M. 2004. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol* 4:348-359.

25. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 369:327-329.

26. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112:1557-1569.

27. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787-793.

28. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441:890-893.

29. Brown, D. M., C. Kamperschroer, A. M. Dilzer, D. M. Roberts, and S. L. Swain. 2009. IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4+ T cells. *Cell Immunol* 257:69-79.

30. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat Rev Immunol* 2:933-944.

31. Le Gros, G., S. Z. Ben-Sasson, R. Seder, F. D. Finkelman, and W. E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in

vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med* 172:921-929.

32. Snapper, C. M., F. D. Finkelman, and W. E. Paul. 1988. Regulation of IgG1 and IgE production by interleukin 4. *Immunol Rev* 102:51-75.

33. Finkelman, F. D., T. A. Wynn, D. D. Donaldson, and J. F. Urban. 1999. The role of IL-13 in helminth-induced inflammation and protective immunity against nematode infections. *Curr Opin Immunol* 11:420-426.

34. Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317:256-260.

35. Horwitz, D. A., S. G. Zheng, and J. D. Gray. 2008. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol* 29:429-435.

36. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287-296.

37. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.

38. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322:271-275.

39. Mempel, T. R., S. E. Henrickson, and U. H. Von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427:154-159.

40. Skokos, D., G. Shakhar, R. Varma, J. C. Waite, T. O. Cameron, R. L. Lindquist, T. Schwickert, M. C. Nussenzweig, and M. L. Dustin. 2007. Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. *Nat Immunol* 8:835-844.

41. Stoll, S., J. Delon, T. M. Brotz, and R. N. Germain. 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296:1873-1876.

42. Miller, M. J., S. H. Wei, I. Parker, and M. D. Cahalan. 2002. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 296:1869-1873.

43. Dustin, M. L. 2008. T-cell activation through immunological synapses and kinapses. *Immunol Rev* 221:77-89.

44. Dustin, M. L. 2008. Hunter to gatherer and back: Immunological synapses and kinapses as variations on the theme of amoeboid locomotion. *Annu Rev Cell Dev Biol* 24:577-596.

45. Kaizuka, Y., A. D. Douglass, R. Varma, M. L. Dustin, and R. D. Vale. 2007. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci U S A* 104:20296-20301.

46. Krummel, M. F., and I. Macara. 2006. Maintenance and modulation of T cell polarity. *Nat Immunol* 7:1143-1149.

47. Ludford-Menting, M. J., J. Oliaro, F. Sacirbegovic, E. T. Cheah, N. Pedersen, S. J. Thomas, A. Pasam, R. Iazzolino, L. E. Dow, N. J. Waterhouse, A. Murphy, S. Ellis, M. J. Smyth, M. H. Kershaw, P. K. Darcy,

P. O. Humbert, and S. M. Russell. 2005. A network of PDZ-containing proteins regulates T cell polarity and morphology during migration and immunological synapse formation. *Immunity* 22:737-748.

48. Lee, J. H., T. Katakai, T. Hara, H. Gonda, M. Sugai, and A. Shimizu. 2004. Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. *J Cell Biol* 167:327-337.

49. del Pozo, M. A., M. Vicente-Manzanares, R. Tejedor, J. M. Serrador, and F. Sanchez-Madrid. 1999. Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. *Eur J Immunol* 29:3609-3620.

50. Smith, A., Y. R. Carrasco, P. Stanley, N. Kieffer, F. D. Batista, and N. Hogg. 2005. A talin-dependent LFA-1 focal zone is formed by rapidly migrating T lymphocytes. *J Cell Biol* 170:141-151.

51. Smith, A., M. Bracke, B. Leitinger, J. C. Porter, and N. Hogg. 2003. LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. *J Cell Sci* 116:3123-3133.

52. Jacobelli, J., S. A. Chmura, D. B. Buxton, M. M. Davis, and M. F. Krummel. 2004. A single class II myosin modulates T cell motility and stopping, but not synapse formation. *Nat Immunol* 5:531-538.

53. Savage, N. D., S. L. Kimzey, S. K. Bromley, K. G. Johnson, M. L. Dustin, and J. M. Green. 2002. Polar redistribution of the sialoglycoprotein CD43: implications for T cell function. *J Immunol* 168:3740-3746.

54. Donnadieu, E., G. Bismuth, and A. Trautmann. 1994. Antigen recognition by helper T cells elicits a sequence of distinct changes of their shape and intracellular calcium. *Curr Biol* 4:584-595.

55. Donnadieu, E., D. Cefai, Y. P. Tan, G. Paresys, G. Bismuth, and A. Trautmann. 1992. Imaging early steps of human T cell activation by antigen-presenting cells. *J Immunol* 148:2643-2653.

56. Delon, J., N. Bercovici, R. Liblau, and A. Trautmann. 1998. Imaging antigen recognition by naive CD4+ T cells: compulsory cytoskeletal alterations for the triggering of an intracellular calcium response. *Eur J Immunol* 28:716-729.

57. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med* 181:577-584.

58. Negulescu, P. A., T. B. Krasieva, A. Khan, H. H. Kerschbaum, and M. D. Cahalan. 1996. Polarity of T cell shape, motility, and sensitivity to antigen. *Immunity* 4:421-430.

59. Imboden, J. B., A. Weiss, and J. D. Stobo. 1985. The antigen receptor on a human T cell line initiates activation by increasing cytoplasmic free calcium. *J Immunol* 134:663-665.

60. Geiger, B., D. Rosen, and G. Berke. 1982. Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J Cell Biol* 95:137-143.

61. Kupfer, A., and G. Dennert. 1984. Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. *J Immunol* 133:2762-2766.

62. Kupfer, A., S. L. Swain, C. A. Janeway, Jr., and S. J. Singer. 1986. The specific direct interaction of helper T cells and antigen-presenting B cells. *Proc Natl Acad Sci U S A* 83:6080-6083.

63. Kupfer, H., C. R. Monks, and A. Kupfer. 1994. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. *J Exp Med* 179:1507-1515.

64. Kupfer, A., S. J. Singer, C. A. Janeway, Jr., and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. *Proc Natl Acad Sci U S A* 84:5888-5892.

65. Monks, C. R., H. Kupfer, I. Tamir, A. Barlow, and A. Kupfer. 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* 385:83-86.

66. Kupfer, A., and S. J. Singer. 1989. The specific interaction of helper T cells and antigen-presenting B cells. IV. Membrane and cytoskeletal reorganizations in the bound T cell as a function of antigen dose. *J Exp Med* 170:1697-1713.

67. Kupfer, A., S. L. Swain, and S. J. Singer. 1987. The specific direct interaction of helper T cells and antigen-presenting B cells. II.

Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. *J Exp Med* 165:1565-1580.

68. Davignon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer. 1981. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. *J Immunol* 127:590-595.

69. Krensky, A. M., F. Sanchez-Madrid, E. Robbins, J. A. Nagy, T. A. Springer, and S. J. Burakoff. 1983. The functional significance, distribution, and structure of LFA-1, LFA-2, and LFA-3: cell surface antigens associated with CTL-target interactions. *J Immunol* 131:611-616.

70. Sanchez-Madrid, F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer. 1982. Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proc Natl Acad Sci U S A* 79:7489-7493.

71. Dustin, M. L., and T. A. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341:619-624.

72. Makgoba, M. W., M. E. Sanders, G. E. Ginther Luce, M. L. Dustin, T. A. Springer, E. A. Clark, P. Mannoni, and S. Shaw. 1988. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature* 331:86-88.

73. Huang, Y., D. D. Norton, P. Precht, J. L. Martindale, J. K. Burkhardt, and R. L. Wange. 2005. Deficiency of ADAP/Fyb/SLAP-130 destabilizes SKAP55 in Jurkat T cells. *J Biol Chem* 280:23576-23583.

74. Wang, H., E. Y. Moon, A. Azouz, X. Wu, A. Smith, H. Schneider, N. Hogg, and C. E. Rudd. 2003. SKAP-55 regulates integrin adhesion and formation of T cell-APC conjugates. *Nat Immunol* 4:366-374.

75. Dustin, M. L., S. K. Bromley, Z. Kan, D. A. Peterson, and E. R. Unanue. 1997. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc Natl Acad Sci U S A* 94:3909-3913.

76. Dustin, M. L., D. E. Golan, D. M. Zhu, J. M. Miller, W. Meier, E. A. Davies, and P. A. van der Merwe. 1997. Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity. *J Biol Chem* 272:30889-30898.

77. Dustin, M. L., M. W. Olszowy, A. D. Holdorf, J. Li, S. Bromley, N. Desai, P. Widder, F. Rosenberger, P. A. van der Merwe, P. M. Allen, and A. S. Shaw. 1998. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* 94:667-677.

78. Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285:221-227.

79. Monks, C. R., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395:82-86.

80. Potter, T. A., K. Grebe, B. Freiberg, and A. Kupfer. 2001. Formation of supramolecular activation clusters on fresh ex vivo CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells. *Proc Natl Acad Sci U S A* 98:12624-12629.

81. Cullinan, P., A. I. Sperling, and J. K. Burkhardt. 2002. The distal pole complex: a novel membrane domain distal to the immunological synapse. *Immunol Rev* 189:111-122.

82. Allenspach, E. J., P. Cullinan, J. Tong, Q. Tang, A. G. Tesciuba, J. L. Cannon, S. M. Takahashi, R. Morgan, J. K. Burkhardt, and A. I. Sperling. 2001. ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. *Immunity* 15:739-750.

83. Sperling, A. I., J. R. Sedy, N. Manjunath, A. Kupfer, B. Ardman, and J. K. Burkhardt. 1998. TCR signaling induces selective exclusion of CD43 from the T cell-antigen-presenting cell contact site. *J Immunol* 161:6459-6462.

84. Roumier, A., J. C. Olivo-Marin, M. Arpin, F. Michel, M. Martin, P. Mangeat, O. Acuto, A. Dautry-Varsat, and A. Alcover. 2001. The membrane-microfilament linker ezrin is involved in the formation of the immunological synapse and in T cell activation. *Immunity* 15:715-728.

85. Faure, S., L. I. Salazar-Fontana, M. Semichon, V. L. Tybulewicz, G. Bismuth, A. Trautmann, R. N. Germain, and J. Delon. 2004. ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. *Nat Immunol* 5:272-279.

86. Delon, J., K. Kaibuchi, and R. N. Germain. 2001. Exclusion of CD43 from the immunological synapse is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. *Immunity* 15:691-701.

87. Round, J. L., T. Tomassian, M. Zhang, V. Patel, S. P. Schoenberger, and M. C. Miceli. 2005. Dlgh1 coordinates actin polymerization, synaptic T cell

receptor and lipid raft aggregation, and effector function in T cells. *J Exp Med* 201:419-430.

88. Eibert, S. M., K. H. Lee, R. Pipkorn, U. Sester, G. H. Wabnitz, T. Giese, S. C. Meuer, and Y. Samstag. 2004. Cofilin peptide homologs interfere with immunological synapse formation and T cell activation. *Proc Natl Acad Sci U S A* 101:1957-1962.

89. Bunnell, S. C., V. Kapoor, R. P. Trible, W. Zhang, and L. E. Samelson. 2001. Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* 14:315-329.

90. Barda-Saad, M., A. Braiman, R. Titerence, S. C. Bunnell, V. A. Barr, and L. E. Samelson. 2005. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat Immunol* 6:80-89.

91. Burkhardt, J. K., E. Carrizosa, and M. H. Shaffer. 2008. The actin cytoskeleton in T cell activation. *Annu Rev Immunol* 26:233-259.

92. Goley, E. D., and M. D. Welch. 2006. The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol* 7:713-726.

93. Kim, A. S., L. T. Kakalis, N. Abdul-Manan, G. A. Liu, and M. K. Rosen. 2000. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* 404:151-158.

94. Cannon, J. L., C. M. Labno, G. Bosco, A. Seth, M. H. McGavin, K. A. Siminovitch, M. K. Rosen, and J. K. Burkhardt. 2001. Wasp recruitment to the T cell:APC contact site occurs independently of Cdc42 activation. *Immunity* 15:249-259.

95. Labno, C. M., C. M. Lewis, D. You, D. W. Leung, A. Takesono, N. Kamberos, A. Seth, L. D. Finkelstein, M. K. Rosen, P. L. Schwartzberg, and J. K. Burkhardt. 2003. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr Biol* 13:1619-1624.

96. Dombroski, D., R. A. Houghtling, C. M. Labno, P. Precht, A. Takesono, N. J. Caplen, D. D. Billadeau, R. L. Wange, J. K. Burkhardt, and P. L. Schwartzberg. 2005. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol* 174:1385-1392.

97. Zeng, R., J. L. Cannon, R. T. Abraham, M. Way, D. D. Billadeau, J. Bubeck-Wardenberg, and J. K. Burkhardt. 2003. SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J Immunol* 171:1360-1368.

98. Zhang, J., A. Shehabeldin, L. A. da Cruz, J. Butler, A. K. Somani, M. McGavin, I. Kozieradzki, A. O. dos Santos, A. Nagy, S. Grinstein, J. M. Penninger, and K. A. Siminovitch. 1999. Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med* 190:1329-1342.

99. Badour, K., J. Zhang, F. Shi, M. K. McGavin, V. Rampersad, L. A. Hardy, D. Field, and K. A. Siminovitch. 2003. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity* 18:141-154.

100. Anton, I. M., M. A. de la Fuente, T. N. Sims, S. Freeman, N. Ramesh, J. H. Hartwig, M. L. Dustin, and R. S. Geha. 2002. WIP deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation. *Immunity* 16:193-204.

101. de la Fuente, M. A., Y. Sasahara, M. Calamito, I. M. Anton, A. Elkhal, M. D. Gallego, K. Suresh, K. Siminovitch, H. D. Ochs, K. C. Anderson, F. S. Rosen, R. S. Geha, and N. Ramesh. 2007. WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). *Proc Natl Acad Sci U S A* 104:926-931.

102. Sims, T. N., T. J. Soos, H. S. Xenias, B. Dubin-Thaler, J. M. Hofman, J. C. Waite, T. O. Cameron, V. K. Thomas, R. Varma, C. H. Wiggins, M. P. Sheetz, D. R. Littman, and M. L. Dustin. 2007. Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell* 129:773-785.

103. Cannon, J. L., and J. K. Burkhardt. 2004. Differential roles for Wiskott-Aldrich syndrome protein in immune synapse formation and IL-2 production. *J Immunol* 173:1658-1662.

104. Nolz, J. C., T. S. Gomez, P. Zhu, S. Li, R. B. Medeiros, Y. Shimizu, J. K. Burkhardt, B. D. Freedman, and D. D. Billadeau. 2006. The WAVE2 complex regulates actin cytoskeletal reorganization and CRAC-mediated calcium entry during T cell activation. *Curr Biol* 16:24-34.

105. Zipfel, P. A., S. C. Bunnell, D. S. Witherow, J. J. Gu, E. M. Chislock, C. Ring, and A. M. Pendergast. 2006. Role for the Abi/wave protein complex in T cell receptor-mediated proliferation and cytoskeletal remodeling. *Curr Biol* 16:35-46.

106. Stradal, T. E., K. Rottner, A. Disanza, S. Confalonieri, M. Innocenti, and G. Scita. 2004. Regulation of actin dynamics by WASP and WAVE family proteins. *Trends Cell Biol* 14:303-311.

107. Nolz, J. C., R. B. Medeiros, J. S. Mitchell, P. Zhu, B. D. Freedman, Y. Shimizu, and D. D. Billadeau. 2007. WAVE2 regulates high-affinity integrin binding by recruiting vinculin and talin to the immunological synapse. *Mol Cell Biol* 27:5986-6000.

108. Gomez, T. S., S. D. McCarney, E. Carrizosa, C. M. Labno, E. O. Comiskey, J. C. Nolz, P. Zhu, B. D. Freedman, M. R. Clark, D. J. Rawlings, D. D. Billadeau, and J. K. Burkhardt. 2006. HS1 functions as an essential actin-regulatory adaptor protein at the immune synapse. *Immunity* 24:741-752.

109. Uruno, T., P. Zhang, J. Liu, J. J. Hao, and X. Zhan. 2003. Haematopoietic lineage cell-specific protein 1 (HS1) promotes actin-related protein (Arp) 2/3 complex-mediated actin polymerization. *Biochem J* 371:485-493.

110. Freiberg, B. A., H. Kupfer, W. Maslanik, J. Delli, J. Kappler, D. M. Zaller, and A. Kupfer. 2002. Staging and resetting T cell activation in SMACs. *Nat Immunol* 3:911-917.

111. Leupin, O., R. Zaru, T. Laroche, S. Muller, and S. Valitutti. 2000. Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr Biol* 10:277-280.

112. Johnson, K. G., S. K. Bromley, M. L. Dustin, and M. L. Thomas. 2000. A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation. *Proc Natl Acad Sci U S A* 97:10138-10143.

113. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc Natl Acad Sci U S A* 86:8959-8963.

114. Pingel, J. T., and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* 58:1055-1065.

115. Trowbridge, I. S., and M. L. Thomas. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol* 12:85-116.

116. Hermiston, M. L., Z. Xu, and A. Weiss. 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* 21:107-137.

117. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55:301-308.

118. Zal, T., M. A. Zal, and N. R. Gascoigne. 2002. Inhibition of T cell receptor-coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. *Immunity* 16:521-534.

119. Balamuth, F., J. L. Brogdon, and K. Bottomly. 2004. CD4 raft association and signaling regulate molecular clustering at the immunological synapse site. *J Immunol* 172:5887-5892.

120. Irvine, D. J., M. A. Purbhoo, M. Krogsgaard, and M. M. Davis. 2002. Direct observation of ligand recognition by T cells. *Nature* 419:845-849.

121. Krogsgaard, M., Q. J. Li, C. Sumen, J. B. Huppa, M. Huse, and M. M. Davis. 2005. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature* 434:238-243.

122. Li, Q. J., A. R. Dinner, S. Qi, D. J. Irvine, J. B. Huppa, M. M. Davis, and A. K. Chakraborty. 2004. CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. *Nat Immunol* 5:791-799.

123. Wülfing, C., C. Sumen, M. D. Sjaastad, L. C. Wu, M. L. Dustin, and M. M. Davis. 2002. Costimulation and endogenous MHC ligands contribute to T cell recognition. *Nat Immunol* 3:42-47.

124. Wang, J. H., R. Meijers, Y. Xiong, J. H. Liu, T. Sakihama, R. Zhang, A. Joachimiak, and E. L. Reinherz. 2001. Crystal structure of the human CD4 N-terminal two-domain fragment complexed to a class II MHC molecule. *Proc Natl Acad Sci U S A* 98:10799-10804.

125. Boniface, J. J., J. D. Rabinowitz, C. Wulfing, J. Hampl, Z. Reich, J. D. Altman, R. M. Kantor, C. Beeson, H. M. McConnell, and M. M. Davis. 1998. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands [corrected]. *Immunity* 9:459-466.

126. Krummel, M. F., M. D. Sjaastad, C. Wülfing, and M. M. Davis. 2000. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* 289:1349-1352.

127. Ehrlich, L. I., P. J. Ebert, M. F. Krummel, A. Weiss, and M. M. Davis. 2002. Dynamics of p56lck translocation to the T cell immunological

synapse following agonist and antagonist stimulation. *Immunity* 17:809-822.

128. Lee, K. H., A. D. Holdorf, M. L. Dustin, A. C. Chan, P. M. Allen, and A. S. Shaw. 2002. T cell receptor signaling precedes immunological synapse formation. *Science* 295:1539-1542.

129. Huppa, J. B., M. Gleimer, C. Sumen, and M. M. Davis. 2003. Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nat Immunol* 4:749-755.

130. Bunnell, S. C., D. I. Hong, J. R. Kardon, T. Yamazaki, C. J. McGlade, V. A. Barr, and L. E. Samelson. 2002. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* 158:1263-1275.

131. Yokosuka, T., K. Sakata-Sogawa, W. Kobayashi, M. Hiroshima, A. Hashimoto-Tane, M. Tokunaga, M. L. Dustin, and T. Saito. 2005. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol* 6: 1253 - 1262.

132. Varma, R., G. Campi, T. Yokosuka, T. Saito, and M. L. Dustin. 2006. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* 25:117-127.

133. Saito, T., and T. Yokosuka. 2006. Immunological synapse and microclusters: the site for recognition and activation of T cells. *Curr Opin Immunol* 18:305-313.

134. Seminario, M. C., and S. C. Bunnell. 2008. Signal initiation in T-cell receptor microclusters. *Immunol Rev* 221:90-106.

135. Campi, G., R. Varma, and M. L. Dustin. 2005. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med* 202:1031-1036.

136. Mossman, K. D., G. Campi, J. T. Groves, and M. L. Dustin. 2005. Altered TCR signaling from geometrically repatterned immunological synapses. *Science* 310:1191-1193.

137. DeMond, A. L., K. D. Mossman, T. Starr, M. L. Dustin, and J. T. Groves. 2008. T cell receptor microcluster transport through molecular mazes reveals mechanism of translocation. *Biophys J* 94:3286-3292.

138. Moss, W. C., D. J. Irvine, M. M. Davis, and M. F. Krummel. 2002. Quantifying signaling-induced reorientation of T cell receptors during immunological synapse formation. *Proc Natl Acad Sci U S A* 99:15024-15029.

139. Qi, S. Y., J. T. Groves, and A. K. Chakraborty. 2001. Synaptic pattern formation during cellular recognition. *Proc Natl Acad Sci U S A* 98:6548-6553.

140. Wulfing, C., and M. M. Davis. 1998. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* 282:2266-2269.

141. Ilani, T., G. Vasiliver-Shamis, S. Vardhana, A. Bretscher, and M. L. Dustin. 2009. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. *Nat Immunol* 10:531-539.

142. Bromley, S. K., A. Iaboni, S. J. Davis, A. Whitty, J. M. Green, A. S. Shaw, A. Weiss, and M. L. Dustin. 2001. The immunological synapse and CD28-CD80 interactions. *Nat Immunol* 2:1159-1166.

143. Sanchez-Lockhart, M., B. Graf, and J. Miller. 2008. Signals and sequences that control CD28 localization to the central region of the immunological synapse. *J Immunol* 181:7639-7648.

144. Sadra, A., T. Cinek, J. L. Arellano, J. Shi, K. E. Truitt, and J. B. Imboden. 1999. Identification of tyrosine phosphorylation sites in the CD28 cytoplasmic domain and their role in the costimulation of Jurkat T cells. *J Immunol* 162:1966-1973.

145. Andres, P. G., K. C. Howland, D. Dresnek, S. Edmondson, A. K. Abbas, and M. F. Krummel. 2004. CD28 signals in the immature immunological synapse. *J Immunol* 172:5880-5886.

146. Wetzel, S. A., T. W. McKeithan, and D. C. Parker. 2002. Live cell dynamics and the role of costimulation in immunological synapse formation. *J Immunol* 169:6092-6101.

147. Tskvitaria-Fuller, I., A. L. Rozelle, H. L. Yin, and C. Wulfing. 2003. Regulation of sustained actin dynamics by the TCR and costimulation as a mechanism of receptor localization. *J Immunol* 171:2287-2295.

148. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed. 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity* 8:723-732.

149. Kovacs, B., R. V. Parry, Z. Ma, E. Fan, D. K. Shivers, B. A. Freiberg, A. K. Thomas, R. Rutherford, C. A. Rumbley, J. L. Riley, and T. H. Finkel. 2005. Ligation of CD28 by its natural ligand CD86 in the absence of TCR stimulation induces lipid raft polarization in human CD4 T cells. *J Immunol* 175:7848-7854.

150. Sadra, A., T. Cinek, and J. B. Imboden. 2004. Translocation of CD28 to lipid rafts and costimulation of IL-2. *Proc Natl Acad Sci U S A* 101:11422-11427.

151. Tavano, R., R. L. Contento, S. J. Baranda, M. Soligo, L. Tuosto, S. Manes, and A. Viola. 2006. CD28 interaction with filamin-A controls lipid raft accumulation at the T-cell immunological synapse. *Nat Cell Biol* 8:1270-1276.

152. Tavano, R., G. Gri, B. Molon, B. Marinari, C. E. Rudd, L. Tuosto, and A. Viola. 2004. CD28 and lipid rafts coordinate recruitment of Lck to the immunological synapse of human T lymphocytes. *J Immunol* 173:5392-5397.

153. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283:680-682.

154. Burack, W. R., K. H. Lee, A. D. Holdorf, M. L. Dustin, and A. S. Shaw. 2002. Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J Immunol* 169:2837-2841.

155. Shaw, A. S. 2006. Lipid rafts: now you see them, now you don't. *Nat Immunol* 7:1139-1142.

156. Holdorf, A. D., K. H. Lee, W. R. Burack, P. M. Allen, and A. S. Shaw. 2002. Regulation of Lck activity by CD4 and CD28 in the immunological synapse. *Nat Immunol* 3:259-264.

157. Huang, J., P. F. Lo, T. Zal, N. R. Gascoigne, B. A. Smith, S. D. Levin, and H. M. Grey. 2002. CD28 plays a critical role in the segregation of PKC theta within the immunologic synapse. *Proc Natl Acad Sci U S A* 99:9369-9373.

158. Yokosuka, T., W. Kobayashi, K. Sakata-Sogawa, M. Takamatsu, A. Hashimoto-Tane, M. L. Dustin, M. Tokunaga, and T. Saito. 2008. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity* 29:589-601.

159. Sanchez-Lockhart, M., E. Marin, B. Graf, R. Abe, Y. Harada, C. E. Sedwick, and J. Miller. 2004. Cutting edge: CD28-mediated transcriptional and posttranscriptional regulation of IL-2 expression are controlled through different signaling pathways. *J Immunol* 173:7120-7124.

160. Praveen, K., Y. Zheng, F. Rivas, and T. F. Gajewski. 2009. Protein kinase Ctheta focusing at the cSMAC is a consequence rather than cause of TCR

signaling and is dependent on the MEK/ERK pathway. *J Immunol* 182:6022-6030.

161. Rudd, C. E., and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol* 3:544-556.

162. Costello, P. S., M. Gallagher, and D. A. Cantrell. 2002. Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nat Immunol* 3:1082-1089.

163. Fabre, S., V. Lang, J. Harriague, A. Jobart, T. G. Unterman, A. Trautmann, and G. Bismuth. 2005. Stable activation of phosphatidylinositol 3-kinase in the T cell immunological synapse stimulates Akt signaling to FoxO1 nuclear exclusion and cell growth control. *J Immunol* 174:4161-4171.

164. Harriague, J., and G. Bismuth. 2002. Imaging antigen-induced PI3K activation in T cells. *Nat Immunol* 3:1090-1096.

165. Park, S. G., J. Schulze-Luehrman, M. S. Hayden, N. Hashimoto, W. Ogawa, M. Kasuga, and S. Ghosh. 2009. The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-kappaB and activate T cells. *Nat Immunol* 10:158-166.

166. Spitaler, M., E. Emslie, C. D. Wood, and D. Cantrell. 2006. Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity* 24:535-546.

167. Sanchez-Lockhart, M., and J. Miller. 2006. Engagement of CD28 Outside of the Immunological Synapse Results in Up-Regulation of IL-2 mRNA Stability but Not IL-2 Transcription. *J Immunol* 176:4778-4784.

168. Egen, J. G., and J. P. Allison. 2002. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* 16:23-35.

169. Collins, A. V., D. W. Brodie, R. J. Gilbert, A. Iaboni, R. Manso-Sancho, B. Walse, D. I. Stuart, P. A. van der Merwe, and S. J. Davis. 2002. The interaction properties of costimulatory molecules revisited. *Immunity* 17:201-210.

170. Pentcheva-Hoang, T., J. G. Egen, K. Wojnoonski, and J. P. Allison. 2004. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 21:401-413.

171. Lee, K. H., A. R. Dinner, C. Tu, G. Campi, S. Raychaudhuri, R. Varma, T. N. Sims, W. R. Burack, H. Wu, J. Wang, O. Kanagawa, M. Markiewicz, P. M. Allen, M. L. Dustin, A. K. Chakraborty, and A. S. Shaw. 2003. The immunological synapse balances T cell receptor signaling and degradation. *Science* 302:1218.

172. Alcover, A., and B. Alarcon. 2000. Internalization and intracellular fate of TCR-CD3 complexes. *Crit Rev Immunol* 20:325-346.

173. Das, V., B. Nal, A. Dujeancourt, M. I. Thoulouze, T. Galli, P. Roux, A. Dautry-Varsat, and A. Alcover. 2004. Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. *Immunity* 20:577-588.

174. Cemerski, S., J. Das, J. Locasale, P. Arnold, E. Giurisato, M. A. Markiewicz, D. Fremont, P. M. Allen, A. K. Chakraborty, and A. S. Shaw.

2007. The stimulatory potency of T cell antigens is influenced by the formation of the immunological synapse. *Immunity* 26:345-355.

175. Cemerski, S., J. Das, E. Giurisato, M. A. Markiewicz, P. M. Allen, A. K. Chakraborty, and A. S. Shaw. 2008. The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* 29:414-422.

176. Maldonado, R. A., D. J. Irvine, R. Schreiber, and L. H. Glimcher. 2004. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature* 431:527-532.

177. Maldonado, R. A., M. A. Soriano, L. C. Perdomo, K. Sigrist, D. J. Irvine, T. Decker, and L. H. Glimcher. 2009. Control of T helper cell differentiation through cytokine receptor inclusion in the immunological synapse. *J Exp Med* 206:877-892.

178. Poo, W. J., L. Conrad, and C. A. Janeway, Jr. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature* 332:378-380.

179. Bossi, G., and G. M. Griffiths. 2005. CTL secretory lysosomes: biogenesis and secretion of a harmful organelle. *Semin Immunol* 17:87-94.

180. Bossi, G., C. Trambas, S. Booth, R. Clark, J. Stinchcombe, and G. M. Griffiths. 2002. The secretory synapse: the secrets of a serial killer. *Immunol Rev* 189:152-160.

181. Huse, M., E. J. Quann, and M. M. Davis. 2008. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nat Immunol* 9:1105-1111.

182. Stinchcombe, J. C., and G. M. Griffiths. 2007. Secretory mechanisms in cell-mediated cytotoxicity. *Annu Rev Cell Dev Biol* 23:495-517.

183. Stinchcombe, J. C., G. Bossi, S. Booth, and G. M. Griffiths. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15:751-761.

184. Wetzel, S. A., T. W. McKeithan, and D. C. Parker. 2005. Peptide-specific intercellular transfer of MHC class II to CD4+ T cells directly from the immunological synapse upon cellular dissociation. *J Immunol* 174:80-89.

185. Hudrisier, D., A. Aucher, A. L. Puaux, C. Bordier, and E. Joly. 2007. Capture of target cell membrane components via trogocytosis is triggered by a selected set of surface molecules on T or B cells. *J Immunol* 178:3637-3647.

186. Stinchcombe, J. C., E. Majorovits, G. Bossi, S. Fuller, and G. M. Griffiths. 2006. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* 443:462-465.

187. Kuhn, J. R., and M. Poenie. 2002. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity* 16:111-121.

188. Krause, M., A. S. Sechi, M. Konradt, D. Monner, F. B. Gertler, and J. Wehland. 2000. Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. *J Cell Biol* 149:181-194.

189. Combs, J., S. J. Kim, S. Tan, L. A. Ligon, E. L. Holzbaur, J. Kuhn, and M. Poenie. 2006. Recruitment of dynein to the Jurkat immunological synapse. *Proc Natl Acad Sci U S A* 103:14883-14888.

190. Shen, A., L. G. Puente, and H. L. Ostergaard. 2005. Tyrosine kinase activity and remodelling of the actin cytoskeleton are co-temporally required for degranulation by cytotoxic T lymphocytes. *Immunology* 116:276-286.

191. Quann, E. J., E. Merino, T. Furuta, and M. Huse. 2009. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* 10:627-635.

192. Anikeeva, N., K. Somersalo, T. N. Sims, V. K. Thomas, M. L. Dustin, and Y. Sykulev. 2005. Distinct role of lymphocyte function-associated antigen-1 in mediating effective cytolytic activity by cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* 102:6437-6442.

193. Somersalo, K., N. Anikeeva, T. N. Sims, V. K. Thomas, R. K. Strong, T. Spies, T. Lebedeva, Y. Sykulev, and M. L. Dustin. 2004. Cytotoxic T lymphocytes form an antigen-independent ring junction. *J Clin Invest* 113:49-57.

194. Krzewski, K., and J. L. Strominger. 2008. The killer's kiss: the many functions of NK cell immunological synapses. *Curr Opin Cell Biol* 20:597-605.

195. O'Keefe, J. P., and T. F. Gajewski. 2005. Cutting edge: cytotoxic granule polarization and cytolysis can occur without central supramolecular activation cluster formation in CD8+ effector T cells. *J Immunol* 175:5581-5585.

196. Faroudi, M., C. Utzny, M. Salio, V. Cerundolo, M. Guiraud, S. Muller, and S. Valitutti. 2003. Lytic versus stimulatory synapse in cytotoxic T

lymphocyte/target cell interaction: Manifestation of a dual activation threshold. *Proc Natl Acad Sci U S A* 100:14145-14150.

197. Wiedemann, A., D. Depoil, M. Faroudi, and S. Valitutti. 2006. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. *Proc Natl Acad Sci U S A* 103:10985-10990.

198. Purbhoo, M. A., D. J. Irvine, J. B. Huppa, and M. M. Davis. 2004. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol* 5:524-530.

199. Kupfer, A., T. R. Mosmann, and H. Kupfer. 1991. Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. *Proc Natl Acad Sci U S A* 88:775-779.

200. Huse, M., B. F. Lillemeier, M. S. Kuhns, D. S. Chen, and M. M. Davis. 2006. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* 7:247-255.

201. Bossi, G., and G. M. Griffiths. 1999. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med* 5:90-96.

202. Koguchi, Y., T. J. Thauland, M. K. Slifka, and D. C. Parker. 2007. Preformed CD40 ligand exists in secretory lysosomes in effector and memory CD4+ T cells and is quickly expressed on the cell surface in an antigen-specific manner. *Blood* 110:2520-2527.

203. Boisvert, J., S. Edmondson, and M. F. Krummel. 2004. Immunological synapse formation licenses CD40-CD40L accumulations at T-APC contact sites. *J Immunol* 173:3647-3652.

204. Barcia, C., A. Gomez, V. de Pablos, E. Fernandez-Villalba, C. Liu, K. M. Kroeger, J. Martin, A. F. Barreiro, M. G. Castro, P. R. Lowenstein, and M. T. Herrero. 2008. CD20, CD3 and CD40L microclusters segregate three-dimensionally in vivo at B Cell-T Cell immunological synapses after viral immunity in primate brain. *J Virol* 82:9978-9993.

205. Revy, P., M. Sospedra, B. Barbour, and A. Trautmann. 2001. Functional antigen-independent synapses formed between T cells and dendritic cells. *Nat Immunol* 2:925-931.

206. Al-Alwan, M. M., G. Rowden, T. D. Lee, and K. A. West. 2001. The dendritic cell cytoskeleton is critical for the formation of the immunological synapse. *J Immunol* 166:1452-1456.

207. Al-Alwan, M. M., R. S. Liwski, S. M. Haeryfar, W. H. Baldridge, D. W. Hoskin, G. Rowden, and K. A. West. 2003. Cutting edge: dendritic cell actin cytoskeletal polarization during immunological synapse formation is highly antigen-dependent. *J Immunol* 171:4479-4483.

208. Brossard, C., V. Feuillet, A. Schmitt, C. Randriamampita, M. Romao, G. Raposo, and A. Trautmann. 2005. Multifocal structure of the T cell - dendritic cell synapse. *Eur J Immunol* 35:1741-1753.

209. Fisher, P. J., P. A. Bulur, S. Vuk-Pavlovic, F. G. Prendergast, and A. B. Dietz. 2008. Dendritic cell microvilli-a novel membrane structure

associated with multifocal synapse and T cell clustering. *Blood* 112:5037-5045.

210. Tseng, S. Y., J. C. Waite, M. Liu, S. Vardhana, and M. L. Dustin. 2008. T cell-dendritic cell immunological synapses contain TCR-dependent CD28-CD80 clusters that recruit protein kinase C theta. *J Immunol* 181:4852-4863.

211. Benvenuti, F., C. Lagaudriere-Gesbert, I. Grandjean, C. Jancic, C. Hivroz, A. Trautmann, O. Lantz, and S. Amigorena. 2004. Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. *J Immunol* 172:292-301.

212. Richie, L. I., P. J. Ebert, L. C. Wu, M. F. Krummel, J. J. Owen, and M. M. Davis. 2002. Imaging synapse formation during thymocyte selection: inability of CD3zeta to form a stable central accumulation during negative selection. *Immunity* 16:595-606.

213. Hailman, E., W. R. Burack, A. S. Shaw, M. L. Dustin, and P. M. Allen. 2002. Immature CD4(+)CD8(+) thymocytes form a multifocal immunological synapse with sustained tyrosine phosphorylation. *Immunity* 16:839-848.

214. Tseng, S. Y., M. Liu, and M. L. Dustin. 2005. CD80 cytoplasmic domain controls localization of CD28, CTLA-4, and protein kinase C-θ in the immunological synapse. *J Immunol* 175:7829-7836.

215. Zambricki, E., T. Zal, P. Yachi, A. Shigeoka, J. Sprent, N. Gascoigne, and D. McKay. 2006. In vivo anergized T cells form altered immunological synapses in vitro. *Am J Transplant* 6:2572-2579.

216. Balamuth, F., D. Leitenberg, J. Unternaehrer, I. Mellman, and K. Bottomly. 2001. Distinct patterns of membrane microdomain partitioning in Th1 and Th2 cells. *Immunity* 15:729-738.

217. Jackman, R. P., F. Balamuth, and K. Bottomly. 2007. CTLA-4 differentially regulates the immunological synapse in CD4 T cell subsets. *J Immunol* 178:5543-5551.

218. Huppa, J. B., and M. M. Davis. 2003. T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol* 3:973-983.

219. Stinchcombe, J. C., and G. M. Griffiths. 2003. The role of the secretory immunological synapse in killing by CD8+ CTL. *Semin Immunol* 15:301-305.

220. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* 182:1591-1596.

221. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275-283.

222. Thomas, M. L., and E. J. Brown. 1999. Positive and negative regulation of Src-family membrane kinases by CD45. *Immunol Today* 20:406-411.

223. Cahir McFarland, E. D., T. R. Hurley, J. T. Pingel, B. M. Sefton, A. Shaw, and M. L. Thomas. 1993. Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc Natl Acad Sci U S A* 90:1402-1406.

224. Graf, B., T. Bushnell, and J. Miller. 2007. LFA-1-mediated T cell costimulation through increased localization of TCR/class II complexes to the central supramolecular activation cluster and exclusion of CD45 from the immunological synapse. *J Immunol* 179:1616-1624.

225. Hsieh, C. S., S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J Exp Med* 181:713-721.

226. Okada, T., M. J. Miller, I. Parker, M. F. Krummel, M. Neighbors, S. B. Hartley, A. O'Garra, M. D. Cahalan, and J. G. Cyster. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol* 3:e150.

227. Hannier, S., C. Bitegye, and S. Demotz. 2002. Early events of TCR signaling are distinct in human Th1 and Th2 cells. *J Immunol* 169:1904-1911.

228. Tamura, T., H. Nakano, H. Nagase, T. Morokata, O. Igarashi, Y. Oshimi, S. Miyazaki, and H. Nariuchi. 1995. Early activation signal transduction pathways of Th1 and Th2 cell clones stimulated with anti-CD3. Roles of protein tyrosine kinases in the signal for IL-2 and IL-4 production. *J Immunol* 155:4692-4701.

229. Itoh, Y., Z. Wang, H. Ishida, K. Eichelberg, N. Fujimoto, J. Makino, K. Ogasawara, and R. N. Germain. 2005. Decreased CD4 expression by polarized T helper 2 cells contributes to suboptimal TCR-induced phosphorylation and reduced Ca2+ signaling. *Eur J Immunol* 35:3187-3195.

230. Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones, and A. D. Kelleher. 2002. Characterization of CD4(+) CTLs ex vivo. *J Immunol* 168:5954-5958.

231. Ju, S. T., H. Cui, D. J. Panka, R. Ettinger, and A. Marshak-Rothstein. 1994. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. *Proc Natl Acad Sci U S A* 91:4185-4189.

232. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J Immunol* 152:1127-1133.

233. Beal, A. M., N. Anikeeva, R. Varma, T. O. Cameron, P. J. Norris, M. L. Dustin, and Y. Sykulev. 2008. Protein kinase C theta regulates stability of the peripheral adhesion ring junction and contributes to the sensitivity of target cell lysis by CTL. *J Immunol* 181:4815-4824.

234. Belkaid, Y., and K. Tarbell. 2009. Regulatory T cells in the control of host-microorganism interactions. *Annu Rev Immunol* 27:551-589.

235. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.

236. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10:1969-1980.

237. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells. *Nat Immunol* 4:330-336.

238. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4:337-342.

239. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor foxp3. *Science* 299:1057-1061.

240. Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A. D. Lapan, J. C. Stroud, D. L. Bates, L. Guo, A. Han, S. F. Ziegler, D. Mathis, C. Benoist, L. Chen, and A. Rao. 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126:375-387.

241. Zheng, Y., S. Z. Josefowicz, A. Kas, T. T. Chu, M. A. Gavin, and A. Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445:936-940.

242. Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 21:267-277.

243. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.

244. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.

245. Zheng, S. G., J. Wang, P. Wang, J. D. Gray, and D. A. Horwitz. 2007. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J Immunol* 178:2018-2027.

246. Benson, M. J., K. Pino-Lagos, M. Rosemblatt, and R. J. Noelle. 2007. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 204:1765-1774.

247. Mucida, D., K. Pino-Lagos, G. Kim, E. Nowak, M. J. Benson, M. Kronenberg, R. J. Noelle, and H. Cheroutre. 2009. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* 30:471-472.

248. Xiao, S., H. Jin, T. Korn, S. M. Liu, M. Oukka, B. Lim, and V. K. Kuchroo. 2008. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* 181:2277-2284.

249. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204:1757-1764.

250. Sun, C. M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells

promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775-1785.

251. Reichardt, P., B. Dornbach, S. Rong, S. Beissert, F. Gueler, K. Loser, and M. Gunzer. 2007. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood* 110:1519-1529.

252. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol* 8:523-532.

253. Mucida, D., N. Kutchukhidze, A. Erazo, M. Russo, J. J. Lafaille, and M. A. Curotto de Lafaille. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest* 115:1923-1933.

254. Tang, Q., and J. A. Bluestone. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 9:239-244.

255. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 164:183-190.

256. Bopp, T., C. Becker, M. Klein, S. Klein-Hessling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M. S. Staege, M. Stassen, H. Jonuleit, and E. Schmitt. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204:1303-1310.

257. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194:629-644.

258. Rubtsov, Y. P., J. P. Rasmussen, E. Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W. R. Henderson, Jr., W. Muller, and A. Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.

259. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190:995-1004.

260. Fahlen, L., S. Read, L. Gorelik, S. D. Hurst, R. L. Coffman, R. A. Flavell, and F. Powrie. 2005. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 201:737-746.

261. Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450:566-569.

262. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M. L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4:1206-1212.

263. Cederbom, L., H. Hall, and F. Ivars. 2000. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 30:1538-1543.

264. Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting edge: human CD4+CD25+ T cells restrain the

maturation and antigen-presenting function of dendritic cells. *J Immunol* 172:4676-4680.

265. Oderup, C., L. Cederbom, A. Makowska, C. M. Cilio, and F. Ivars. 2006. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* 118:240-249.

266. Onishi, Y., Z. Fehervari, T. Yamaguchi, and S. Sakaguchi. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 105:10113-10118.

267. Tadokoro, C. E., G. Shakhar, S. Shen, Y. Ding, A. C. Lino, A. Maraver, J. J. Lafaille, and M. L. Dustin. 2006. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 203:505-511.

268. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7:83-92.

269. Sarris, M., K. G. Andersen, F. Randow, L. Mayr, and A. G. Betz. 2008. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 28:402-413.

270. Kaye, J., N. J. Vasquez, and S. M. Hedrick. 1992. Involvement of the same region of the T cell antigen receptor in thymic selection and foreign peptide recognition. *J Immunol* 148:3342-3353.

271. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77-92.

272. Lewis, R. S. 2001. Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol* 19:497-521.

273. Sehrawat, S., S. Suvas, P. P. Sarangi, A. Suryawanshi, and B. T. Rouse. 2008. In vitro-generated antigen-specific CD4+ CD25+ Foxp3+ regulatory T cells control the severity of herpes simplex virus-induced ocular immunoinflammatory lesions. *J Virol* 82:6838-6851.

274. Schneider, H., J. Downey, A. Smith, B. H. Zinselmeyer, C. Rush, J. M. Brewer, B. Wei, N. Hogg, P. Garside, and C. E. Rudd. 2006. Reversal of the TCR stop signal by CTLA-4. *Science* 313:1972-1975.

275. Schneider, H., X. Smith, H. Liu, G. Bismuth, and C. E. Rudd. 2008. CTLA-4 disrupts ZAP70 microcluster formation with reduced T cell/APC dwell times and calcium mobilization. *Eur J Immunol* 38:40-47.

276. Maillard, M. H., V. Cotta-de-Almeida, F. Takeshima, D. D. Nguyen, P. Michetti, C. Nagler, A. K. Bhan, and S. B. Snapper. 2007. The Wiskott-Aldrich syndrome protein is required for the function of CD4(+)CD25(+)Foxp3(+) regulatory T cells. *J Exp Med* 204:381-391.

277. Marangoni, F., S. Trifari, S. Scaramuzza, C. Panaroni, S. Martino, L. D. Notarangelo, Z. Baz, A. Metin, F. Cattaneo, A. Villa, A. Aiuti, M. Battaglia, M. G. Roncarolo, and L. Dupre. 2007. WASP regulates suppressor activity

of human and murine CD4(+)CD25(+)FOXP3(+) natural regulatory T cells. *J Exp Med* 204:369-380.

278. Snapper, S. B., F. S. Rosen, E. Mizoguchi, P. Cohen, W. Khan, C. H. Liu, T. L. Hagemann, S. P. Kwan, R. Ferrini, L. Davidson, A. K. Bhan, and F. W. Alt. 1998. Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* 9:81-91.

279. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.

280. Dustin, M. L. 2009. The cellular context of T cell signaling. *Immunity* 30:482-492.

281. Miller, M. J., O. Safrina, I. Parker, and M. D. Cahalan. 2004. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med* 200:847-856.

282. O'Keefe, J. P., K. Blaine, M. L. Alegre, and T. F. Gajewski. 2004. Formation of a central supramolecular activation cluster is not required for activation of naive CD8+ T cells. *Proc Natl Acad Sci U S A* 101:9351-9356.

283. Gondek, D. C., L. F. Lu, S. A. Quezada, S. Sakaguchi, and R. J. Noelle. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174:1783-1786.

284. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21:589-601.

285. Sugimoto, N., T. Oida, K. Hirota, K. Nakamura, T. Nomura, T. Uchiyama, and S. Sakaguchi. 2006. Foxp3-dependent and -independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis. *Int Immunol* 18:1197-1209.