

**THE DENDRITIC CELL CYTOSKELETON AND
THE IMMUNOLOGICAL SYNAPSE**

by

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requirements for the degree of

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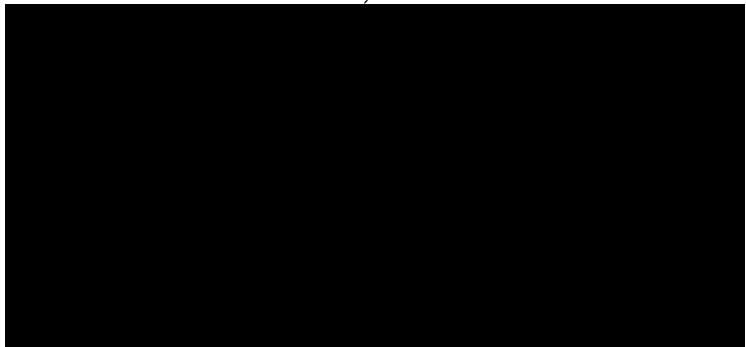
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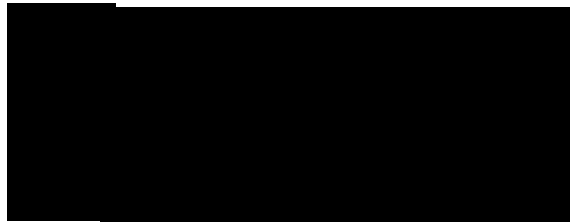
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DEDICATION

To my family without whom I would not be where I am today.
To my fiancée, Maha, I dedicate this dissertation with all my love.
Despite the distance, her endless encouragement gave me the
strength and support to achieve my goals.

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ABSTRACT

Generation of an antigen-specific immune response is dependent on T cell activation. A fundamental step for productive activation of T cells is the formation of an immunological synapse at the interface between a T cell and an antigen-presenting cell (APC). Previous studies have demonstrated that synapse formation is a dynamic process depending on active rearrangement of the T cell's cytoskeleton. However, the prevailing view in the literature is that the APC's cytoskeleton plays a passive role in this process. This conclusion was derived from studies that used preactivated T cells with surrogate APCs or APCs such as B cells, but the role of the APC's cytoskeleton in this process has not been examined using naïve T cells.

Activation of naïve T cells is a key step in the generation of primary immune responses. In contrast to preactivated T cells that can be stimulated by all APCs, naïve T cells can only be efficiently clustered and activated by dendritic cells (DCs). In this study, I used primary cells to evaluate the role of the APC's cytoskeleton during interactions between DCs and resting/naïve T cells. The data demonstrates that in contrast to previous studies, DCs form synapses with T cells. This synapse is characterized by polarization of the DC actin cytoskeleton toward the contact point with the T cells. In contrast to T cells, polarization of the DC's cytoskeleton occurs in an antigen-dependent fashion. Most importantly, DC actin cytoskeletal rearrangement, which is mediated via MHC class II ligation, is critical for antigen-dependent T cell binding and activation.

The study provides the first evidence of an active role for the APC/DC's cytoskeleton in the establishment of the synapse with naïve T cells. This finding has revolutionized the way the synapse may be perceived. A better knowledge of the mechanisms that control formation of the synapse between DCs and naïve T cells will increase our understanding of how to regulate the initiation of immune responses in various diseases.

ABBREVIATIONS

APC(s)	Antigen-presenting cell(s)
BSA	Bovine serum albumin
CCR(s)	Chemokine receptor(s)
CD	Cluster of differentiation
cDNA	Complementary DNA
cRPMI	Complete RPMI
cSMAC	Central supramolecular activation cluster
CTL(s)	Cytotoxic T lymphocyte(s)
CytD	Cytochalasin D
DAG	Diacylglycerol
DC(s)	Dendritic cell(s)
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DH ₂ O	Deionized water
EBV	Epstein-Barr virus
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorter
F-actin	Filamentous actin
FCS	Fetal calf serum
FDC(s)	Follicular dendritic cell(s)

FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol
HEV	High endothelial venules
ICAM	Intercellular adhesion molecule
IDC(s)	Interdigitating dendritic cells
IFN- γ	Interferon-gamma
IL	Interleukin
ILR(s)	Interleukin receptor(s)
IP ₃	Inositol 1,4,5-triphosphate
I-Synapse	Immunological synapse
Jasp	Jasplakinolide
LatA	Latrunculin A
LC(s)	Langerhans cell(s)
LFA	Lymphocyte function-associated antigen
MCF	Monocyte chemotactic protein
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
mRNA	Messenger RNA
MTOC	Microtubule organizing center
NF-AT	Nuclear factor of activated T cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PIP ₂	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PLC- γ 1	Phospholipase γ 1
PMA	Phorbol myristic acetate
pSMAC	Peripheral supramolecular activation cluster
PTK	Protein tyrosine kinase
RmGM-CSF	Recombinant mouse Granulocyte/macrophage colony stimulating factor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCF	Stem cell factor
SDF	Stromal cell-derived factor
SMAC(s)	Supramolecular activation cluster(s)
TCR(s)	T cell receptor(s)
TDC(s)	Thymic dendritic cell(s)

TECK	Thymus-expressed chemokine
TGF- β	Transforming growth factor-beta
Th	T helper cell
TNF- α	Tumor necrosis factor alpha
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein

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INTRODUCTION

1.1 Background

The immune system has evolved to provide responses against a wide range of nonself entities including pathogenic products and abnormally developed cells. These responses occur in a tightly regulated manner to prevent damage to normal (self) cells. Thus the immune system is uniquely designed to discriminate between self and nonself in order to produce immune responses in an antigen-specific fashion. The hallmark of generating an antigen-specific immune response is the activation of T cells, the organizers and effectors of the adaptive immune response. T cell activation occurs in a well-coordinated multi-step process. This process is initiated when a T cell receptor (TCR) on a T cell interacts with a processed antigenic peptide in the context of a major histocompatibility complex (MHC) molecule on the surface of an antigen-presenting cell (APC) (Zinkernagel and Doherty, 1974; Townsend et al., 1990). Through the high antigen-specificity of the TCR, T cells are able to discriminate between self and nonself antigenic epitopes, which may differ by only one or two amino acids (Corr et al., 1994; Schneck et al., 1989). Once the TCR is triggered by the proper peptide-MHC complexes, a variety of other T cell adhesion and accessory membrane molecules interact with their counter-receptors on the APC. This step in turn initiates a cascade of biochemical events that trigger various genes that are involved in the

activation, proliferation and clonal expansion of responding T cells into effector cells.

While antigens may be perceived in the periphery, generation of an antigen-specific immune response occurs mainly in the secondary lymphoid organs. This is consistent with the fact that the APCs and T cells are both mobile. The APCs migrate from the periphery through the lymphatic system to secondary lymphoid organs, where they accumulate in the regions of the lymph nodes that are rich in trafficking T cells *i.e.* near the high endothelial venules; (HEV). Naïve T cells circulate in the blood, entering lymph nodes across HEV and thereby congregate in the regions rich in APCs. Once in the node, T cells probe the surface of many APCs but stop only when they recognize the proper antigen-MHC complexes, thereby allowing antigen-specific T cells to receive the activation signals from the APC. This continuous mobility of both cells is believed to maximize the interaction between a rare antigen-specific T cell with the proper antigen-MHC complexes displayed on the surface of an APC.

In a physiological system, T cell activation requires the interaction of the TCR with its natural ligand, the peptide-MHC complex on an APC. However, T cells may be activated by other means such as bacterial superantigens or crosslinking antibodies to the TCR-CD3 complex (Kappler et al., 1989; Tax et al., 1983). In addition, T cell activation may be achieved without TCR crosslinking. This has been demonstrated by the use of agents such as calcium ionophore and phorbol myristic acetate (PMA). Calcium ionophore, which mimics the effects of inositol 1,4,5-triphosphate (IP₃), allows extracellular Ca²⁺ to enter the cell. PMA,

which mimics the effects of diacylglycerol (DAG), activates protein kinase C (PKC). Activation of T cells triggers the translocation of nuclear factor of activated T cells (NF-AT) and NF- κ B into the nucleus, which in turn result in the activation of interleukin (IL)-2 and IL-2 receptor (IL-2R) genes. Up-regulation of both IL-2 and IL-2R expression is a key component in T cell proliferation and differentiation. In summary, activation of T cells is a key step in the generation of antigen-specific immune responses such as stimulating antibody production by B cells and helping cytotoxic T cells to kill virally infected cells or tumor cells (Guinan et al., 1994; Harlan et al., 1995; June et al., 1994; Linsley and Ledbetter, 1993). Therefore, understanding the mechanisms that control T cell activation will provide valuable information on how to regulate the antigen-specific immune response in health and diseases.

1.2 T cell activation

1.2.1 The two-signal model

It is well established that T cell activation is a multi-step process that requires the interaction between the T cell surface molecules and their counterparts on APCs. More than 3 decades ago, Bretscher and Cohn proposed that activation of T lymphocytes requires delivery of two distinct signals, neither one of which is sufficient to achieve full activation (Bretscher and Cohn, 1970). This model specifies that antigen-specific induction of proliferation and full activation of T cells requires a primary signal delivered through the TCR recognizing peptide in the context of MHC molecule plus a second antigen-

independent (costimulatory) signal. The costimulatory signals lowers the TCR activation threshold and enhances T cell tyrosine phosphorylation pathways (Viola and Lanzavecchia, 1996; Viola et al., 1999). In addition, costimulation stabilizes IL-2 mRNA as well as IL-2R expression (Wells et al., 1997). On the other hand, TCR engagement by specific antigen in the absence of costimulatory signals results in unresponsiveness to subsequent challenge with the antigen. This implies that the second signal plays a pivotal role in determining the fate of a T cell when it encounters an antigen.

The legitimacy of the two-signal model of T cell activation has been supported by experimental observations from many laboratories. A variety of receptor-ligand interactions that may provide the costimulatory second signals for T cell activation have been identified. The reported costimulatory pairs include CD2 (LFA-2)-CD58 (LFA-3) (Goldbach-Mansky et al., 1992; Moingeon et al., 1989), CD11a/CD18 (LFA-1)-CD54 (ICAM-1) (Damle et al., 1992; Maraskovsky et al., 1992; Van Severter et al., 1990), CD154 (CD40L)-CD40, CD4-MHC class II, and CD28 or CTLA-4-CD80 (B7-1) or CD86 (B7-2) (Galvin et al., 1992; Harding and Allison, 1993; Lenschow et al., 1993). Among these, costimulatory signals delivered through CD28 binding to B7-1 or B7-2 have been shown to be the principle costimulators, whereas other ligands may function more in increasing the avidity of the interactions. In the presence of CD28 costimulation, T cell activation may be achieved at a lower threshold of TCR triggering, suggesting that CD28 may amplify the signals transduced by each triggered TCR. In the human Jurkat T cell clone, it has been estimated that proliferation

and cytokines production requires the triggering of ~8000 TCR, which represents ~30% of the total TCR number per cell (Viola and Lanzavecchia, 1996). This number of triggered receptors is significantly reduced to ~1000 TCR when CD28 is engaged. In summary, the T cell senses fine differences in the peptide-MHC complexes, including the density of the antigen, length of exposure of T cells to antigen and presence of costimulatory molecules (Lanzavecchia and Sallusto, 2001). All of these factors contribute to the degree of phosphorylation, which translate into different downstream events. Several studies have demonstrated that the requirement for costimulation (signal 2) depends on the extent of TCR stimulation (signal 1) and the differentiation state of T cells *i.e.* naïve or primed (Croft et al., 1994; Horgan et al., 1990; Luqman and Bottomly, 1992).

It has been demonstrated that activation of T cells via a combination of TCR signals and costimulatory signals is strictly dependent on the T cell subset. While the response of all T cells to a strong TCR signal (e.g. immobilized anti-CD3 antibody) may be enhanced by the presence of APC-expressing costimulatory molecules, some T cells require less costimulation for optimal activation. Naïve T cell respond weakly to anti-CD3 triggering and require much more costimulation for maximal proliferation and cytokine secretion (Croft et al., 1994; Horgan et al., 1990; Luqman and Bottomly, 1992). However, memory T cells respond moderately to anti-CD3 stimulation and require less costimulation, and effectors respond well to anti-CD3 and need even fewer additional signals. When APCs were used, Steinman and colleagues showed that dendritic cells (DCs) were

superior in stimulating proliferation and cytokine production by naïve allogeneic CD4⁺ T cells (Inaba and Steinman, 1984; Metlay et al., 1989). They demonstrated that while resting B cells or macrophages were inefficient, activated B cells were able to activate naïve CD4⁺ T cells, although less effectively than DCs. Subsequent study has shown that DCs are able to stimulate allogeneic resting CD8⁺ T cells and induce cytotoxic T lymphocytes (CTLs), whereas unstimulated macrophages are ineffective (Inaba et al., 1987). In contrast, memory CD8⁺ T cells were induced to differentiate into CTL effectors by both DCs and macrophages, although macrophages were less efficient stimulators (Macatonia et al., 1989). On the other hand, resting or activated B cells can only efficiently stimulate memory but not naïve CD8⁺ T cells (Fuchs and Matzinger, 1992).

The differential requirement for costimulation by different T cell subsets explains why DCs are more efficient than other APC and can stimulate all T cells with high potency, whereas resting B cells and macrophages are poor stimulators and are only active with more differentiated T cells. DCs constitutively express high levels of B7-1, B7-2, ICAM-1, MHC class II, whereas resting B cells and macrophages express low to undetectable levels of these molecules (Boussiotis et al., 1993; Ding et al., 1993; Freedman et al., 1991; Larsen et al., 1992; Lenschow et al., 1993; Liu et al., 1992). Activation of B cells and macrophages induces the expression of B7 and MHC molecules. Thus it likely that naïve T cells are not activated by resting B cells or macrophages because of the lack of the necessary costimulation. This is supported by the fact that naïve T cells may be stimulated by anti-CD3 if costimulus is provided by anti-CD28 (Horgan et al., 1990). In

summary, memory and effector T cells, which must have received a high level of costimulation when first activated at the naïve stage, are much more susceptible to TCR triggering, require weaker cosignaling, and thus respond to APCs expressing even low levels of costimulatory molecules.

Altogether, T cell activation is initiated when the TCR recognize a specific antigen in the context of MHC. This step provides “signal one”, which is necessary but not enough to induce a productive T cell activation. A second signal is required to generate full T cell activation. The requirement for costimulation depends on the differentiation stage of T cells (naïve or primed). However, it appears that the overall activation process of T cells requires more than the mere interaction of surface receptors on a T cell with their ligands on an APC.

1.2.2 Different mechanisms

While T cell activation requires the engagement of the TCR by the peptide-MHC complex, TCR engagement *per se* does not necessarily result in a productive T cell activation. A number of factors have been implicated as playing roles in determining the fate of TCR triggering. A critical factor in this process is the duration of TCR engagement, which must be sufficient to promote the signal transmission from the triggered TCR to downstream pathways (Kersh et al., 1998a; McKeithan, 1995). A second factor that is critical for productive T cell activation is the number of TCR that need to be triggered. The number has been estimated to be $\sim 20 \times 10^3$ TCR on a single T cell (Valitutti et al., 1995b). While large numbers of TCR need to be triggered, the actual number of specific peptide-

MHC complexes for a T cell activation event is as few as 100 complexes (Christinck et al., 1991; Harding and Unanue, 1990; Sykulev et al., 1996). This observation raises the question of how so few peptide-MHC complexes on an APC can trigger so many TCRs on a T cell. Many studies have been carried out to provide a reasonable explanation for this ambiguity.

Two independent studies have reported that interactions between TCRs and peptide-MHC complexes have a very low affinity and fast disassociation rates (Matsui et al., 1991; Weber et al., 1992). A subsequent study by Valitutti *et al* demonstrated that once a bound TCR is triggered, it quickly dissociated and is internalized to allow another TCR to be triggered (Valitutti et al., 1995b). This observation is in agreement with another study that showed downregulation of TCR expression from the cell surface following triggering (Valitutti et al., 1997). The extent of TCR downregulation defines the strength of TCR-mediated "signal 1" which correlates with the spectrum of effector functions activated within the T cell. This indicates that activation of different T cell functions requires the triggering of distinct numbers of TCR. Furthermore, the interaction between a single TCR and its ligand occurs over a time frame of a few seconds, while the interaction between a T cell and an APC has a time course of several hours. These findings have led to the proposed "serial triggering model" of T cell activation (Valitutti et al., 1995b). In this model, the low affinity binding coefficient of the TCRs enable them to dissociate from their ligands once they have been triggered thus allowing the serial triggering of other TCRs by the same ligand. The dissociation of TCR once triggered may contribute to signal transduction by facilitating the encounter

between receptors and intercellular signaling molecules. Alternatively, it may contribute to the termination of cellular responses by reducing the number of receptors on the cell surface. This model fits well with most findings that link downregulation of the TCR complexes to T cell activation. However, a report by San Jose *et al* demonstrated that the number of down-regulated TCR does not necessarily reflect the number of engaged receptors (San Jose et al., 2000). They suggested that T cells might have evolved a system to amplify downregulation signals to nonengaged receptors when T cells are activated by a low number of ligands.

Another model proposes that upon initial binding with peptide-MHC complexes the TCR-CD3 complex undergoes conformational changes (Ding et al., 1999; Janeway-CA, 1995). This model assumes a simultaneous engagement of adjacent TCR with their ligands. Upon engagement, TCR undergo conformational changes that alter their affinity for the ligands and thereby allow for sustained TCR signaling. There is as yet very little support for this model since no structural changes have been reported for TCR upon peptide-MHC binding. Other groups have proposed a “segregation model” of T cell activation (Davis and van der Merwe, 1996; Shaw and Dustin, 1997; Thomas, 1999). This model suggests that peptide-MHC binding leads to triggering by holding the TCR/CD3 complex in a membrane environment in which tyrosine kinases have been segregated from tyrosine phosphatases. Both models do not fit with the serial triggering model since they do not explain the ability of few peptide-MHC complexes to engage many TCR. It is also important to keep in mind that the experiments on which all

of these models are based were performed using either soluble peptide-MHC complexes or surrogate APCs (beads or glass-supported lipid bilayers). It is difficult, therefore, to predict the possible relevance of these models under physiological conditions. However, one may speculate from these studies that besides the simple ligand-receptor interaction, other cellular structures might be involved in the process of T cell activation.

1.2.3 Other models

1) The lipid raft model

The immediate consequence of TCR engagement is the recruitment of protein tyrosine kinase (PTK) proteins, such as Lck and Fyn, into close association with the cytoplasmic tail of the TCR/CD3 complex. The association between these signaling molecules and TCRs is considered as an early sign of T cell signaling. However, it appears that these signaling molecules and TCRs are present in different microdomains in the T cell membranes and thus one might wonder how do they get together? Recent studies have provided both direct and indirect evidence that support the pivotal role of lipid rafts in mediating this interaction and thus facilitating T cell signaling.

A number of signaling molecules have been shown to constitutively accumulate in lipid rafts, including Lck, Fyn, LAT, protein kinase C θ , Ras, PIP₂, and G-coupled protein receptors (Casey, 1995; Cinek and Horejsi, 1992; Hope and Pike, 1996). The role of LAT localization in lipid rafts in T cell signaling was elegantly demonstrated using a mutant form of LAT. This mutant, which remains

attached to the plasma membrane but is excluded from lipid rafts, fails to become tyrosine phosphorylated after TCR stimulation (Zhang et al., 1998). Phospholipase γ 1 (PLC- γ 1) and the Grb2/SOS complex are recruited by LAT to lipid rafts, where they encounter their substrates phosphatidylinositol 4,5-bisphosphate (PIP₂) and Ras respectively, these being constitutively associated with lipid rafts (Zhang et al., 1998). Molecules that negatively regulate the kinase activity (CD45, CD43) within the T cell membranes are excluded from lipid rafts microdomains (Cerny et al., 1996; Rodgers and Rose, 1996). In addition, the localization of several glycosylphosphatidylinositol (GPI)-anchored proteins to lipid rafts in T cells has been reported and their importance for T activation demonstrated because cells deficient in GPI biosynthesis show inhibited TCR signaling (Romagnoli and Bron, 1997).

It has been demonstrated that TCR/CD3 complexes are normally present outside of rafts in resting T cell membranes (Montixi et al., 1998). Upon activation, TCR/CD3 complexes are recruited into lipid rafts where they interact with lipid raft-associated signal-transducing molecules (Montixi et al., 1998; Xavier et al., 1998). Recruitment of TCR into lipid rafts is a critical step for efficient TCR signal transduction. T cell activation is inhibited when the raft integrity is perturbed using raft-disrupting drugs such as M β CD, filipin or nystatin. Disruption of raft structure abrogates T cell activation, indicating that membrane compartmentation is a prerequisite for TCR signal transduction. It is unlikely that signaling molecules in lipid rafts are involved in TCR recruitment, since TCR are still associated with lipid rafts in Lck deficient cells or in cells pretreated with PP1 (Src-family kinase

inhibitor) (Janes et al., 1999). Similarly to the TCR, the hyperphosphorylated ζ chain and activated ZAP-70 accumulate in raft microdomains upon TCR triggering (Zhang et al., 1998). In fact, the phosphorylation of ζ chain, ZAP-70, and PLC- γ 1 following TCR engagement occurs principally in the rafts (Xavier et al., 1998).

Most importantly, lipid raft involvement in signaling depends on the activation state of T cells. Loretta *et al* demonstrates that naïve T cells have few raft domains organized on their cell membrane and that they require CD28 costimulation in order to amplify TCR signaling by recruiting more signaling molecule-containing rafts to the triggered TCR (Tuosto et al., 2001). Lipid rafts as well as Lck are targeted to the plasma membrane of memory or CD3/CD28-activated T cells and thus these cells are less dependent on CD28-mediated recruitment of rafts in order to amplify the TCR signaling. This is consistent with the fact that the engagement of CD28 increases T cell proliferation and cytokine production as well as tyrosine phosphorylation (Tuosto and Acuto, 1998). In addition, Viola *et al* reported that CD28 costimulation enhances raft redistribution to the site of TCR engagement, resulting in higher levels of phosphorylation and increased recruitment of Lck to the TCR-containing raft fraction (Viola et al., 1999). This explains why optimal T cell activation may be achieved at a lower threshold of TCR triggering when costimulation is present. Therefore, the low level of lipid rafts expressed in naïve T cell membranes is in line with their extra need for costimulation as compared with primed T cells.

Taken together, the constitutive localization in, and the exclusion of certain proteins from, lipid rafts is consistent with the hypothesis that rafts provide an

appropriate environment where rare molecules may interact and mediate signaling. Lipid rafts may function as platforms for signal transduction by concentrating TCR/CD3 molecules around kinase-enriched rafts, from which the tyrosine phosphatase molecule (CD45) is removed. Overall, these observations suggest an important role of such membrane compartmentation in signal transduction in T cells. In addition, rafts may be involved in negative regulation of signaling pathways in T cells, by creating an environment that isolates certain signaling proteins from their substrates.

2) The dynamic model

Structural studies have demonstrated that the distance spanned by the TCR-MHC complex is approximately 15 nm (Garboczi et al., 1996; Garcia et al., 1996). By definition, therefore, the distance between the T cells and APC plasma membrane in the area of contact between TCR and MHC must be approximately 15 nm. The problem is that the TCR are buried between significantly taller molecules (45nm) such as CD45 and CD43, which are abundant on the surface of T cell (Cyster et al., 1991). These taller molecules will hamper TCR from engaging its ligand, the peptide-MHC complex. Significant reorganization of proteins at the contact between the T cell and the APC must, therefore, occur to permit TCR engagement of peptide-MHC. Indeed, tall molecules such as CD43 and CD45 are excluded from the contact point between T cells and APC (Leupin et al., 2000; Sperling et al., 1998). There is also compelling evidence that the redistribution of cell surface molecules as well as signaling molecules during T cell antigen

recognition is an active process. A number of studies have shown that crosslinking of the TCR/CD3 complex with antibodies can trigger redistribution “capping” of TCR/CD3 as well as other molecules (Kupfer and Singer, 1989a; Linsley et al., 1996). Furthermore, inhibition of the cytoskeletal rearrangement inhibits redistribution of these molecules (Grakoui et al., 1999; Wulfiging et al., 1998; Wulfiging and Davis, 1998). These cytoskeletal-dependent changes are essential for effective T cell activation.

TCR engagement on a T cell with the appropriate peptide-MHC complex on an APC results in the formation of specialized junction between the interfaces of two cells. The formation of this delicate junction, which is referred to by Dustin *et al* as an “immunological synapse” (I-Synapse), is critical for a productive T cell activation (Grakoui et al., 1999). TCR, adhesion, costimulatory and signaling molecules are segregated within the IS into distinct regions, which is referred to by Monks *et al* as supramolecular activation clusters (SMACs) (Monks et al., 1998). LFA-1 and talin are organized into a peripheral ring (pSMAC) surrounding a central ring (cSMAC) containing the TCR, CD3 and Lck, Fyn and protein kinase C. The study of the I-Synapse had been pioneered by Abraham Kupfer and colleagues more than 15 years ago (Kupfer and Dennert, 1984; Kupfer et al., 1986). They studied the I-Synapse at the single cell level using immunofluorescence technique of fixed cell conjugates (Kupfer and Dennert, 1984; Kupfer et al., 1986). Subsequent studies have also visualized the surface molecule movement and formation of the I-Synapse in living cells (Grakoui et al., 1999; Krummel et al., 2000; Wulfiging et al., 1998). Previous studies have demonstrated that these

changes are dynamic and are dependent on the T cell cytoskeletal rearrangement (Kupfer and Singer, 1989b; Pardi et al., 1992). These changes may be achieved via costimulation since stimulation of T cells with anti-CD3 plus anti-CD28 induces a reorganization of lipid rafts to the synapse, whereas stimulation with anti-CD3 alone fails (Viola et al., 1999). It is believed that cytoskeletal reorganization serve to stabilize the contact between the two cells and thus permit the sustained TCR triggering that is critical for optimal T cell activation.

The initial interaction between a T cell and an APC is mediated by integrins such as LFA-1 that are relatively large in size compared with TCR. These adhesion molecules, therefore, need to be excluded from the I-Synapse to facilitate the interaction of short molecules such TCR. Although of low affinity, integrins facilitate antigen sampling on APCs by the TCRs on T cells. On antigen recognition, TCR triggering may activate LFA-1 by direct phosphorylation of the β -chain of LFA-1 (Chatila and Geha, 1988), or alternatively, by phosphorylation of a cytoskeletal protein such as talin (Burn et al., 1988). It is possible that integrins through their association with the cytoskeleton unzip from the I-Synapse area giving the opportunity to the short TCRs to sample more peptide-MHC complexes on the APCs. Recognition of specific peptide-MHC complex on an APC by the TCR on a T cell might initiate a signal that simultaneously recruits kinases and excludes negative regulatory phosphatases from the I-Synapse. Formation of an I-Synapse with an APC may provide the T cell with the appropriate environment that facilitates the interaction between the engaged TCRs and kinases, thereby triggering the phosphorylation cascade. It may also exclude larger molecules from

the T cell-APC contact and thus position the membranes at the optimal distance for TCR-peptide-MHC. This process may provide an optimal density of peptide-MHC complexes and facilitate their interaction with TCRs.

In summary, formation of the I-Synapse may result in the local concentration of signal transduction molecules and the exclusion of the negative regulator molecules, thus facilitating TCR signal transduction. It may furthermore provide a stable structure that overcomes the problems of low TCR-ligand affinity, low ligand concentration, and the small size of the TCR extracellular domain relative to other surface molecules. Collectively, the interaction between a T cell and an APC results in the formation of I-Synapse, which is critical for full T cell activation.

1.3 The T cell's cytoskeleton and the I-Synapse

The formation of an I-Synapse between the interface of a T cell and an APC is critical for the initiation of an immune response (reviewed in (Bromley et al., 2001)). The I-Synapse develops as a multi-step process that is initiated by engagement of the TCR on a T cell with the appropriate peptide-MHC complex on the surface of an APC. Although the initial interaction sets the appropriate background for the initiation of T cell activation, additional signals mediating sustained TCR engagement are required for a productive T cell activation (Grakoui et al., 1999; Iezzi et al., 1998; Valitutti et al., 1995a). Maintenance of a stable I-Synapse is critical for the activation process. Studies on the T cell side of the synapse have clearly demonstrated that the formation of stable I-Synapse

between the T cell and the APC results from reorganization of the T cell's actin cytoskeleton (Kupfer and Singer, 1989b; Pardi et al., 1992; Dustin et al., 1998; Grakoui et al., 1999; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Sedwick et al., 1999; Stowers et al., 1995). This is characterized by the accumulation of F-actin and other cytoskeletal proteins in the T cell at the contact point with the APC. These active changes in the T cell's cytoskeleton result in the dynamic clustering of T cell surface receptors and signaling molecules into SMAC at the interface with the APC (Grakoui et al., 1999; Monks et al., 1997; Monks et al., 1998). The SMAC organizes the TCR, adhesion, costimulatory and signaling molecules into pSMAC of LFA-1 and talin surrounding cSMAC containing the TCR, CD3 and p56^{lck}, p59^{lyn} and protein kinase C. Stimulation of T cells with immobilized anti-CD3 antibodies also results in marked morphological changes characterized by a spreading process and the formation of pseudopodia associated with rearrangement of the actin cytoskeleton (Pardi et al., 1992; Parsey and Lewis, 1993). However, T cell activation by soluble anti-CD3 antibodies is actin cytoskeleton-independent (Valitutti et al., 1995a), suggesting that the T cell's cytoskeleton is important only when they are activated by an immobilized surface such as APC.

The importance of the cytoskeleton integrity for T cell activation has also been evaluated using more physiological approaches. Wiskott-Aldrich syndrome (WAS) is a rare X-linked immunodeficiency that is characterized by recurrent infections (Brickell et al., 1998). Several lines of evidence indicate that the Wiskott-Aldrich syndrome protein (WASp) is involved in regulating the actin

cytoskeleton. Indeed, T cells from WAS patients and WASp knockout mice are defective in both antigen receptor-induced proliferation and regulation of the actin cytoskeleton (Molina et al., 1993; Gallego et al., 1997; Snapper et al., 1998). Furthermore, overexpression of WASp inhibits TCR signaling-mediated T cell proliferation (Sato et al., 2001). These findings further emphasize the importance of the T cell's cytoskeleton for their optimal activation.

The dynamics of the I-Synapse formation between T cells and APCs has also been monitored in living cells using green fluorescent protein (GFP)-tagged proteins expressed in either T cells or APCs. Using a B cell hybridoma expressing ICAM-1-GFP, Wulfing *et al* have demonstrated the accumulation of ICAM-1 during antigen-specific T-B cell interactions (Wulfing et al., 1998). Similarly, CD3 ζ -GFP and CD4-GFP were used to monitor their movement during antigen-specific T cell-APC interactions (Krummel et al., 2000). While both molecules moved into the I-Synapse, CD3 remained in and CD4 migrated away from the center zone of the synapse. Studies of the I-Synapse available from fixed and living cells demonstrated that the movement of proteins into and out of the I-Synapse is an active process mediated via T cell cytoskeletal rearrangement. It is believed that these cytoskeletal changes in T cells serve to stabilize the contact between TCR and MHC, thus providing an optimal environment for signaling molecules downstream of the TCR (Penninger and Crabtree, 1999). In summary, previous studies have clearly demonstrated that T cell's cytoskeleton is critical for the formation of I-Synapse, and thus cytoskeletal reorganization is a key step for productive T cell activation.

1.4 Problems with previous studies

The knowledge gained from previous studies on the I-Synapse has confirmed how critical this process is in determining the fate of T cells following stimulation. However, these studies have focused on the T cell side of the synapse and how critical is the formation of a stable I-Synapse for productive T cell activation (Bromley et al., 2001). Despite the fact that the T cell's cytoskeleton plays an active role in synapse formation (Penninger and Crabtree, 1999), the prevailing view in the literature is that APCs play a passive role in this process (Valitutti et al., 1995a). This conclusion was based on studies that used B cells as APCs and observed no rearrangement of their cytoskeleton during interaction with T cells (Kupfer and Singer, 1989b; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Pardi et al., 1992; Valitutti et al., 1995a; Wulfing et al., 1998). In addition, inhibition of APC/B cell cytoskeletal rearrangement has no effect on APC-T cell binding or T cell activation (Valitutti et al., 1995a; Wulfing et al., 1998). Other evidence suggesting a passive role of the APC's cytoskeleton in the synapse formation came from studies where T cells were observed to form SMAC when lipid bilayers were used as surrogate APCs (Grakoui et al., 1999). However, previous studies in this area have only used activated T cells (Dustin et al., 1998; Grakoui et al., 1999; Kupfer and Singer, 1989b; Kupfer et al., 1991; Larsen et al., 1990; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Sedwick et al., 1999; Stowers et al., 1995). None of these studies, therefore, investigated the role of the APC's cytoskeleton during interaction with naïve T cells, which have different requirements for

activation as compared with primed T cells. Indeed, stability of the I-Synapse is probably more critical for the optimal activation of unprimed T cells, since they need to interact with the APC for longer time periods as compared to antigen-experienced T cells (Iezzi et al., 1998).

All APCs may activate previously primed T cells, although DCs are the most potent. However, clustering and activation of naïve T cells is a unique feature of DCs. While resting B cells or macrophages were inefficient in activating naïve T cells, DCs exhibit superior potency (Austyn et al., 1988b; Inaba and Steinman, 1984; Metlay et al., 1989; Inaba et al., 1987; Macatonia et al., 1989). These studies clearly demonstrated significant differences between APCs with respect to their capability to stimulate naïve T cells. They also illustrated that DCs are more efficient than other APCs in activating primed T cells. In addition, the communication between DCs and T cells is mutual rather than being dominated by one side (Banchereau and Steinman, 1998). This suggests that the formation of the I-Synapse between a DC and a naïve T cell is likely to be different to what has been observed between B cells or surrogate APC and primed T cells. The use of primed T cells in the previous I-Synapse studies may have simply overlooked the role of the APC's cytoskeleton in this process.

1.5 DCs and immune responses

1.5.1 Origin of DCs

DCs are bone marrow derived leukocytes characterized by a highly irregular shape with numerous cell membrane processes (reviewed in

(Banchereau et al., 2000)). Almost 3 decades have past since Steinman and Cohn first identified DCs from murine spleen and named them after their characteristic morphology (Steinman and Cohn, 1973). Subsequent studies have shown that DCs are widely distributed in the body except in the brain, cornea of the eye and testes (Daar and Fabre, 1983; Hart and Fabre, 1981). In spite of their wide distribution, DCs are relatively rare cells accounting for less than 1% of the total leukocyte population in most tissues studied (Steinman and Nussenzweig, 1980). The paucity of DCs, the absence of DC specific markers and the complexity in the isolation of these cells has hampered extensive study of their role in immune modulation. It has also been difficult to understand how these rare cells may be involved in the induction of T cell-mediated immunity. All of these factors combined, along with the fact that the macrophage was regarded as the major professional APC, lead many immunologists to underestimate the role of DC in the immune system.

The success achieved in the field of DC isolation and enrichment in vitro and in vivo has lead to remarkable progress in the study of DCs and their role in health and disease. Studies have shown that tissue DCs are heterogeneous with regard to their origin, maturation state and rate of turnover (Salomon et al., 1998; Shortman and Liu, 2002). This heterogeneity has been reflected in the molecules they express and the nature of the functions they perform. However, there is a general agreement that DCs are derived from bone marrow precursors in spite of their heterogeneity and controversy over their lineage derivation. In both humans and mice, it has been reported that there are at least two distinct pathways of DC

development, representing myeloid-derived and lymphoid-derived populations.

The bulk of the evidence for the myeloid origin of DCs comes from *in vitro* studies in which DCs have been grown from bone marrow, cord blood progenitors, and peripheral blood using granulocyte/macrophage colony stimulating factor (GM-CSF) (Inaba et al., 1992; Caux et al., 1992; Inaba et al., 1993; Lutz et al., 1999). The concept that DCs are of lymphoid origin comes from studies on the thymus where they have been found to express markers normally associated with lymphoid cells, including CD8 α , CD4, CD2, and CD25 (Vremec et al., 1992; Wu et al., 1995). Furthermore, lymphoid-derived DCs lack a requirement for GM-CSF, an essential cytokine required for the development of myeloid DCs, to stimulate their proliferation and differentiation (Saunders et al., 1996; Shortman and Caux, 1997). Although more recent studies suggest a common precursor for both (Del Hoyo et al., 2002), there is an ongoing dispute in the literature concerning the exact function(s) of the lymphoid and myeloid DCs.

DCs differ not only in their lineage derivation but also in the nature of function that they perform. Follicular dendritic cells (FDCs), for example, are another lineage of DCs that play an essential role in maintaining the integrity of the germinal center of a lymph node. They have been shown to directly contribute to B cell proliferation and to maturation into memory cells (MacLennan, 1994). Thymic dendritic cells (TDCs) are another DC type that function not to initiate a T cell response, but rather to induce cell death of any of the developing T cells which are potentially reactive with self-antigens (Ardavin, 1997; Sprent et al., 1988). Like the developing T cell population, TDCs turn over rapidly consistent with their function

in negative selection (Kampinga et al., 1990; Wu et al., 1995). Langerhans cells (LCs) are probably the first studied member of the DC family and often referred to as the “classical” immature DC (Rowden, 1981). They were first described by Paul Langerhans in 1868 and were initially regarded as neuronal cells until Breathnach *et al* ruled out their neuronal origin (Breathnach et al., 1968). Subsequent studies demonstrating Birbeck granules in LCs confirmed their migration from skin to afferent lymphatics (Silberberg et al., 1976). Furthermore, the detection of MHC class II expression on LCs more than 25 years ago provided the first evidence linking these cells to antigen presentation (Rowden et al., 1977). In skin explants, LCs were observed to migrate from the epidermis through the dermis and into culture medium (Larsen et al., 1990). Using radiolabeled LCs, Fossum *et al* demonstrated LC migration from skin into the T cell areas of lymph nodes, where they acquire the characteristic appearance of interdigitating dendritic cells (IDCs) (Fossum, 1988). Upon in vitro culture, they mature into IDC-like cells (Schuler and Steinman, 1985).

1.5.2 The life cycle of DCs

One of the characteristic features of DCs at the various differentiation stages is their mobility (Austyn et al., 1988a). This feature allows them to migrate from the site of inflammation, where they pick up the antigens, into the secondary lymphoid organs, where they present antigen to and activate T cells. The capacity of DCs to carry out various tasks at the different stages of their life cycle has attracted the attention of many investigators in recent years. These

studies proposed that there are three stages in the development of DCs from committed bone marrow progenitors (reviewed in (Banchereau et al., 2000)). 1) DC precursors that circulate from the bone marrow to various nonlymphoid tissues, where they eventually become resident cells. 2) Tissue-residing immature DCs that are poor in antigen presentation but have high endocytic capacity, thus permitting antigen uptake. 3) Secondary lymphoid organ mature DCs that have poor endocytic capacity but are very efficient in antigen presentation.

It is now well accepted that DC migration is strictly dependent on their maturation stage (Banchereau et al., 2000). DC precursors leave the bone marrow, circulate in the blood and eventually exit into peripheral tissues. In the periphery, they reside as immature DCs, which are characterized by a low level of MHC, adhesion, and costimulatory molecule expression. While these DCs are very poor in antigen presentation, they have a very active endocytic and processing capacity (Mommaas et al., 1999; Tan et al., 1997; Steinman, 1991; Hart, 1997; Caux et al., 1995). Exposure of immature DCs to a danger signal such as microbial products or inflammatory cytokines results in their maturation and migration to draining lymph nodes (Mommaas et al., 1995; Austyn, 1996; McWilliam et al., 1994; Roake et al., 1995). Cytokines such as GM-CSF, tumor necrosis factor alpha (TNF- α) (Caux et al., 1992), stem cell factor (SCF) (Young et al., 1995) and IL-4 (Rosenzwajg et al., 1996) play an important role in DC maturation. In addition, FLT3 ligand (Shurin et al., 1997) as well as CD40 ligand (Flores-Romo et al., 1997) have been shown to selectively induce DC maturation

from bone marrow precursors. Other factors such as transforming growth factor-beta (TGF- β) appears to be essential for LC differentiation since TGF- β -/- mice lack LCs (Borkowski et al., 1996).

DC maturation is associated with a reduced antigen-processing ability and enhanced expression of MHC, adhesion, and the costimulatory molecules (Banchereau et al., 2000). Thus, DC maturation results in the conversion of cells carrying out antigen surveillance into a highly efficient APC with the ability to activate resting T cells. This conversion may result from a remarkable increase in the MHC class II half-life in mature compared with immature DCs (Chow et al., 2002; Turley et al., 2000), which allows the accumulation and persistence of the peptide-MHC class II complexes formed (Inaba et al., 2000). It is interesting to note that the distribution of immature DCs in the sites of high antigen exposure such as the skin, the gastrointestinal tract, the airways and the interstitial spaces of most organs especially suited for their function to capture antigens. Conversely, the localization of mature DCs in the T cell area of the lymphoid organs is compatible with their function in antigen presentation and T cell activation.

Chemokines are one of the key factors that regulate the trafficking of immature DC into the sites of inflammation and mature DCs into secondary lymphoid organs (Sozzani et al., 1995; Xu et al., 1996). Chemokines act via binding to unique cell surface receptors that belong to the seven transmembrane, G protein-coupled Rhodopsin superfamily (Murphy, 1994). Many believe that the changes in migratory behavior of DCs are associated with a switch in the expression of chemokine receptors, which permits them to respond to different

chemokines. Therefore, several studies have examined the expression of chemokine receptors on DCs during their maturation. Immature DCs, for example, have been reported to express high levels of the chemokine receptors CCR1 and CCR5, which are dramatically decreased upon maturation (Yanagihara et al., 1998). The expression of CCR1 and CCR5 makes them responsive to several chemokines such as monocyte chemoattractant protein (MCP)-3, MCP-4, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , RANTES, stromal cell-derived factor (SDF)-1 and thymus-expressed chemokine (TECK). The expression of these chemokines in high levels at sites of inflammation is consistent with the presence of their ligands on immature DCs. However, they have been reported to exert chemotactic activity on other leukocytes that also express their corresponding ligands and thus they are not solely chemotactic for DCs.

Chemokine receptors such as CCR6 and CCR7 are selectively expressed on DCs (Greaves et al., 1997; Yoshida et al., 1997). These chemokine receptors are the only known receptors for MIP-3 α and MIP-3 β , respectively. The expression of CCR6 and CCR7 has been found to be regulated during DC maturation (Dieu et al., 1998; Sallusto et al., 1998). Immature DCs express high levels of CCR6 and migrate in response to MIP-3 α . Upon maturation, they sharply down-regulate the expression of CCR6 and up-regulate the expression of CCR7. This is linked to the loss of response to MIP-3 α and the acquisition of a sustained responsiveness to MIP-3 β . The expression of MIP-3 α at the site of inflammation and MIP-3 β in the T cell area of the lymph node fits well with the expression of

CCR6 and CCR7 on immature and mature DCs, respectively. Furthermore, it has been reported that naïve T cells also express CCR7 and respond to MIP-3 β (Sallusto and Lanzavecchia, 2000). The MIP-3 β -dependent recruitment of both mature DCs and naïve T cells to the same area of the lymph node fits well with the role of DC in the initiation of primary immune responses. The physical interactions between DCs and naïve T cells have also been demonstrated in vivo (Ingulli et al., 1997; Ingulli et al., 2002; Stoll et al., 2002). DCs not only respond to different chemokine signals, but they also secrete various chemokines such as MIP-1 α , MIP-1 β , RANTES, MCP-1 and IL-8 (Zhou and Tedder, 1995). This suggests that chemokines regulate the trafficking of DCs, which in turn secrete different chemokines to facilitate the migration of other leukocytes. In summary, maturation of DCs is a critical step for their ability to function as potent APCs. This maturation is influenced by the expression of different chemokine receptors that in turn plays a critical role in regulating their trafficking from the site of inflammation into the secondary lymphoid organs, where they stimulate T cell proliferation.

1.5.3 Properties of DCs

DCs possess several features that distinguish them from other professional APCs. It has been reported that the expression of MHC class II on DCs is 10-100 times higher than that on B cells or macrophages (Inaba et al., 1997). At a single cell level, the expression of MHC class II on a DC may reach more than 10⁶ molecules (Steinman, 1996). In addition to the quantitative

differences with regard to MHC class II expression on the various APCs, DCs exhibit some qualitative differences. In culture, the peptide-MHC class II complexes are retained for prolonged periods (1-2 days) on DCs (Cella et al., 1997; Pierre et al., 1997; Pure et al., 1990), whereas B cells and macrophages have a turnover measured in hours (Harding et al., 1989). It has also been reported that mature DCs have an increased ability to load MHC class II with antigenic peptide as compared with self peptide (Inaba et al., 2000). Mature DCs have also been reported to up-regulate the expression of costimulatory molecules such as B7-1, B7-2 and CD40, which provide an essential signal for T cell stimulation (Inaba et al., 1994; Caux et al., 1994). The expression of CD40 on DCs has been demonstrated to be critical for DC survival and function besides its role in costimulation. Indeed, crosslinking of CD40 rescues DCs from apoptosis by up-regulating the anti-apoptotic gene Bcl-2 (Caux et al., 1994; Wong et al., 1997), and provides important signals for IL-12 production (Cella et al., 1996; Koch et al., 1996). DCs express LFA-1, the ligand for a variety of adhesion molecules including ICAM-1, ICAM-2 and ICAM-3. They also express LFA-3 adhesion molecules, which bind to the CD2 molecules on T cells. These adhesion molecules are not only important for DC-T cell binding, but also play an essential costimulatory function. Indeed, antibodies to LFA-1 and LFA-3 have been reported to inhibit DC-stimulated allogeneic T cell (MLR) responses (Prickett et al., 1992).

It is now well established that mature DCs are the most potent professional APCs (Faustman et al., 1984; Forbes et al., 1986; Larsen et al.,

1990; Steinman, 1991). Although other APCs can stimulate previously primed T cells, DCs have a unique ability to cluster and activate multiple unprimed T cells (Austyn et al., 1988b; Metlay et al., 1989). The clustering of T cells by DCs has been observed to occur not only *in vitro* but also *in vivo*, emphasizing the physiological significance of this binding for T cell activation (Ingulli et al., 1997; Ingulli et al., 2002; Stoll et al., 2002). Most importantly, DCs are able to bind naïve T cells in both an antigen-dependant and -independent fashion (Inaba and Steinman, 1986), whereas other APCs can not. It is believed that the ability of DCs to bind T cells in an antigen-independent fashion is to permit T cells to scan the different peptide-MHC class II complexes on the DC surface. This process, thus, facilitates the binding of antigen-specific TCR on a T cell with rare peptide-MHC class II complexes on a DC, which in turn involves the interaction of various costimulatory receptor ligand pairs resulting in T cell activation (Banchereau and Steinman, 1998; Austyn et al., 1988b; Flechner et al., 1988).

The initial antigen-independent binding of resting T cells by DCs has recently been demonstrated to occur via DC-SIGN, a DC-specific receptor (Geijtenbeek et al., 2000). Interestingly, the DC's DC-SIGN bind to ICAM-3, which is expressed on resting T cells. After the DC-SIGN/ICAM-3 binding establishes the antigen-independent interaction between the DC and the T cell, other receptor ligand interactions become involved to mediate the antigen-dependant clustering (Geijtenbeek et al., 2000; Inaba and Steinman, 1987; Hauss et al., 1995; Starling et al., 1995; Scheeren et al., 1991). These receptor-ligand pairs include LFA-1/ICAM-3, ICAM-1/LFA-1 and LFA-3/CD2. LFA-1 is

present in its inactive form on DCs and has low affinity for ICAM-1 and -3 on the T cell (Geijtenbeek et al., 2000). However, the involvement of DC's LFA-1 in clustering increased drastically after 15 min of interaction with resting allogeneic T cells. This is consistent with previous reports where the blockage of LFA-1 was found to affect cluster stability but not initial clustering (Inaba and Steinman, 1987). This specialized ability of DCs to cluster resting T cells suggests that the I-Synapse between DCs and resting T cells is substantially different than between other APCs and activated T cells.

1.6 Preliminary evidences demonstrating the involvement of DC's cytoskeleton in the I-Synapse formation

While other APC's cytoskeletons have been shown to play a passive role in the I-Synapse formation, there is some preliminary evidence in the literature that might suggest the involvement of the DC's cytoskeleton in this process. Almost 15 years ago, Austyn *et al* presented a scanning electron micrographs of a DC-T cell conjugate showing the DC's dendrites wrapped around the T cell (Austyn et al., 1988b). This micrograph provided direct evidence of changes in the DC shape during binding to a T cell, but it failed to demonstrate whether this binding was mediated via the DC's cytoskeleton. However, they reported that treatment of DCs with formaldehyde, which among other effects interferes with cytoskeletal rearrangement, inhibited their ability to activate T cells (Austyn et al., 1988b). This suggests that the integrity of the DC's cytoskeleton is an important factor for T cell activation.

Another study using electron microscopy demonstrated that while formation of DC-T cell conjugates is antigen-independent, contact area between the two cells increased significantly in the presence of antigen (Delon et al., 1998b). Interestingly, the contact areas that formed between a DC and a T cell conjugate are larger as compared with those formed between a B cell and a T cell conjugate. While this is not a definitive proof, the fact that the contact areas in DC-T cell conjugates are larger than B-T cell conjugates might suggest that this increase resulted from DC rather than T cell actin cytoskeletal rearrangement.

WASp, which is known to be involved in regulating the actin cytoskeleton, has been mostly studied in T cells. Previous studies demonstrated T cells from WAS patients and WASp knockout mice have defects in both antigen receptor-induced proliferation and regulation of the actin cytoskeleton (Molina et al., 1993; Gallego et al., 1997; Snapper et al., 1998). Interestingly, DCs from WAS patients which have a phenotype very similar to normal DCs, exhibit cytoskeletal abnormalities that impede their motility, a key step for DC function as APCs (Binks et al., 1998). This suggests that the integrity of both DC's and T cell's cytoskeleton is important for effective T cell activation.

Finally, I investigated the expression of fascin during the maturation of mouse DCs. Fascin is a 55-kDa, actin-bundling protein, that bundles actin filaments into tertiary structures (Edwards and Bryan, 1995). Its expression in humans has only been detected in DCs, and not in any other leukocytes examined, including monocytes, B cells and T cells (Mosialos et al., 1996). Furthermore, fascin expression was found to be restricted to IDCs, which are

present in the T cell areas of the lymph nodes. I have demonstrated that fascin is expressed only in mature DCs. Similar to human, its expression was restricted to IDCs in the lymph node. I and others have also shown that fascin is involved in the development of dendrites in DCs (Al Alwan et al., 2001a; Ross et al., 1998). When I inhibited fascin expression by 70%, using an anti-sense oligonucleotide, there was a pronounced effect on DC cytoskeletal structure with 85% of DCs losing their dendrites. Most importantly, inhibition of fascin expression, which did not affect MHC class II or B7-2 expression, markedly reduced the ability of DCs to activate T cells. This was the first evidence directly linking the DC's actin cytoskeleton to their ability to activate T cells and suggested that the DC's actin cytoskeleton might be involved in formation of the I-Synapse (Al Alwan et al., 2001a).

1.7 Rationales and Objectives

It has now become clear that the formation of a stable I-Synapse between a T cell and an APC is crucial for productive T cell activation (Bromley et al., 2001). While the T cell's cytoskeleton plays an active role in the I-Synapse formation, it has been widely believed that the APC's cytoskeleton play a passive role in this process (Valitutti et al., 1995a). However, this conclusion has been based on studies of activated T cells with B cell or surrogate APCs (Grakoui et al., 1999; Kupfer and Singer, 1989b; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Pardi et al., 1992; Valitutti et al., 1995a; Wulfiging et al., 1998). DCs are different from other APCs in that they are superior in stimulating

activated T cells and have a unique capability to cluster and stimulate resting T cells, leading to the initiation of a primary immune response (Austyn et al., 1988b; Metlay et al., 1989). In addition, it has been demonstrated that the communication between DCs and T cells is mutual rather than being dominated by one side (Banchereau and Steinman, 1998). This suggests that the formation of the I-Synapse between a DC and a T cell is substantially different from that seen in activated T cell-APC interaction. Since DCs and T cells communicate in a bi-directional fashion and previous studies have already provided convincing evidence of the critical role that T cell's cytoskeletal changes play in their activation, it is possible that corresponding changes occur at the DC level. DC cytoskeletal changes may play a role in providing the appropriate microenvironment for optimal T cell activation.

The overall objectives of the proposed study are: 1) to examine whether the DC's cytoskeleton plays an active role in the establishment and stability of I-Synapse with resting/naïve CD4⁺ T cells; and 2) to evaluate the mechanisms by which such cytoskeletal reorganization may contribute to T cell activation and the signals that mediate these changes. The results are presented in a style of two chapters. The first chapter is entitled "Dendritic cell actin cytoskeletal rearrangement is critical for the formation of the immunological synapse". This chapter shows the role of the DC's cytoskeleton in the establishment of the I-Synapse with resting CD4⁺ T cells using an allogeneic system. The second chapter is entitled "Dendritic cell actin cytoskeletal participation in the immunological synapse formation is highly antigen-dependent". Using an OVA-

specific transgenic system, this chapter addresses that in contrast to CD4⁺ T cells, DC cytoskeletal participation in the I-Synapse occurs in an antigen-dependent fashion and is mediated via MHC class II crosslinking.

MATERIALS AND METHODS

2.1 Animal

Adult BALB/c and C57BL/6 mice were purchased from Charles River Canada and housed in the Carleton Animal Care Facility (Sir Charles Tupper Medical Building, Dalhousie University, Halifax). The DO11.10 transgenic mice expressing MHC class II (IA^d)-restricted TCR specific for the chicken OVA₃₂₃₋₃₃₉ were obtained from NIH/NIAID (Bethesda, MD).

All animals were housed in compliance with the guidelines established by the Canadian Council on Animal Care and were given standard rodent chow and water *ad libitum*.

2.2 Media

RPMI-1640 liquid medium (Sigma-Aldrich.) was supplemented with 10% heat-inactivated (30 min, 65⁰C) fetal calf serum (FCS; Gibco BRL), 100U/ml penicillin, 100ug/ml streptomycin, 50μM of 2-mercaptoethanol (BDH) under aseptic conditions. This complete RPMI 1640 (cRPMI) was stored at 4⁰C and only used for DC culture. For all other cultures, RPMI-1640 liquid medium containing 5% heat-inactivated FCS, 100U/ml penicillin and 100ug/ml streptomycin, was used unless otherwise specified.

Purified and deionized water (dH₂O; Milli-QTM Water System, Corning) was sterilized by autoclaving in glass bottles. Phosphate buffered saline (PBS; pH 7.4) was prepared as a 25x stock containing 3.1M NaCl (BDH Inc), 1.1M K₂HPO₄

(Sigma-Aldrich) and 239mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (Fisher Scientific) in dH_2O . The 25X stock was stored at room temperature. Before use, the 25X stock was diluted 25 times in sterile dH_2O and the 1X PBS stored at room temperature.

2.3 Reagents

Solutions of 1% (w/v) paraformaldehyde (Fisher Scientific) in PBS, 0.1% saponin (Sigma-Aldrich) in PBS were filtered with Whatman #1 filter papers (Fisher Scientific) and stored at 4°C . A solution of 0.1% Triton-X (Sigma-Aldrich) in PBS was also prepared and stored at 4°C . The 1% bovine serum albumin (BSA) solution was made fresh on the day of use, whereas the 1% paraformaldehyde, the 0.1% saponin and the 0.1% Triton-X were discarded after approximately two wk.

Acetate buffer solution (100mM; pH5.2) was prepared by mixing 100mM $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (Sigma-Aldrich) and 100mM CH_3COOH (Fisher Scientific) and stored at 4°C . The 100mM acetate buffer was prepared fresh every week. Glycine-Lysine buffer was prepared by mixing 10mM of L-Lysine (Sigma-Aldrich) and 100mM of Glycine (Gibco BRL) in 1 liter of dH_2O . Dextran-Tris buffer pH 7.4 was prepared by dissolving 130 μM of dextran (PharMacia Fine Chemical) in 1 liter of 50mM Tris-base (Boehringer Mannheim Corporation). The Glycine-Lysine buffer and the Dextran-Tris buffer were kept at 4°C and discarded after approximately a month. Acetate buffered formalin (10%) was prepared by mixing 200ml of 37% formaldehyde and 350mM $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 2 liters of dH_2O .

All actin polymerization inhibitors were dissolved in DMSO, stored at -20°C and used in the dark. Cytochalasin D was purchased from Sigma-Aldrich, while Jasplakinolide and Latrunculin A were purchased from Molecular Probes. The agonist (OVA₃₂₃₋₃₃₉; ISQAVHAAHAEINEAGR) and the control (OVA₃₂₄₋₃₃₄; SQAVHAAHAEI) peptides were purchased from Bethyl Laboratory, USA. The purity of the peptides was greater than 98% as assessed by HPLC.

2.4 Antibodies

A panel of mouse, rat, or hamster antibodies was used in the immunocytochemistry and flow cytometry studies. All antibodies were titered and the optimal dilution was used. The anti-fascin monoclonal antibody (mouse IgG1) was purchased from DAKO. Anti-CD4 (GK1.5; rat IgG2b), PE-anti-CD44 (IM7.8.1; rat IgG2b), PE-anti-CD62L (MEL-14; rat IgG2a), anti-Fc γ RII/III (CD32/CD16; mouse IgG2a), anti-Thy1.2; (CD90; mouse IgG2b), anti-CD11c (N418; hamster IgG), anti-B7-2 (RMMP-2; rat IgG2a), anti-CD11b (Mac-1; rat IgG2b), FITC-anti-B220 (CD45R; rat IgG2a) and FITC/PE conjugated secondary antibodies were purchased from Cedarlane, Canada. Anti-LFA-1 α (CD11a; rat IgG2a) and anti-ICAM-1 (CD54; hamster IgG) monoclonal antibodies were purchased from Endogen, USA. PE- anti-MHC class II (2G9; rat IgG2a) antibody was purchased from BD PharMingen. Anti-talin (8D4; mouse IgG1), FITC-anti- β tubulin (TUB2.1; mouse IgG1) and anti- γ tubulin (GTU-88; mouse IgG1) monoclonal antibodies were from Sigma-Aldrich. FITC-anti-phosphotyrosine

(4G10; mouse IgG2b) monoclonal antibody was from Upstate Biotechnology. FITC-antigenotypic (KJ1-26; mouse IgG2a) TCR monoclonal antibody that recognizes OVA₃₂₃₋₃₃₉-specific transgenic TCR was purchased from Caltag, USA. AlexaTM 488 (green) and 594 (red) goat anti mouse IgG and Alexa FluorTM 568 (red) and 488 (green) phalloidin were purchased from Molecular Probes. All isotype control antibodies were purchased from Cedarlane Canada Ltd. The polyclonal anti-actin (rabbit IgG) and the peroxidase-conjugated anti-rabbit antibody were from Sigma-Aldrich. For negative controls, either isotype-specific IgG or PBS supplemented with 1% BSA were substituted for the primary antibody.

2.5 Flow cytometric analysis

Cells were suspended in 100 μ l of PBS supplemented with 1% BSA. The cell suspension was then incubated with the appropriate primary antibody. All antibodies were incubated for 30-45 min at 4⁰C. The Alexa, FITC- and PE-labeled primary or secondary antibodies were incubated in the dark.

Fascin is a cytoplasmic protein and thus permeabilization was required prior to the addition of the anti-fascin monoclonal antibody. For permeabilization, cells were incubated with 100% methanol (BDH Inc) for 30 min at room temperature. For all other surface marker staining, cells were directly incubated with the primary antibodies followed by appropriate secondary antibody. Cells were washed twice with 1% BSA-PBS after each step and fixed with 1% paraformaldehyde-PBS. Fluorescence and mean channel number were analyzed on a total of 10,000 cells

per sample using a flow cytometer (Becton Dickinson FACScan). WEHI164 cells (ATCC; CRL-1751) were maintained in the lab and used as a positive control for fascin staining (Ross et al., 1998).

2.6 Cell preparation

DCs were prepared from BALB/c bone marrow essentially as previously described (Lutz et al., 1999). Briefly, marrow was flushed out of mouse femurs and tibias using cold PBS. Red blood cells were removed by hypo-osmotic shock (4ml of 0.2% NaCl/20 sec + 4ml of 1.6% NaCl to bring solution to an isotonic equilibrium) and washed 3 times with PBS. Red blood cell-free bone marrow cells were cultured in 100mm diameter petri dish (Falcon/ Becton Dickinson) at 2×10^6 in 10ml of cRPMI supplemented with 200U/ml recombinant mouse GM-CSF (rmGM-CSF; Peprotech). On day 3, the cultures were fed with fresh 10ml of cRPMI supplemented with 200U/ml rmGM-CSF. On day 6, 10ml of the supernatant was aspirated from the petri dish and spun down. The supernatant was removed and the pellet was resuspended in fresh 10ml of cRPMI supplemented with 200U/ml rmGM-CSF and return into the culture. On day 9 of culture, the non-adherent cells were aspirated, spun down and the pellet was resuspended in a new petri dish containing 10ml of cRPMI supplemented with 100U/ml rmGM-CSF plus 1ug/ml LPS (Sigma-Aldrich). The non-adherent cells were harvested after 24 h and evaluated for DC phenotype. Flow cytometry examination confirmed that the resulting DCs were routinely > 94% CD11c, MHC Class II, B7-2, LFA-1, ICAM-1 and CD48 positive (Fig. 1).

For B cell enrichment, BALB/c spleen was homogenized to achieve a single cell suspension and red blood cells were lysed. Cells were then resuspended in 2% FCS/PBS and loaded into a commercially available mouse B cell recovery column kit (Cedarlane, Canada). B cells were negatively selected through the column as recommended by the manufacturer. B cells purity was routinely > 92% using B220 (anti-CD45R) on FACS analysis (Fig 2).

T cells were purified by homogenizing C57BL/6 (allogeneic) or BALB/c (syngeneic) spleen to achieve a single cell suspension. After red blood cells were lysed, the cells were suspended in 5% FCS-RPMI and loaded into a nylon wool column. Following 1 h incubation at 37°C, cells were collected from the column and T cell purity was routinely 92% using CD90 staining on FACS analysis. For resting allogeneic or syngeneic CD4⁺ T cell enrichment, nylon wool-purified T cells were further passed through a commercially available mouse CD4 cell recovery column kit (Cedarlane, Canada). This kit enriched CD4⁺ T cells by negative selection. CD4 purity was routinely > 90% using GK1.5 (anti-CD4) on FACS analysis (Fig 3). To generate activated CD4⁺ T cells, resting CD4⁺ T cells were treated with phorbol 12-myristate 13-acetate (PMA, 15ng/ml) and ionomycin (500ng/ml) for 48 h.

For naive DO11.10 transgenic CD4⁺ T cell enrichment, nylon wool-purified spleen T cells were further passed through a naive CD4 recovery column (R&D Systems). This kit enriched naive CD4⁺ T cells by negative selection. The resulting CD4⁺ cells were naive as assessed by CD62L⁺ and CD44^{low} expression on FACS analysis (Fig. 4). Routinely, greater than 82% of the resulting CD4⁺ T

cells express the TCR transgene as assessed by FACS analysis using KJ1-26, an anticonotypic TCR monoclonal antibody that recognize OVA₃₂₃₋₃₃₉-specific transgenic TCR (Fig. 5).

Figure 1. A-F, Purity of bone marrow-derived DCs. Day 10 mature DCs were stained with anti-CD11c (A), anti-MHC class II (B), anti-B7-2 (C), anti-LFA-1 (D), anti-ICAM-1 (E) and anti-CD48 (F) antibodies followed by the appropriate fluorescence-conjugated secondary antibodies. The fluorescence of 10^4 cells was then analyzed by FACS. Filled and open areas indicate isotype control and positive staining, respectively.

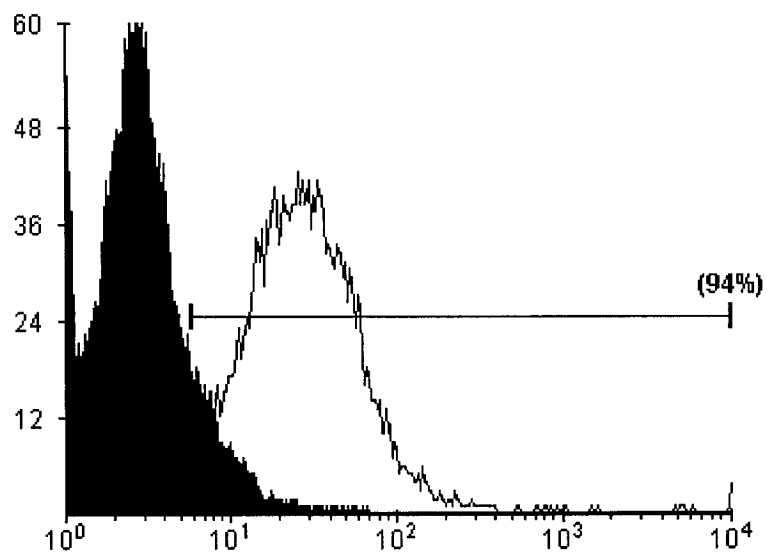


Fig. 1A

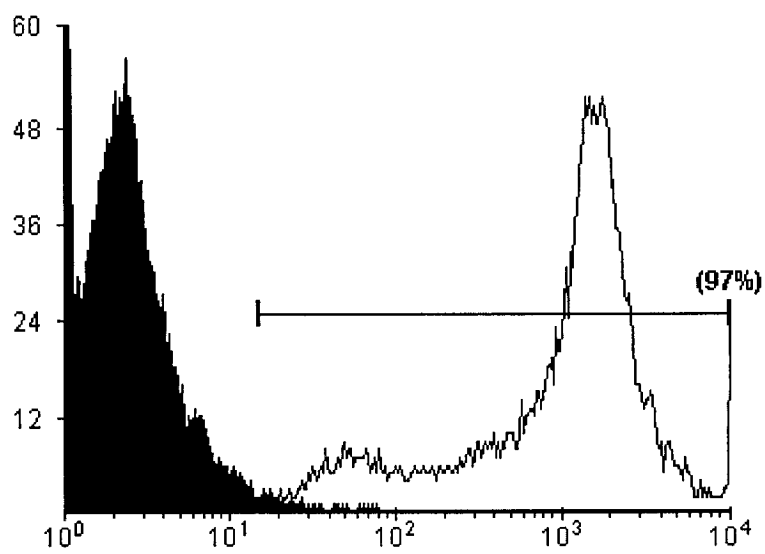


Fig. 1B

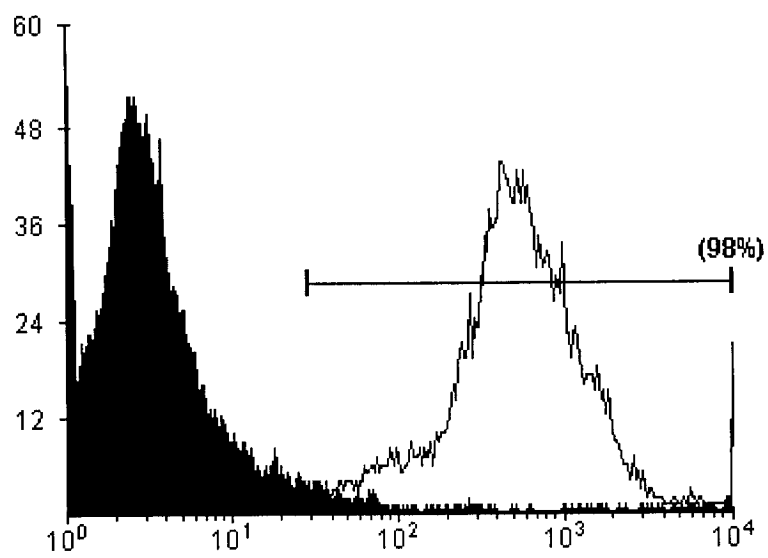


Fig. 1C

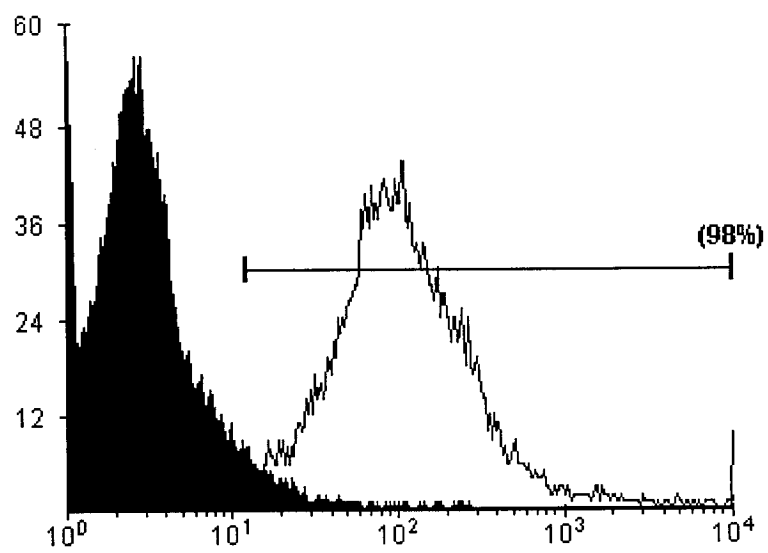


Fig. 1D

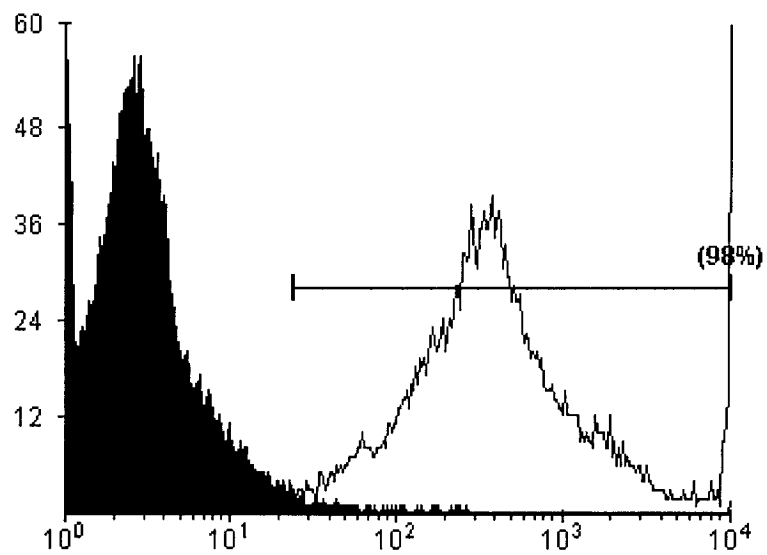


Fig. 1E

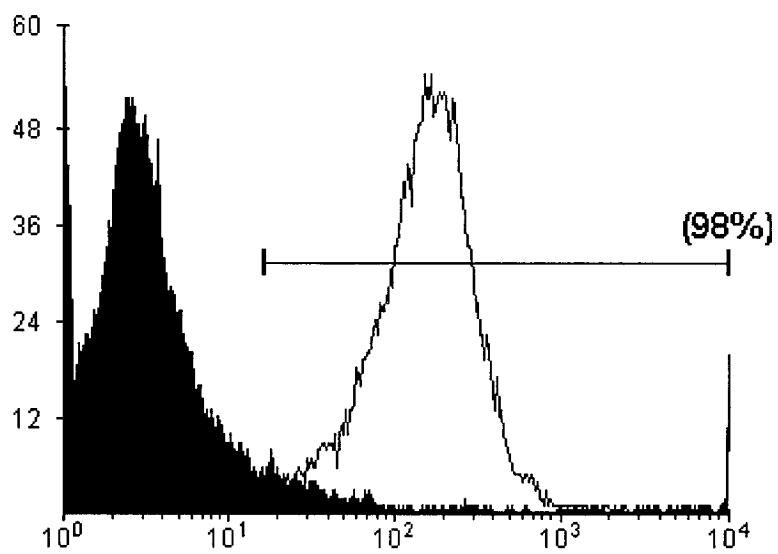


Fig. 1F

Figure 2. Enrichment of B cells from spleen. Negatively selected cells from the B cell enrichment column were stained with FITC-anti-CD45R (B220). The fluorescence of 10^4 cells was then analyzed by FACS. Filled and open areas indicate isotype control and positive staining, respectively.

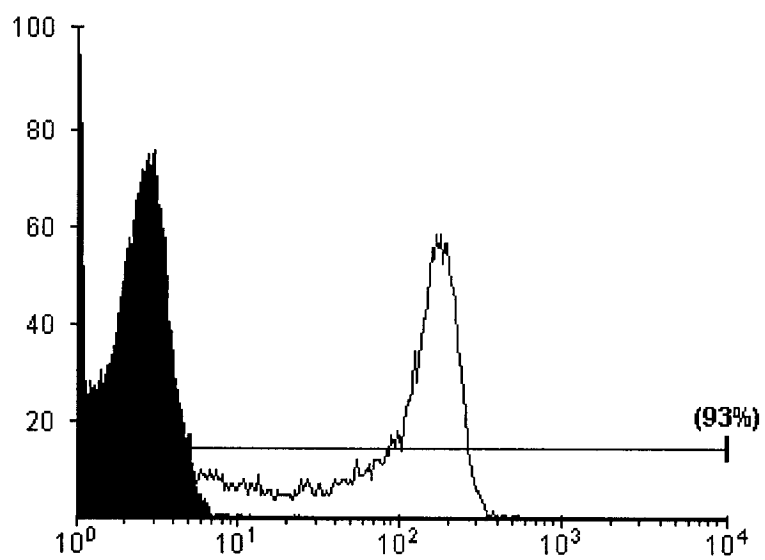


Fig. 2

Figure 3. Enrichment of CD4⁺ T cells from spleen. Negatively selected cells from the CD4⁺ T cell enrichment column were stained with FITC-anti-CD4 (GK1.5). The fluorescence of 10⁴ cells was then analyzed by FACS. Filled and open areas indicate isotype control and positive staining, respectively.

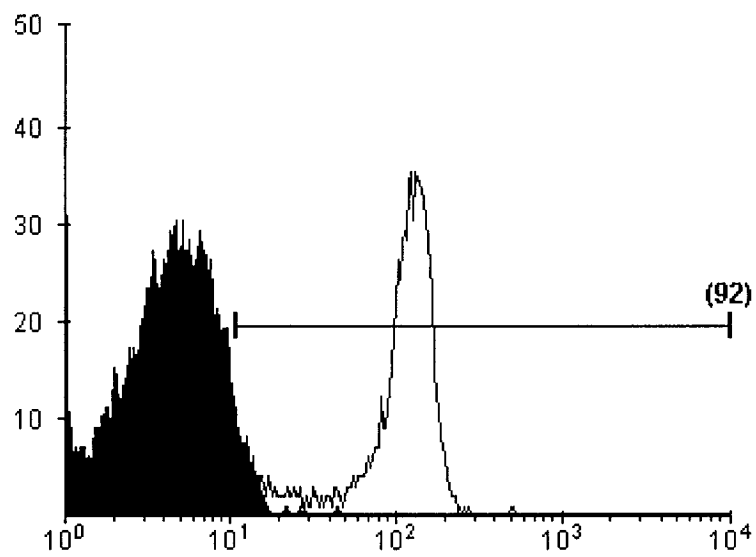


Fig. 3

Figure 4. A and B, Enrichment of naive CD4⁺ cells from DO11.10 spleen.

Negatively selected cells from naive CD4⁺ T cell enrichment column were stained with PE-anti-CD44 (A) or PE-anti-CD62L (B). The fluorescence of 10⁴ cells was then analyzed by FACS. Filled and open areas indicate isotype control and positive staining, respectively.

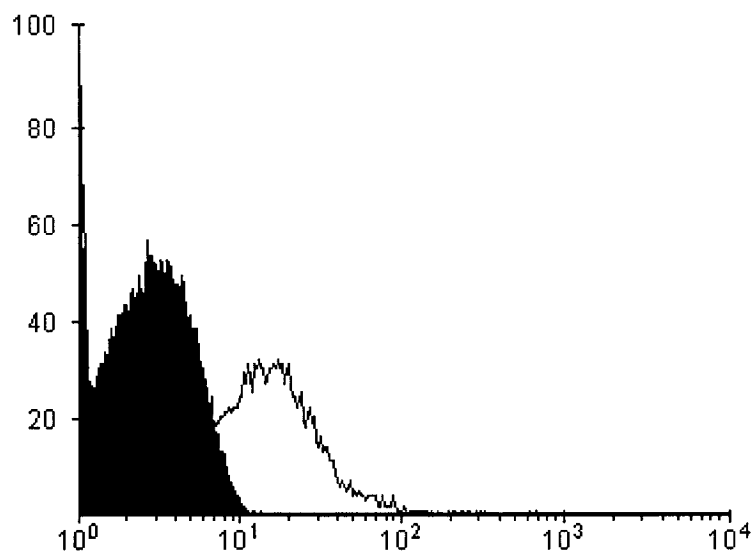


Fig. 4A

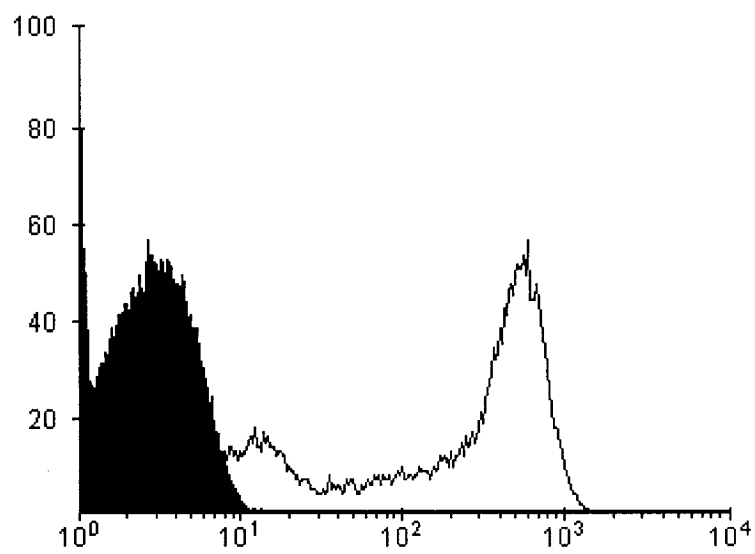


Fig. 4B

Figure 5. Expression of the TCR transgene on the DO11.10 T cells. Negatively selected cells from naive CD4⁺ T cell enrichment column were stained with FITC-anticlonotypic (KJ1-26). The fluorescence of 10⁴ cells was then analyzed by FACS. Filled and open areas indicate isotype control and positive staining, respectively.

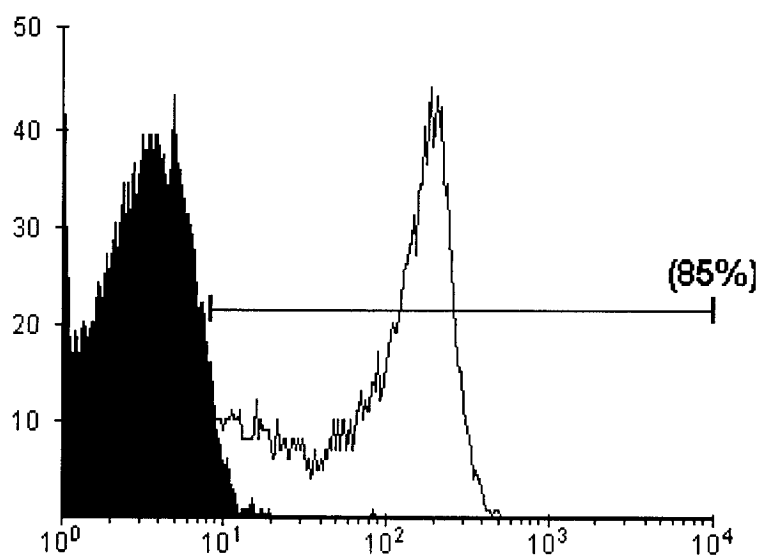


Fig. 5

2.7 DC-T cell clustering assay

DCs or T cells were treated with different doses of the actin polymerization inhibitors for 1 h at 37°C and washed three times before mixing. DCs were labeled with CellTracker green CFMDA (Molecular Probes) and CD4⁺ T cells were labeled with CellTracker CM-Dil dye (Molecular Probes). DCs were centrifuged with syngeneic or allogeneic (resting or activated) T cells (1:3) at 50 x g for 5 min at 4°C in a conical tube. B cells were sometimes used as APCs and were treated with the actin polymerization inhibitors, washed extensively and labeled with CellTracker green CFMDA before mixing with allogeneic T cells as above. After centrifugation, DC-T cells or B cells-T cells were incubated at 37°C in a water bath for 30 min and then resuspended and transferred into flat-bottom 96-well plates (Nunc). DCs were also cocultured, without centrifugation, with allogeneic T cells at a ratio of 1:3 in flat-bottom 96-well plates and clusters were harvested at 0.5, 1, 2, 6, and 24 h. Cocultures were examined using an inverted fluorescent microscope and DC-T cell clusters (defined as DC binding to one or more T cells) were expressed as the percentage of binding DC as follows: percent binding DC = (clustered DC/total DC) x 100. In addition, the numbers of T cells binding to 100 DCs were also expressed in some experiments.

In addition, DCs pulsed with media (control) or various doses of peptide (2 h at 37°C) were pretreated with the different actin polymerization inhibitors (1 h at 37°C) and washed extensively. Pretreated DCs were labeled with CellTracker green CFMDA prior to mixing with CellTracker CM-Dil dye prelabeled naïve DO11.10 transgenic CD4⁺ T cells. DC-T cell conjugates were formed and

analyzed as above. For time course clustering, DCs prepulsed with peptide were mixed with naïve DO11.10 transgenic CD4⁺ T cells and incubated at 37°C in a water bath. Conjugates were formed as above and harvested at the different time points (5, 10, 15, 30, 60 min) followed by an immediate fixation with 1% paraformaldehyde. DC-T cell conjugates were analyzed as above.

2.8 Immunostaining and confocal microscopy

DC-T cell conjugates were formed by centrifuging DCs with T cells (1:3) at 50 x g for 5 min at 4°C in a conical tube. Cells were then incubated at 37°C in a water bath for 30 min, resuspended and plated on poly-L-lysine (Sigma-Aldrich)-coated slides. For time course polarization, DCs prepulsed with peptide were mixed with naïve DO11.10 transgenic CD4⁺ T cells and incubated at 37°C in a water bath. Conjugates were harvested at the different time points (5, 10, 15, 30, 60 min), resuspended 3 times and fixed immediately with 1% paraformaldehyde for 10 min at room temperature. The fixative was then removed by spinning and cell pellets were resuspended in PBS followed by plating on poly-L-lysine coated slides.

For all intracellular staining, slides were fixed in 10% acetate-buffered formalin and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. Slides were then washed before incubation with different antibodies (anti-fascin, anti-talin, anti-β tubulin, anti-γ tubulin, anti-phosphotyrosine) for overnight followed by incubation with the appropriate secondary antibodies for 1 h at room temperature. For double staining of fascin and F-actin, slides were first incubated

with the anti-fascin antibody followed by Alexa 488 (green) goat anti-mouse IgG before incubation with Fluor 546 (red) phalloidin for 30 min. Following staining, all slides were mounted with CITIFLUOR mounting media (Molecular Probes), which is a glycerol/PBS solution containing an anti-fading agent. Fluorescence signals were detected with a Zeiss LSM510 confocal laser-scanning microscope. Conjugates that had T cells binding to only one-half of the DC were captured. The DC's cytoskeleton was scored as polarized if the intensity of staining was greater in the region adjacent to the binding T cell than in unbound regions. At least 50 conjugates were evaluated blindly in each treatment group.

2.9 DC-bead cluster analysis

Carboxylated polystyrene beads (10micron; Polysciences) were first coated with the appropriate secondary antibodies. Beads (4×10^6) were suspended in 1ml of PBS/well in a 24 flat-bottom plate. The secondary antibody (40 μ g) was then added and incubated for 2 h at 37 $^{\circ}$ C with shaking every 30 min. Following incubation, beads were harvested and washed 3 times with PBS (10.000 x g at 4 $^{\circ}$ C for 15 min). In the meantime, DCs were incubated with anti-MHC class II (MK-D6), anti-MHC class I (34-5-8S), anti-B7-2 (RMMP-2), anti-LFA-1 (FD441.8) or no primary antibodies for 45 min at 4 $^{\circ}$ C. The cells were then washed 3 times with PBS to remove excess antibodies. DCs coated with antibodies against individual surface proteins were incubated with beads pre-labeled with the appropriate secondary antibodies to crosslink the

corresponding surface receptors. DC-beads (2:1) were incubated in a 37°C water bath for 30 min, resuspended 3 times and plated on poly-L-lysine-coated slides. Slides were fixed, stained for F-actin and analyzed for polarization as above.

2.10 T cell activation

In the allogeneic study, the different groups of DC were treated with 25µg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C. Treated DCs were then applied in graded doses to the different groups of allogeneic CD4⁺ T cells (2 x 10⁵). Cultures were maintained in 200µl cRPMI in U-shaped 96-well plates (Nunc) for 4 days. The cells were pulsed with 1µCi/ml of [³H] thymidine (ICN Pharmaceuticals) in the last 18 h of incubation. T cell proliferation was assessed by harvesting the cells on filtermats using a cell harvester (Skatron) and measuring the [³H] thymidine uptake in a liquid scintillation counter (Beckman Coulter).

In the transgenic study, DCs pulsed with media (control) or various doses of peptide were treated with 25µg/ml mitomycin C for 30 min at 37°C. Treated DCs were then added to the different groups of naïve DO11.10 transgenic CD4⁺ T cells (10⁵). Cultures were maintained in 200µl cRPMI in U-shaped 96-well plates for 3 days. The cells were pulsed with 1µCi/ml of [³H] thymidine in the last 18 h of incubation. T cell proliferation was assessed as above.

2.11 Western blot:

Actin was analyzed by Western blotting essentially as described by Patterson *et al* (Patterson et al., 1999). DCs were treated with different doses of the actin polymerization inhibitors or DMSO (control) for 1 h at 37°C. Cells were washed 3 times with cRPMI and returned to culture. After 45-60 min, cells were washed 3 times with cold PBS and lysed for 30 min at 4°C with 100µl of lysis buffer (1% Triton X-100, 20mM HEPES-NaOH [pH 7.2], 100mM NaCl, 1mM sodium orthovanadate, 50mM NaF, 1mM PMSF, 1mM aprotinin, 1mM leupeptin). Cells were sonicated for 4 min at room temperature to disrupt membranes. The lysate was spun at 10,000 x g for 20 min. Supernatants containing the detergent-soluble cell fraction were transferred into another tubes and labeled as G-actin. Pellets were dissolved for 1 h at 4°C with 100µl of RIPA buffer (15mM HEPES-NaOH [pH 7.5], 0.15mM NaCl, 1% Triton- X-100, 1% sodium deoxycholate, 0.1% SDS, 10mM EDTA, 1mM DTT, 1mM sodium orthovanadate, 1mM PMSF, 1mM aprotinin, 1mM leupeptin). This step gave the detergent-insoluble fraction (F-actin). Protein was assayed in both solutions (G-actin and F-actin) using the standard lowery method. Equal amounts of protein (25µg) were run on precast gradient (4-20% SDS-PAGE) gels (BIO RAD). Western analysis was performed using a 1:1000 dilution of the polyclonal actin antibody followed by peroxidase-conjugated secondary antibody and visualized by chemiluminescence.

2.12 Semiquantitative reverse transcriptase-polymerase chain reaction

(RT-PCR):

OVA₃₂₃₋₃₃₉ peptide (3-300nM) pulsed DCs were pretreated with actin polymerization inhibitors or DMSO (control) and washed extensively before incubation with naïve transgenic CD4⁺ T cells. Total RNA was isolated from culture at the different time points (0, 2, 4, 6, 8 h) as previously described (Makrigiannis et al., 2001) using TRIzol reagent (Life Technologies) as recommended by the manufacturer. To determine IL-2 and GAPDH mRNA expression, cDNA was synthesized by reverse transcription of ~1µg RNA with 200U Moloney murine leukemia virus in the presence of 1µg random hexanucleotide primers and 0.5mM dNTPs. The reaction mixture was incubated for 1 h at 37°C and for 10 min at 94°C. At the end of the incubation, the volume was adjusted to 0.1ml with pyrogen-free water. The quantity of RNA employed in the RT-PCR reaction was electrophoresed and bands of 18S and 28S ribosomal RNA were used as a visual control for equal template loading.

For PCR, each reaction used a 50µL volume of cDNA, 2.5U Taq DNA polymerase (Life Technologies), 0.2mM dNTPs, and 50mM each primer pair in a 1:10 dilution of PCR buffer [2M KCl, 1M Tris-HCl, pH 8.4, 1M MgCl₂, 1mg/ml bovine serum albumin (BSA)]. The PCR mixtures were overlaid with 100µl mineral oil. The amplification protocol for GAPDH (30 cycles), and IL-2 (29 cycles) was as follows: denaturation at 92°C (GAPDH) or 94°C (IL-2) for 30 sec, annealing at 57°C for 30 sec, and primer extension at 72°C for 1 min (IL-2) or 1.5

min (GAPDH). The number of PCR cycles used was determined previously to generate PCR product during the exponential phase of amplification. GAPDH was normalized for all samples before running the IL-2 mRNA. The primers used were: GAPDH (F) 5'-ACTCACGGCAAATTCAACGGC-3'; GAPDH (R) 5'-ATCACAAACATGGGGGCATCG-3'; IL-2 (F) 5'-TGATGGACCTACAGGAGCTCCTGAG-3'; IL-2 (R) 5'-GAGTCAAATCCAGAACATGCCGCAG-3'. PCR products were visualized by electrophoresis across an ethidium bromide-stained 1.5% agarose gel. The PCR product size for GAPDH (247 bp) and IL-2 (170 bp) was confirmed with controls.

2.13 IL-2 bioassay:

The IL-2-dependent CTLL-2 cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in complete medium supplemented with 10% FCS and 20U/ml recombinant mouse IL-2 (rmIL-2; R&D Systems). Prior to use in experiments, CTLL-2 cells were washed twice and resuspended in IL-2-free medium. Cells were subsequently incubated at 37°C for 1 h, washed and seeded into a 96-well flat-bottomed plate (Sarstedt) at 10^4 cells/well. DCs were pretreated with the different actin polymerization inhibitors and washed extensively before incubation with OVA₃₂₃₋₃₃₉ (300nM) and naïve transgenic CD4⁺ T cells in U-bottomed 96-well plates. After 12-24 h, cell-free supernatants were collected from these cultures and immediately added at 50µl aliquots into IL-2-deprived CTLL-2 cells and the final volume was adjusted to 200µl/well. RmIL-2 was also used at 1-50U/ml in parallel as a control. The

cultures were incubated at 37°C for 24 h. The cells were pulsed with 0.5 μ Ci/ml of [³H] thymidine for the final 8 h of the cultures and [³H] thymidine uptake by CTLL-2 cells was determined as a measure of IL-2 bioactivity in the culture supernatants.

2.14 Statistical analysis:

Statistical significance was assessed using a one-way ANOVA (GraphPad InStat; GraphPad, San Diego, CA). Where not significant (ns) indicates a *p* value of >0.05, * indicates a *p* value <0.05, ** indicates a *p* value <0.01, and *** indicates a *p* value <0.001.

CHAPTER 1

“Dendritic cell actin cytoskeletal rearrangement is critical for the formation of the immunological synapse”

3.1 Preamble

T cell activation is a complex process that occurs in a well-coordinated manner to ensure specificity and efficacy. This process is initiated when a TCR on a T cell recognizes a processed antigenic peptide in the context of an MHC molecule on an APC (Zinkernagel and Doherty, 1974). While this step is critical for the initiation of T cell activation, recognition of peptide-MHC complex by TCR does not necessarily result in a complete T cell activation. Sustained TCR engagement, which is essential for productive T cell activation, requires the formation of a specialized junction called an I-Synapse at the interface between a T cell and an APC (reviewed in (Bromley et al., 2001)). Formation of the I-Synapse is a dynamic process resulting from active T cell actin cytoskeletal rearrangement (Kupfer and Singer, 1989b; Pardi et al., 1992). It is characterized by the accumulation of F-actin, other cytoskeletal proteins such as talin and MTOC, and surface molecules such as CD4 and LFA-1 in the T cell at the contact point with the APC (Kupfer and Singer, 1989b). Following T cell stimulation with anti-CD3 antibodies localized increases in Ca^{+2} fluxes were observed in the area where the TCR polarized (Khan et al., 1992). This finding further emphasizes the importance of the surface and signaling molecule polarization.

Subsequent studies have shown that these active changes in the T cell's cytoskeleton result in the dynamic clustering of cell surface receptors and signaling molecules into a SMAC at the interface with the APC (Grakoui et al., 1999; Monks et al., 1997; Monks et al., 1998). The SMAC is a specialized structure that

organizes proteins in and on a T cell into two distinct regions. Peripheral zone (pSMAC) is composed of LFA-1 and talin surrounding a central zone (cSMAC) containing the TCR, CD3 and p56^{lck}, p59^{fyn} and protein kinase C. Drugs such as CytD, which disrupt actin cytoskeletal rearrangement (Carter, 1967; Flanagan and Lin, 1980), were employed to examine the role of the T cell's actin cytoskeletal rearrangement in this process. The integrity of the T cell's cytoskeleton is of major functional importance, because treatment of T cells with CytD prevents surface receptor clustering, IL-2 production and proliferation (Holsinger et al., 1998; Kong et al., 1998; Wulfing et al., 1998). In summary, productive T cell activation depends on T cell actin cytoskeletal rearrangement leading to formation of the I-Synapse. These active changes in the T cell's cytoskeleton serve to increase TCR signaling by concentrating both TCR and peptide-MHC complexes as well as signaling molecules rich-lipid rafts in the center of the synapse (Bromley et al., 2001; Delon and Germain, 2000; Penninger and Crabtree, 1999). The stability of this structure encourages sustained TCR engagement and provides an optimal microenvironment for downstream signaling events that lead to an effective activation of T cells (Grakoui et al., 1999; Valitutti et al., 1995a). Finally, formation of the I-Synapse may facilitate the engagement of small molecules such as TCR and MHC, thereby enhancing antigen recognition by T cells.

While the T cell's cytoskeleton plays an active role in formation of the I-Synapse, the APC's cytoskeleton has been thought to play only a passive role in this process (Valitutti et al., 1995a). However, all previous studies of the I-Synapse have used Jurkat T cell lines (Dustin et al., 1998; Lowin-Kropf et al.,

1998; Sedwick et al., 1999), T cell clones (Kupfer and Singer, 1989b; Kupfer et al., 1991; Monks et al., 1997; Monks et al., 1998), T cell hybridomas (Stowers et al., 1995) or activated T cells (Grakoui et al., 1999). In addition, the APCs used in previous studies were mostly either B cell lymphoma or surrogate APCs. B cell's cytoskeleton did not polarize during interaction with T cells (Kupfer and Singer, 1989b; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Pardi et al., 1992; Valitutti et al., 1995a; Wulfing et al., 1998). Furthermore, inhibition of the B cell cytoskeletal rearrangement had no effect on T cell binding or activation (Valitutti et al., 1995a; Wulfing et al., 1998). Finally, the role of the cytoskeletal reorganization in the establishment of the I-Synapse has been mostly examined in situations where target cells or the APCs (B cells) required mediators released by T cells. The study of the I-Synapse under these conditions may have mistakenly lead to the conclusion that the APC's cytoskeleton does not actively participate in T cell activation. These studies may have underestimated the importance of the APC's cytoskeleton especially since none of these studies were carried out using primary naïve T cells and potent APCs such as DCs.

Primed T cells may be activated by all professional APCs, whereas naïve T cells may only be clustered and activated by DCs (Inaba and Steinman, 1986). The potency of DCs is not due to one surface or secreted molecule. Although DCs are known to be very efficient in forming MHC-peptide complexes (Inaba et al., 2000; Turley et al., 2000), this mechanism alone does not account for their potency as APCs. Activated B cells express similar levels of MHC class II and

yet they are less efficient than DCs as APCs (Metlay et al., 1989), suggesting differences between DCs and B cells in the array of peptides that they presented or the context in which they presented them. In comparison with B cells and macrophages, DCs have the ability to retain the peptide-MHC class II complexes on their surface for prolonged periods (Cella et al., 1997; Pierre et al., 1997; Pure et al., 1990; Harding et al., 1989), which in turn enhances their recognition by TCRs on T cells. Furthermore, previous studies showed that newly formed peptide-MHC complexes and CD86 are transported together in vesicles during DC development (Inaba et al., 2000; Turley et al., 2000). Upon arrival at the surface of mature DCs, peptide-MHC and CD86 are deposited as stable clusters. The expression of special accessory molecules may be a contributing factor to the efficacy of DCs as APCs, but these molecules are also shared with other APCs that are not as potent as DCs. This suggests that DCs may possess other mechanisms that enhance their ability to bind and activate naïve T cells more specifically. The specialized ability of DCs to cluster and activate naïve T cells suggests that the I-Synapse between DC and naïve T cells is substantially different (Metlay et al., 1989).

In this study, I demonstrate that in contrast to the results seen with B cells, DCs actively polarize their F-actin and fascin, an actin-bundling protein, during binding with CD4⁺ T cells. DC actin cytoskeletal rearrangement was critical for the clustering and activation of resting, but not activated T cells. These findings indicate an important role for the DC's actin cytoskeleton in the establishment of the I-synapse with resting CD4⁺ T cells.

3.2 Results

3.2.1 Polarization of the DC's actin cytoskeleton during I-Synapse formation with allogeneic CD4⁺ T cells

Polarization of surface receptors would not be indicative of an active process because it occurs in B cells and surrogate APCs (Grakoui et al., 1999). Therefore, I evaluated the role of the DC's cytoskeleton during interaction with allogeneic CD4⁺ T cells by examining the localization of F-actin and fascin. Fascin is an actin-bundling protein that is expressed primarily in mature DCs and has an important role in dendrite formation (Ross et al., 1998) and T cell activation (Al Alwan et al., 2001a). F-actin (Fig. 1, A and B) and fascin (Fig. 1, C and D) were uniformly distributed around the periphery of the cell in unclustered DCs. When DCs were clustered with CD4⁺ T cells, there was a pronounced focal polarization of both F-actin (Fig. 1, E and F) and fascin (Fig. 1, G and H) toward the contact site with the T cells. Colocalization of fascin, which is only expressed in DCs, with F-actin demonstrated that the F-actin was accumulating in the DC not just the T cell at the point of contact (Fig. 1I). All clustered DCs in the cocultures were scored as polarized or nonpolarized by comparison of F-actin distribution in T cell contact areas with areas that did not contact T cells. Focal polarization occurred as early as 30 min and was present at all time points examined in the cocultures (0.5, 1, 2, 6, and 24 h). These results demonstrate that unlike other APCs, DCs are actively involved in formation of the I-Synapse through rearrangement of their actin cytoskeleton.

DCs are potent at clustering syngeneic T cells in an antigen-independent manner which likely allows sampling of MHC peptide by the T cell (Inaba and Steinman, 1986). When syngeneic T cells clustered with DCs, there was very little polarization of F-actin (Fig. 2A) or fascin (Fig. 2B). Focal polarization of DC's F-actin was present in 84% of the clustered allogeneic DC group but only in 22% of syngeneic DCs (Fig. 2C). This suggests that DC formation of the I-Synapse is an antigen-dependent event.

3.2.2 The DC's actin cytoskeleton is crucial for the binding of resting allogeneic CD4⁺ T cells

To evaluate the functional significance of DC actin cytoskeletal rearrangements, I examined the effects of inhibiting DC F-actin formation with CytD before the clustering of DCs and T cells. CytD is a cell-permeable fungal toxin, which is a potent inhibitor of F-actin formation and cytoskeletal function (Cooper, 1987), and inhibition of T cell actin polymerization with CytD prevents I-Synapse formation (Wulfing et al., 1998). I generated clusters by centrifuging DCs and T cells together at low speed and clusters were counted at 30 min. The level of F-actin in the CytD-pretreated DCs was reduced by 47%, as assessed by Western blot at 1 h after the treatment, compared with control DCs (Appendix Fig. 1). When T cells were pretreated with CytD, their ability to cluster with DCs was reduced by 46% as has been previously described (Fig. 3A) (Wulfing et al., 1998). Importantly, pretreatment of DCs with CytD reduced clustering with untreated T cells by 76% (Fig. 3A). This is in direct contrast to similar

experiments conducted previously using B cells as APCs in which pretreatment of the B cells with CytD had no effect on cluster formation or T cell activation (Wulfing et al., 1998). In our hands, the overall clustering of B cells with resting allogeneic CD4⁺ T cells was very poor, with no significant difference seen in the percentage of B cells clustered between the control ($3.5 \pm 2.5\%$) and the CytD-pretreated group ($4.1 \pm 1.4\%$). To confirm that the observed inhibition was due to the effect of CytD on DC actin cytoskeletal rearrangement, DCs were also pretreated with Jasplakinolide (Jasp), which prevents actin rearrangement by stabilizing the F-actin rather than inhibiting its formation (Bubb et al., 1994). Pretreatment of DCs with Jasp reduced clustering with untreated T cells by 68% (Fig. 3B). Clustering of syngeneic T cells was not affected by pretreatment of DCs with CytD (Appendix Fig. 2), which is consistent with the lack of polarization observed in the DCs in syngeneic DC-T cell clusters.

I next evaluated the functional effects of the DC's actin cytoskeleton on the clustering of resting or activated T cells. DCs are much more potent at clustering resting T cells than other APCs; however, activated T cells are capable of clustering with B cells and macrophages as well as with DCs (Metlay et al., 1989). Clustering of resting T cells was significantly inhibited by pretreatment of DCs with CytD, whereas clustering of activated T cells was not significantly inhibited (Fig. 3C). Similar results were seen with Latrunculin A (LatA; (Spector et al., 1989)), which inhibits actin cytoskeletal rearrangement by a different mechanism (Appendix Fig. 3). This suggests that although activated T cells can form an I-

Synapse with any APC, resting T cells require active participation of the DC's cytoskeleton.

3.2.3 The DC's actin cytoskeleton is critical for resting allogeneic T cell activation

To evaluate the role of the DC's actin cytoskeleton in T cell activation, I performed an MLR after pretreatment of DCs or T cells or both with CytD. Consistent with previous studies, pretreatment of the T cells significantly inhibited the MLR (Wulfing et al., 1998). However, I found that inhibition of the MLR was greater when the DCs were pretreated (Fig. 4A). DCs treated with CytD or LatA for 1 h did not differ significantly compared with untreated DCs in the expression of MHC class II, B7-2, or fascin as detected by flow cytometry (Appendix Fig. 4, A-C). MLR inhibition was seen at all doses of CytD used (Fig. 4B) and at different stimulator to responder ratios (Fig. 4C). There was also a dose response with greater doses of CytD resulting in greater inhibition of T cell proliferation (Fig. 4B). There was a strong correlation ($r = 0.99$) between the clustering seen and the degree of T cell activation seen in the different treatment groups. Pretreatment of DCs with LatA resulted in a similar inhibition of T cell activation (Appendix Fig. 5, A and B). In contrast to DCs, the overall proliferation of resting CD4⁺ T cells with B cells was very poor, consistent with the clustering data, but there was no significant difference between the control and the CytD-pretreated B cell group (Appendix Fig. 6, A and B).

Figure 1. a–i, DC clustering allogeneic CD4⁺ T cells show polarization of fascin and F-actin. DC were clustered with allogeneic CD4⁺ T cells by centrifugation at low speed in a conical tube followed by incubation at 37°C for 30 min. Unclustered DC or DC clustering CD4⁺ T cells were allowed to settle on poly-L-lysine-coated slides. Slides were then fixed, stained, and examined under confocal microscopy for the distribution of F-actin and fascin. F-actin (a) and fascin (c) localization in unclustered DC. F-actin (e) and fascin (g) polarization in DC clustering CD4⁺ T cells. Copolarization of fascin and F-actin (i) in DC clustering CD4⁺ T cells. Right column micrographs (b, d, f, and h) indicate the staining intensity (blue to red = low to high).

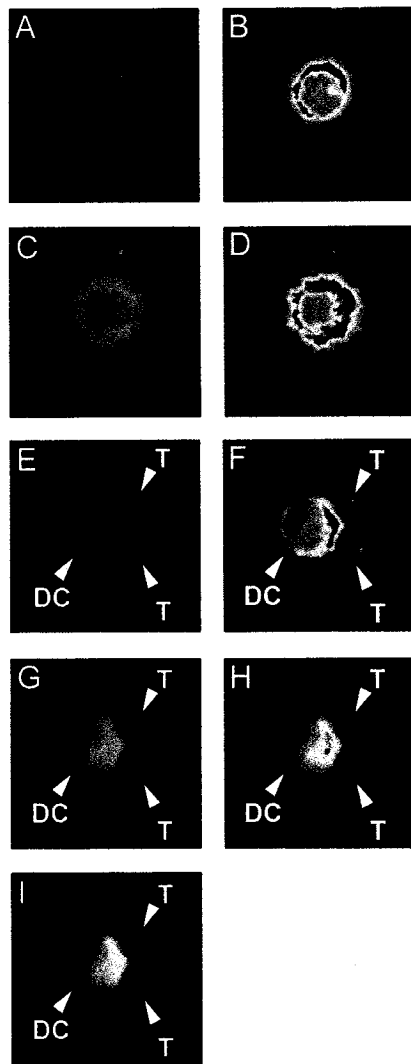


Fig. 1 (A-I)

Figure 2. a–c, DC clustering syngeneic CD4⁺ T cells have markedly reduced F-actin and fascin polarization. DC were clustered with syngeneic CD4⁺ T cells by centrifugation at low speed in a conical tube followed by incubation at 37°C for 30 min. Clusters were then evaluated for F-actin and fascin localization under confocal microscopy. Distribution of F-actin (a) or fascin (b) was observed in DC clustering syngeneic CD4⁺ T cells. The percentage of DC with F-actin polarization toward the T cell (c) during interaction with either allogeneic or syngeneic CD4⁺ T cells. At least 50 conjugates were evaluated in each treatment group in three independent experiments.

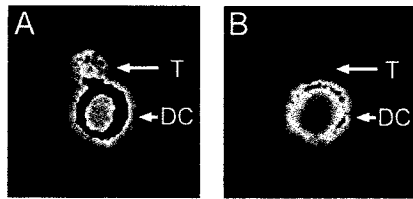


Fig. 2 (A-B)

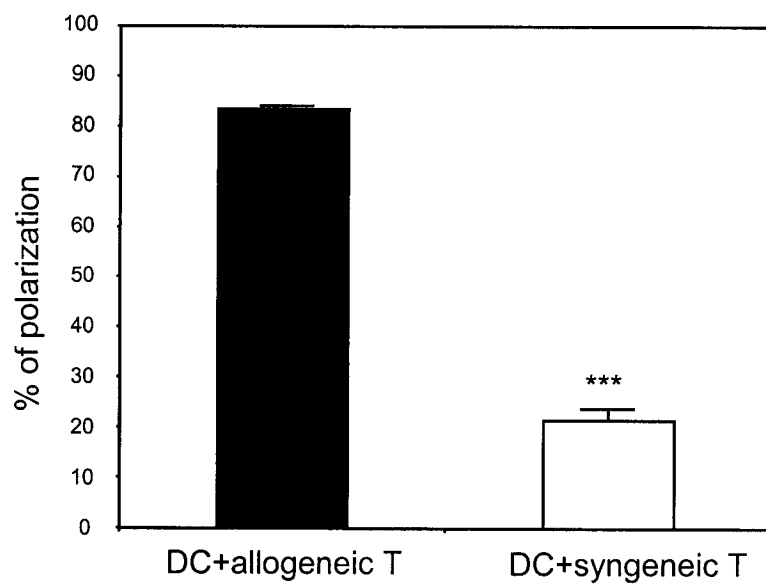


Fig. 2C

Figure 3. a–c, Pretreatment of DC with CytD inhibits their ability to cluster resting but not activated allogeneic CD4⁺ T cells. DC were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. a, Control or CytD (20 μM)-pretreated DC were mixed with control or CytD (20 μM)-pretreated resting CD4⁺ T cells. b, Control or Jas (10 μM)-pretreated DC were mixed with resting CD4⁺ T cells. c, Control or CytD (20 μM)-pretreated DC were mixed with either resting or activated CD4⁺ T cells. Clusters were formed by low-speed centrifugation of DC and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean ± SD of the percentage of the DC clustering CD4⁺ T cells and are representative of three independent experiments.

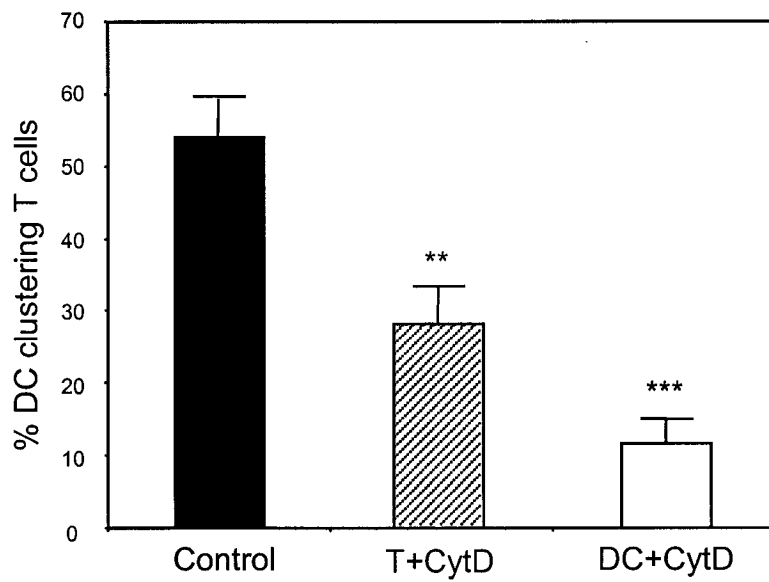


Fig. 3A

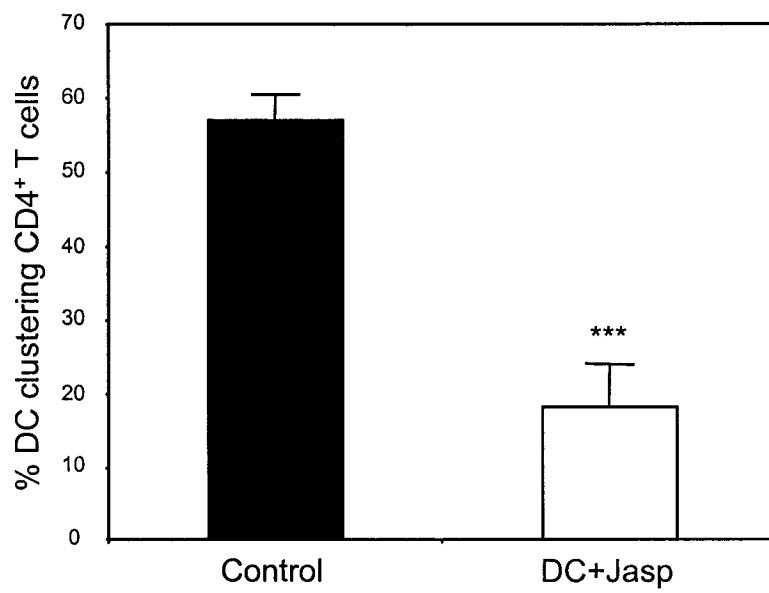


Fig. 3B

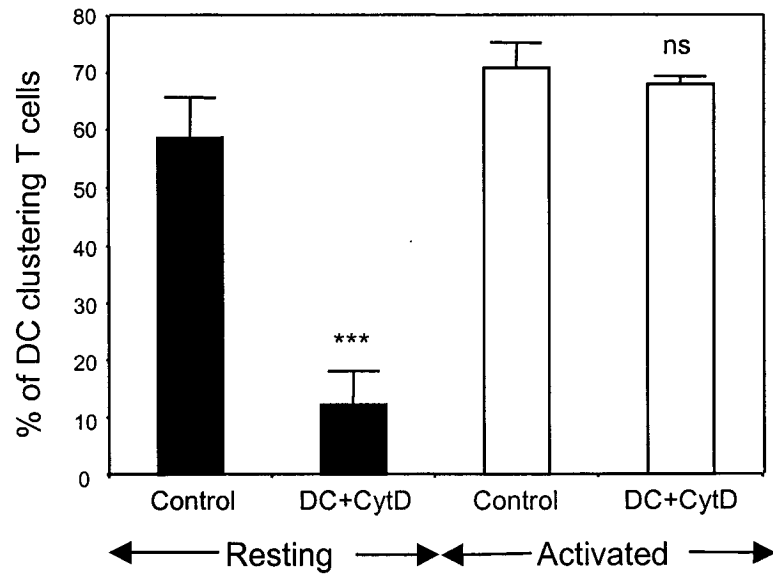


Fig. 3C

Figure 4. a–c, Pretreatment of DC with CytD inhibits their ability to activate CD4⁺ T cells. a, Control or CytD (20 μM)-pretreated DC were mixed with control or CytD (20 μM)-pretreated CD4⁺ T cells. b, Either DC or CD4⁺ T cells or both were pretreated with different doses of CytD (2–40 μM) before mixing. c, Control or CytD (20 μM)-pretreated DC were mixed with CD4⁺ T cells at different DC:T cell ratios. The cells were pulsed with [³H]thymidine in the last 18 h of the 4-day MLR. T cell proliferation was assessed by measuring the [³H]thymidine uptake in a liquid scintillation counter. The results are expressed as mean dpm ± SD and are representative of five independent experiments.

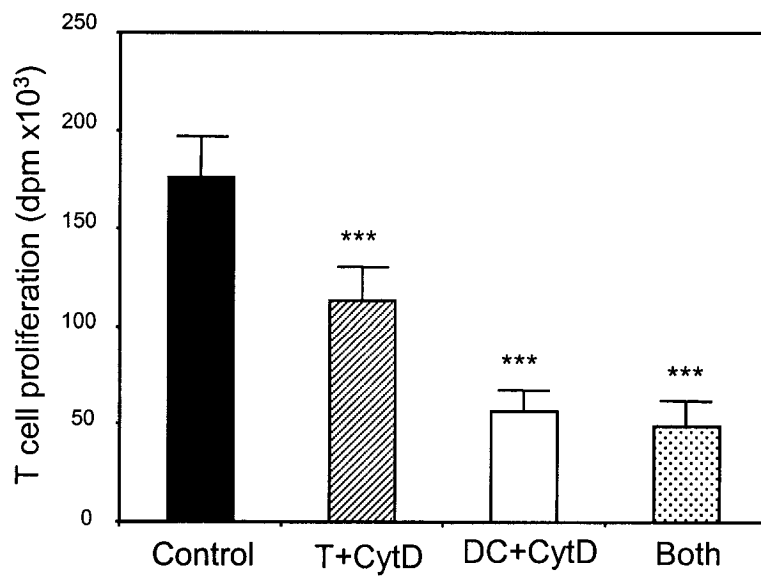


Fig. 4A

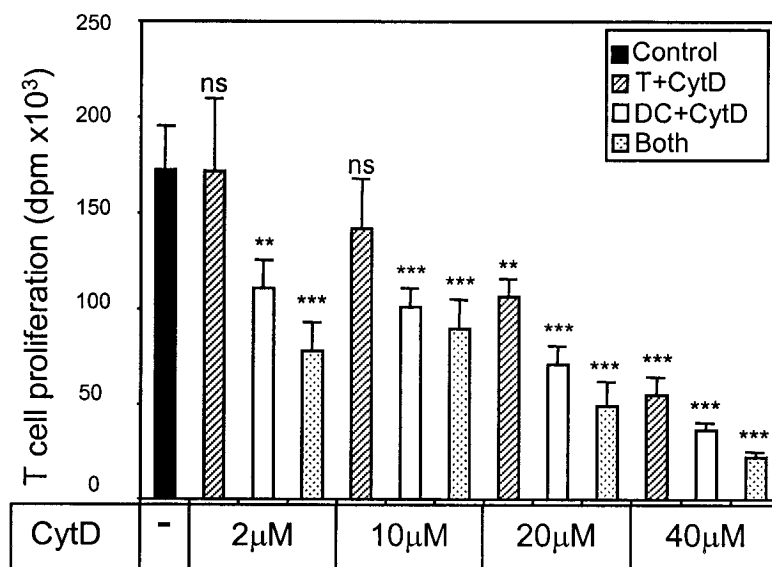


Fig. 4B

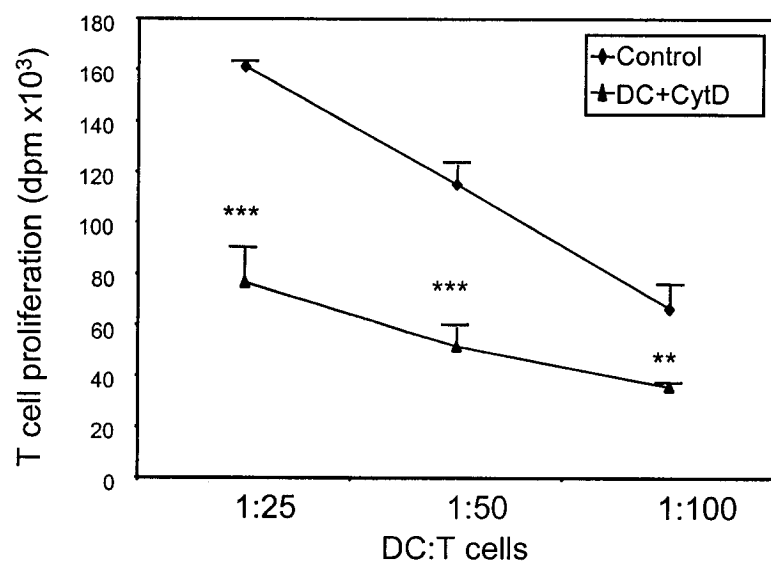


Fig. 4C

3.3 Discussion

Previous studies provided compelling evidence that emphasized the importance of T cell actin cytoskeletal rearrangement for formation of the I-Synapse, as a critical step for productive T cell activation. While the T cell's cytoskeleton plays an active role in formation of the I-Synapse, the APC's cytoskeleton has been thought to play only a passive role in this process (Valitutti et al., 1995a). However, careful analysis of these studies may explain why the APC's cytoskeleton was not perceived as being actively involved in formation of the I-Synapse. Firstly, all previous studies of the I-Synapse have used Jurkat T cell lines (Dustin et al., 1998; Lowin-Kropf et al., 1998; Sedwick et al., 1999), T cell clones (Kupfer and Singer, 1989b; Kupfer et al., 1991; Monks et al., 1997; Monks et al., 1998), T cell hybridomas (Stowers et al., 1995) or activated T cells (Grakoui et al., 1999). Activation of previously primed T cells has different requirements than that of the naïve T cells. Most importantly, the required duration of binding between an APC and activated T cell (1 hour) is much less than that between an APC and naïve T cells (20 hours) (Iezzi et al., 1998). The stability of the I-Synapse is probably more critical for the optimal activation of unprimed T cells because they need to interact with the APC for a longer time period. Therefore, it is conceivable that the APC's cytoskeleton might play an essential role to establish a stable I-Synapse with unprimed compared with previously primed T cells. Therefore, the use of activated T cells in these studies may have mistakenly lead to the conclusion that the APC's cytoskeleton does not actively participate in T cell activation.

Secondly, the APC used in previous studies were mostly either B cell lymphoma or surrogate APCs. Neither of these were potent APCs and they may not necessarily reflect physiological APCs. In the studies where B cells were used as APCs, their cytoskeleton did not polarize during interaction with T cells (Kupfer and Singer, 1989b; Lowin-Kropf et al., 1998; Monks et al., 1997; Monks et al., 1998; Pardi et al., 1992; Valitutti et al., 1995a; Wulfing et al., 1998). Most of these studies used talin as a surrogate marker for F-actin. Their rationale for this is never discussed. However, T cells have a very small amount of cytoplasm and thus it is hard to visualize their F-actin. It may be that examination of F-actin in some of these situations would have shown polarization. However, consistent with the lack of cytoskeletal polarization observed in B cells, inhibition of their cytoskeletal rearrangement had no effect on T cell binding or activation (Valitutti et al., 1995a; Wulfing et al., 1998).

In addition to the studies with B cells, others created surrogate APCs by plating fluorescently labeled (green) peptide-MHC class II complexes and (red) ICAM-1 onto a glass-supported planar bilayer membrane (Grakoui et al., 1999). Using real-time imaging, Grakoui *et al* showed that T cells are able to form synapses when plated on these membranes. The I-Synapse develops over a period of min following the interactions of the T cells and the antigen-presenting membrane. Initially (first 0.5 min), TCR was engaged by peptide-MHC complex and LFA-1 was engaged by ICAM-1 in the periphery and center of the synapse, respectively. Within 5 min, the engaged peptide-MHC complexes moved to the center of the synapse (cSMAC), while engaged ICAM-1 moved to the periphery

(pSMAC). Movement of molecules into and out of the synapse is a dynamic process dependent on the T cell's actin cytoskeleton, because it was inhibited by CytD treatment. In this particular study, Grakoui *et al* used super-activated T cells and the peptide concentration was extremely high (100 μ M). It should also be noted that the density of the agonist peptide-MHC complexes that induced formation of the I-Synapse and correlated with full T cell activation was ≥ 60 molecules/ μm^2 , whereas 0.2 molecules/ μm^2 was enough to trigger T cell proliferation. At such a high dose and density (100 molecules/ μm^2) of specific peptide, which may not normally be found on APCs, the results reported above may not necessarily reflect changes that would occur under physiological conditions.

Finally, the role of the cytoskeletal reorganization in the establishment of the I-Synapse has been mostly examined in situations where target cells or the APCs (B cells) required mediators released by T cells. Therefore, T cell cytoskeletal rearrangement may facilitate the polarized secretion by T cells of mediators directed toward B cells or target cells. This may explain the polarization of the T cell's but not the B cell's or target cell's cytoskeleton during I-Synapse formation. Furthermore, B cells do not bind well to naïve T cells, rather they interact with primed T cells and thus may not need to rearrange their cytoskeleton. Formation of the I-Synapse between naïve T cells and APCs is likely to be different. In this situation, one would predict that the APC will rearrange its cytoskeleton to enhance binding to and polarized secretion of

activation mediators toward naïve T cells, thereby resulting in improved stimulation signals. Such changes might have significant impact on effective naïve T cell activation and the generation of primary immune responses.

Altogether, previous studies demonstrated that formation of the I-Synapse is a dynamic process critical for productive T cell activation. While formation of these specialized structures depends on the active T cell's cytoskeletal rearrangement, it was widely believed that the APC's cytoskeleton plays only a passive role in this process. However, one should keep in mind that the basis for these conclusions relied on studies with activated T cells and/or B cell lymphoma or surrogate APCs. While these findings have increased our understanding of the I-Synapse and its significance for efficient T cell activation, they may have underestimated the importance of the APC's cytoskeleton especially since none of these studies were carried out using primary naïve T cells and potent APCs such as DCs.

The results summarized in this chapter demonstrate that unlike other APCs, the DC's actin cytoskeleton is actively involved in forming I-Synapse with resting allogeneic CD4⁺ T cells. The dynamic involvement of the DC's cytoskeleton in the establishment of I-Synapse is characterized by focal polarization of F-actin and fascin, a DC-specific actin-bundling protein, at the interface with the T cell. DC actin cytoskeletal rearrangement is critical for their function as APC, because drugs that inhibit DC's actin polymerization reduced their ability to cluster and activate resting allogeneic T cells.

3.3.1 Focal polarization of DC's cytoskeleton during I-Synapse formation with allogeneic T cells

Previous studies have demonstrated that formation of the I-Synapse results in polarization of surface receptors on both T cells and B cells/surrogate APCs (reviewed in (Bromley et al., 2001)). However, disruption of the T cell's and not the APC's cytoskeletal rearrangement inhibited the polarization of surface receptors on APCs (Monks et al., 1998; Wulfing et al., 1998). This suggests that while the movement of the surface receptors on T cells is a dynamic process facilitated by the T cell's cytoskeletal rearrangement, polarization of surface receptors on the APC occurs passively because it was inhibited only when the T cell's cytoskeletal rearrangement was perturbed. This suggestion is further supported by the fact that T cells were able to form I-Synapse and polarize their surface receptors even when they were mixed with surrogate APCs (Grakoui et al., 1999). Consistent with the study of T-B cell conjugates, polarization of the T cell's surface receptors at the interface with surrogate APCs was inhibited when the T cell's cytoskeleton was disrupted. The APC cell surface receptors may be pulled into the I-Synapse passively by simply binding to their corresponding ligands on the T cells (Wulfing et al., 1998). Alternatively, the geometry of attachment to T cell may simply cause the APC surface receptor movement into the synapse without cytoskeletal reorganization (Qi et al., 2001). Therefore, the movement of a cell surface receptor into the I-Synapse *per se* is not indicative of the active involvement of this cell in the I-Synapse formation. Rather, reorganization of a cell actin cytoskeleton leading to

surface receptor polarization is a sign of the active involvement of this cell in the process.

DCs possess several features that distinguish them from other APC, suggesting that they play a dynamic role during their interactions with resting T cells (Steinman, 2000). In order to evaluate whether the DC is actively involved in the I-Synapse formation, I examined whether DCs alter their actin cytoskeletal distribution during interaction with resting allogeneic CD4⁺ T cells. I evaluated the distribution of F-actin, which is the basic structural unit of the cytoskeleton. However, F-actin is expressed in both DC and T cells and thus it was important to resolve whether these changes occurred in the DC or the T cell. To eliminate any confusion, I used fascin, an actin-bundling protein, which is expressed in DCs but not in T cells. In a previous study, I and others demonstrated that the expression of fascin is restricted to mature DC and it is also involved in dendrite formation (Al Alwan et al., 2001a; Ross et al., 1998). In addition, I found that fascin expression in mature DC is critical for T cell activation (Al Alwan et al., 2001a), providing the first direct evidence that the DC's cytoskeleton is critical for their function as APCs. Therefore, the use of fascin along with F-actin to examine cytoskeletal changes not only allows DC's cytoskeleton to be discriminated from T cells, but it is also an excellent marker of mature DCs, which are the most potent in T cell clustering and binding (Banchereau et al., 2000).

During binding to resting allogeneic CD4⁺ T cells, DCs polarize their F-actin at the interface with the T cells. In addition, fascin also polarizes and

colocalizes with F-actin, indicating that the F-actin was accumulating in the DCs at the point of contact. Fascin polarization at the interface with the T cell may suggest a role for dendrite formation in T cell activation such as increasing the surface area of contact between the DC and the T cell. This suggestion is further supported by scanning electron micrographs showing DC's dendrites wrapping around the T cells (Austyn et al., 1988a). The expression of fascin in mature DCs may also allow the extension of adhesion receptor rich-lamellipodia (Adams, 1997) towards the T cells, providing a better environment for binding and signal transduction between the two cells. Epstein-Barr virus (EBV)-transformed B cells, which express fascin, are able to bind resting CD4⁺ T lymphocytes in antigen-independent fashion (Mazerolles et al., 1990) and initiate their activation (Kuntz et al., 1976; Mosialos et al., 1994). Thus, the expression of fascin in DCs only when they mature and its recruitment at the interface with the T cells suggests an essential role for fascin in formation of the I-Synapse. The observed polarization is not an artifact of DC binding to T cells, because both F-actin and fascin were uniformly distributed around the periphery of unclustered DC. Furthermore, no polarization was observed when a DC binds to another DC. Interestingly, while DC can cluster syngeneic T cells (Inaba and Steinman, 1986), very little polarization of F-actin was seen in DC binding syngeneic as compared with allogeneic CD4⁺ T cells. Although not a definitive proof, the data suggests that DC's actin cytoskeletal participation in the I-Synapse occurs in an antigen-dependent manner.

The role of antigen in the T cell's actin cytoskeleton polarization is somewhat controversial, but it is widely believed that these changes occur in an antigen-dependent manner (Grakoui et al., 1999; Kupfer et al., 1986; Kupfer et al., 1987; Kupfer and Singer, 1989b; Valitutti et al., 1995a). It is thus likely that the antigen-dependent involvement of both the DC's and T cell's actin cytoskeleton in formation of the I-Synapse is to ensure firm binding and promote sustained TCR triggering in the presence of the appropriate antigen. Altogether, the data implies that contrary to previous studies that used other APCs, the cytoskeleton of DCs is actively involved in forming the I-Synapse. Most importantly, participation of DC's cytoskeleton in the I-Synapse appears to occur in a tightly regulated fashion, because it occurs with allogeneic but not with syngeneic T cells.

3.3.2 Clustering of resting allogeneic CD4⁺ T cells is dependent on DC actin cytoskeletal rearrangement

Among APCs, DCs have a unique ability to cluster T cells in both an antigen-dependent and -independent manner (Inaba and Steinman, 1986). Consistent with previous studies, DC clustering of syngeneic T cells in my experiments is much less than clustering of allogeneic T cells. This data along with the fact that I note polarization of the DC's actin cytoskeleton only with allogeneic T cells, suggests a functional link between the observed changes in the DC's cytoskeleton and their increased ability to bind allogeneic T cells. I, therefore, inhibited DC's F-actin formation using CytD, which has been shown to

prevent I-Synapse formation (Wulfing et al., 1998), and examined the effect on their ability to bind T cells. When CytD-pretreated DCs were mixed with untreated T cells, there was a more pronounced inhibition in DC-T cell clustering than when CytD-pretreated T cells were mixed with untreated DCs. My results with DCs are in direct contrast with the Wulfing's findings, where they used B cell lymphoma as APCs and showed that pretreatment with CytD had no effect on clustering or activation of T cells (Wulfing et al., 1998). Consistent with the Wulfing's findings and in spite of the very poor clustering of resting allogeneic T cells by the B cells I used, I observed that pretreatment of B cells with CytD had no effect on T cell clustering or activation. Therefore, unlike B cells, DC are not only reorganizing their cytoskeleton during clustering with T cells, but these rearrangements are critical for their binding ability.

To isolate the observed result to the effect of CytD on the cytoskeleton as opposed to a non-specific effect, I used LatA and Jasp, which inhibit actin rearrangement by different mechanisms. F-actin is a polymer that grows in one direction (the head) through the addition of G-actin monomers. In contrast, F-actin depolymerization results from the removal of a G-actin (monomer) from the tail. CytD binds to the fast growing end of F-actin, resulting in actin depolymerization (Cooper, 1987). LatA induces conformational changes in the fast growing head of F-actin, leading to actin depolymerization (Spector et al., 1989). CytD and LatA may release integrins from the cytoskeleton and prevent them from reattaching, which is likely to be important in creating a firm binding with their corresponding ligands. However, Jasp binds to the tail of F-actin and

prevents actin depolymerization, which ultimately prevents actin polymerization since there will be no free G-actin to be polymerized to the head of F-actin (Bubb et al., 1994). Jasp may act opposite to CytD and LatA by preventing the release of integrins from the cytoskeleton, thereby reducing their lateral movement and binding to the corresponding ligands. Therefore, the three drugs that I used throughout my study interfere with actin rearrangement through different mechanisms.

The observed inhibition in binding was due to the effect of CytD on the DC's actin cytoskeletal rearrangement, because pretreatment of DC with Jasp also reduced clustering with untreated resting allogeneic T cells. In addition, the level of F-actin was significantly inhibited when DCs were pretreated with CytD consistent with CytD's role in inhibiting F-actin formation (Cooper, 1987). In agreement with previous studies, I found that DCs were able to cluster syngeneic T cells, although the percentage of DC-syngeneic T cell clusters were less than that of DC-allogeneic T cell clusters. Interestingly, consistent with the lack of polarization observed in DC during binding with syngeneic T cells, pretreatment of DC with CytD did not affect their ability to cluster syngeneic T cells. This indicates that while DCs are able to bind both syngeneic and allogeneic T cells they rearrange their actin cytoskeleton only during binding with allogeneic T cells. This suggests that DC actin rearrangement is critical for antigen-dependent binding, because CytD treatment inhibits allogeneic but not syngeneic T cell binding.

In contrast to resting T cells, activated T cells may be clustered by all APCs (Metlay et al., 1989) and require less interaction time with the APCs for stimulation (Iezzi et al., 1998). Consistent with my hypothesis, pretreatment of DC with CytD inhibits their ability to cluster resting but not activated allogeneic T cells. The inhibition in binding was a cytoskeleton-dependent event because I obtained similar results with LatA. In addition, this inhibition was not due to cell death, because the drug dose I used did not affect the cell viability. It is unlikely that the inhibition in binding I observed was due to the effect of CytD on the chemokine gradient that mediates DC-T cell interaction, because the DC and T cells were brought together by a low speed centrifugation. Furthermore, CytD increases $[Ca^{2+}]$ mobilization when T cells were stimulated with anti-CD3 crosslinking antibodies (DeBell et al., 1992; Valitutti et al., 1995a), indicating that CytD does not affect the TCR signal transduction machinery. However, in a situation where resting T cells are activated by APC/DCs and thus need to form I-Synapse, pretreatment with CytD of either the DCs or the T cells may reduce the formation of new contacts. Alternatively, CytD may reduce the reassociation of new TCRs with peptide-MHC complexes that have been transiently disassociated from other TCRs.

In contrast to resting T cells, when activated T cells are pretreated with CytD prior to their mixing with untreated DC, there is no inhibition in DC-T cell binding. This suggests that the binding of activated T cells to DCs is less dependent on the cytoskeleton. Alternatively, the combination of PMA plus ionomycin that I used to activate the T cells rather than activation *per se* may

circumvent the need of the T cell's cytoskeleton to bind DC. Indeed, CytD did not inhibit interferon (IFN)- γ production when preactivated T cells were stimulated by PMA plus ionomycin or crosslinking anti-CD3 antibodies (Valitutti et al., 1995a). Moreover, the significance of the cytoskeleton integrity for T cell function has been examined using Vav1-deficient mice. Vav1 is a member of the Rho family guanine nucleotide exchange factors (GEF) that acts upstream of the WASp to regulate the cytoskeletal reorganization following TCR triggering, which is also essential for integrin function (Krawczyk et al., 2002). Following PMA activation, Vav1^{-/-} and Vav1^{+/+} T cells adhere to extracellular matrix (ECM) proteins or recombinant ICAM-1 at a comparable level. However, there was a reduction in Vav1^{-/-} T cell adhesion as compared with Vav1^{+/+} T cells after activation with anti-CD3 crosslinking antibodies. This indicates that PMA bypasses the TCR-proximal signaling events (Pardi et al., 1992) and thus may induce stronger signals than anti-CD3 crosslinking-mediated T cell activation. These signals may allow the PMA-activated T cell binding to be less dependent on the cytoskeleton or alternatively it may restore cytoskeletal integrity. It is important to note that PMA and anti-CD3 crosslinking antibodies are surrogate approaches of T cell activation and are likely to induce different signals than that produced from TCR engagement by peptide-MHC complexes. It is, therefore, possible to envisage that previous studies may have underestimated the need for APC's cytoskeleton when they used activated T cells.

Several mechanisms may account for cytoskeletal rearrangement-mediated binding. Cytoskeletal reorganization may align the cell surface

membranes, thereby increasing the ability of multiple receptor-ligand pairs to bind simultaneously. The cytoskeleton is also known to play a key role in regulating integrin clustering through enhancing their avidity rather than affinity (van Kooyk et al., 1999). Integrins especially the LFA-1:ICAM-1 interactions have been shown to be critical in mediating DC-T cell binding and I-Synapse formation. Although the expression levels of LFA-1 are not different on activated versus resting T cells, LFA-1 is inactive on resting T cells. Activation of LFA-1 on T cells are mediated through CD3 crosslinking (Dustin and Springer, 1989; van Kooyk et al., 1989) or treatment with PMA, which may bypass the TCR-proximal signaling events (Pardi et al., 1992). This process results in a conformational change in the α/β heterodimer of the LFA-1 (Dransfield and Hogg, 1989; van Kooyk et al., 1991), leading to an enhanced binding to its ligand ICAM-1. Therefore, it is possible that the lack of inhibition in binding when PMA-activated T cells were pretreated with CytD stems from the fact that their integrins have already been activated (Pardi et al., 1992; van Kooyk et al., 1999). In addition, LFA-1 activity is thought to be regulated through attachment to the actin cytoskeleton (Hughes et al., 1996; Lu and Springer, 1997; O'Toole et al., 1995). It appears that this regulation occurs in two steps. Firstly, attachment of LFA-1 to the actin cytoskeleton keeps it in an inactive state. Secondly, a temporary release from the cytoskeleton, which may be achieved using CytD (Bleijns et al., 2001), facilitates later diffusion of LFA-1 into clusters, which is an important step for its ability to bind ICAM-1. Following liberation, reattachment to the cytoskeleton is required to mediate and enhance LFA-1 binding to its ligand (van

Kooyk and Figdor, 2000). This has been demonstrated using various concentrations of CytD, which have been shown to have a different effect on the release/attachment of LFA-1 from/to the cytoskeleton. It is, therefore, possible that the CytD dose used in my study freed already activated integrins from the cytoskeleton of activated T cells and thereby the binding to DC was not inhibited. In line with this assumption, Valitutti *et al* showed that addition of CytD to preformed conjugates (preactivated T cells and B cells) has no effect on the number of conjugates (Valitutti *et al.*, 1995a). Furthermore, treatment of the Vav1^{-/-} T cells with Mg²⁺, which activate integrins, restores their binding ability following anti-CD3 triggering (Krawczyk *et al.*, 2002). This not only indicates that the decreased binding of Vav1^{-/-} T cells was integrin-mediated, but it also demonstrates that this inhibition was not due to a defect in integrins *per se* since it may be restored by treatment with Mg²⁺.

The fact that the TCR and MHC molecules are short in size and have weak avidity suggests that the binding of a TCR to a peptide-MHC complex is not the signal that initiates the interactions between a T cell and an APC. In addition, the number of peptide-MHC complexes per cell is very limited (10-1000 copies/cell) and thus one might wonder how so few peptide-MHC complexes on an APC may trigger many antigen-specific TCR on a T cells. On the other hand, while T cells display ~30,000-40,000 TCRs on their surface, only 50-100 peptide-MHC complexes on APC are required for T cell activation. This is in line with the serial triggering model, where the low affinity binding coefficient of the TCRs enable them to dissociate from their ligands (peptide-MHC complexes) once they

have been triggered thereby allowing the serial triggering of other TCRs by the same ligand. Interestingly, DC have the ability to display an increased frequency of MHC molecules loaded with pathogenic-peptide compared with those loaded with self-peptide (Manickasingham and Reis e Sousa, 2000). Therefore, antigen-independent binding between T cells and APCs must precede the TCR signal. Several studies have suggested that ICAM-3, which is expressed at high levels on resting T cells, as being responsible for establishing the initial DC-T cell interactions (De Fougerolles and Springer, 1992; Hauss et al., 1995; Starling et al., 1995; Vilella et al., 1990). In addition to its ability to mediate adhesion, ICAM-3 may also be important for providing the accessory signals necessary for T cell activation (Hernandez-Caselles et al., 1993). Indeed, antibody directed against ICAM-3 results in elevations of intracellular calcium levels and indications of tyrosine phosphorylation (Juan et al., 1994). The integrin LFA-1 has been described as the primary receptor for ICAM-3 (Dustin and Springer, 1989; van Kooyk et al., 1989). However, its affinity for ICAM-3 even after activation is rather low (Binnerts et al., 1994; Van, V et al., 1995).

Geijtenbeek *et al* identified the DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) to be responsible for antigen-independent interactions that take place between human DCs and resting T cells (Geijtenbeek et al., 2000). They demonstrated that although DCs express both DC-SIGN and LFA-1, only DC-SIGN was able to bind ICAM-3. The lack of LFA-1 binding to ICAM-3 was due to the inactivity of LFA-1, because the human T lymphoblastoid cell line (HSB), which expresses similar levels of an active form of LFA-1 (Binnerts and van

Kooyk, 1999) binds ICAM-3 very well. Importantly, DC-SIGN dependent clustering is transient reaching a maximum at 19 min at which time LFA-1 become involved. The significance of DC-SIGN was demonstrated using blocking antibodies. These antibodies inhibited the proliferation of resting T cells, which express ICAM-3, but not activated T cells, which lack the expression of ICAM-3. The DC-SIGN-mediated antigen-independent binding is likely to be a critical step that precedes and promotes TCR signaling. Therefore, DC-SIGN-mediated binding may promote the TCRs to scan the DC surface to identify these small amounts of peptide-MHC complexes, which then enhance this interaction. Subsequently, T cells may respond vigorously to antigens presented by other APCs. The ability of one adhesion molecule to bind various ligands and the multiple interactions make it difficult to investigate the role of a particular adhesion molecule in mediating this binding. Finally, other molecules such as neuropilin-1, a neuronal receptor recently identified in human DCs and resting T cells (Tordjman et al., 2002), may participate in antigen-independent binding.

Integrins, including LFA-1, are thought to be the crucial anchors for the maintenance of stable conjugates between the APCs and T cell in order for proper signaling to occur (Bromley et al., 2001; Dustin and Cooper, 2000). In lymphocytes, the TCR signaling activates integrins by inducing clustering, which increases the integrin avidity for its ligand (Dustin and Springer, 1989; Kucik et al., 1996; van Kooyk and Figdor, 2000). This antigen-dependent binding facilitates a stable conjugate and allows T cell activation to proceed. A similar process may be occurring during the DC formation of the I-Synapse. When an

allogeneic (or antigen specific) T cell is encountered the DC may be receiving a signal analogous to the TCR triggering seen in T cells. This signal to the DC may allow cytoskeletal rearrangement and activation of integrins. This might mediate higher avidity contacts between the DC and the T cell, thereby enhancing conjugate stability and T cell activation.

In summary, the data reported herein not only indicates that DCs rearrange their actin cytoskeleton, but it also demonstrates that this reorganization is important for their ability to bind allogeneic T cells. It also demonstrates that while activated T cells may form an I-Synapse with any APCs, formation of the I-Synapse with resting T cells requires active participation of the DC's cytoskeleton.

3.3.3 DC's actin cytoskeletal rearrangement is critical for T cell activation

Clustering with DCs is known to precede and to be essential for T cell activation (Austyn et al., 1988b). One might, therefore, expect to see a parallel inhibition in T cell activation when the cytoskeleton is perturbed. Indeed, disruption of either the DC's or the T cell's cytoskeleton using CytD significantly inhibits the MLR. However, inhibition of the MLR was always greater when the DC's cytoskeleton was disrupted. The inhibition of the MLR seen when the T cell's actin cytoskeleton was disrupted is consistent with previous studies (Wulfing et al., 1998). In contrast with previous reports, I not only showed that the APC's cytoskeleton is critical for T cell activation, but I also demonstrated that inhibition of the APC/DC's actin cytoskeleton results in a more pronounced effect

than that of the T cells. The MLR inhibition was not due to drug diffusing from DCs to T cells, because at all the drug doses I used the inhibition was always greater when DCs were pretreated as compared to when T cells were pretreated. Furthermore, DCs have a larger cytoplasm and much more F-actin per cell volume unit than T cells, and thus one would expect that disruption of the DC's actin cytoskeleton would be more difficult.

Consistent with Austyn *et al* findings (Austyn et al., 1988b), which demonstrated the clustering with DC to be critical for T cell activation, I found a strong correlation between the levels of DC-T cell clustering and the degree of T cell activation in the MLR. The observed inhibition in the MLR following DC pretreatment with CytD was due to the effect of the drug on the cytoskeletal rearrangement rather than inhibition of proteins important for T cell stimulation. I obtained similar results using LatA and DC expression of MHC class II, B7-2, or fascin was not altered following pretreatment with CytD or LatA. However, the drugs were working because actin polymerization as assessed by measuring the level of F-actin was significantly reduced following treatment. When day 3 or 6 immature DC were pretreated with CytD (1 h followed by extensive washing) and returned to culture in order to mature, these DCs matured and became as potent as control DCs. This data is in line with the reversible effect of CytD (Cooper, 1987) and rules out the possibility that the drug might affect the cell viability or protein expression. Furthermore, pretreatment with CytD inhibited the MLR in a dose-dependent fashion as well as at different stimulator to responder ratios. Finally, while these drugs had not previously been used on DCs, the dose used

in our experiment was within the normal range used by other groups (Mc et al., 2002; Valitutti et al., 1993).

As mentioned above, DC are unique among other APC in their ability to cluster and activate resting T cells (Inaba and Steinman, 1984; Inaba et al., 1985; Nussenzweig and Steinman, 1980). Consistent with the weak APC ability of B cells, proliferation of resting allogeneic T cells was very poor when resting B cells were used as APCs. This is consistent with the observed low level of T cell clustering with B cells, which was not further reduced when the B cell's actin cytoskeleton was disrupted. In line with my clustering data, the MLR was not inhibited when B cells were pretreated with CytD. This data is in agreement with previous studies, where it was reported that there was no cytoskeletal rearrangement in B cells during binding to T cells and that disruption of the B cell's cytoskeleton with CytD did not affect their ability to cluster and stimulate T cells (Valitutti et al., 1995a; Wulfing et al., 1998). Altogether, my data provides a reasonable explanation for the observed differences between DC and B cells in the antigen-dependent clustering and activation of resting T cells. In contrast with B cells, DC reorganize their actin cytoskeleton to enhance their ability to cluster resting allogeneic T cells, thereby leading to enhanced T cell activation. Furthermore, DC rearranged their actin cytoskeleton during interaction with activated T cells, but this reorganization was not necessary for clustering or activation. Therefore, the conclusion derived from previous studies that used activated T cells with B cells or surrogate APC underestimated the significance of

the APC's cytoskeleton when it is needed the most for clustering and activation of resting T cells.

CHAPTER 2

“Dendritic cell actin cytoskeletal participation in the immunological synapse formation is highly antigen-dependent”

4.1 Preamble

The recognition of peptide-MHC complexes on the APC by TCRs on the T cell is facilitated by the formation of a specialized junction between the two cells referred to as the I-Synapse (reviewed in (Bromley et al., 2001)). Formation of the I-Synapse is a dynamic process that results in clustering of T cell surface receptors and signaling molecules into specialized domains and is dependent on actin-myosin motors and cytoskeletal proteins such as talin, ezrin and moesin that act as a bridge between transmembrane molecules and the cortical actin cytoskeleton (Monks et al., 1998; Delon et al., 2001; Wulfing and Davis, 1998). T cell actin cytoskeletal reorganization is critical for their activation because inhibition of T cell actin formation with CytD, prevents surface receptor clustering, T cell proliferation and IL-2 production (Holsinger et al., 1998; Kong et al., 1998; Wulfing et al., 1998). Although participation of the T cell's actin cytoskeleton in the I-Synapse formation was thought to occur in a highly antigen-specific fashion, a recent study demonstrated that naive CD4⁺ T cells may form antigen-independent synapses with DCs (Revy et al., 2001).

In contrast to other APCs, DCs have unique ability to bind and activate naïve T cells (Nussenzweig and Steinman, 1980). In a previous study, I demonstrated that DCs focally polarize their F-actin and fascin, a DC specific actin-bundling protein, towards the interface with resting allogeneic CD4⁺ T cells (Al Alwan et al., 2001b). However, this study did not address the specificity of the DC involvement in the I-Synapse. Therefore, I used a TCR transgenic system to address this issue. Using this system, I demonstrated that the DC actin

cytoskeletal reorganization leading to formation of the I-Synapse with naive CD4⁺ T cells is highly antigen-dependent. In contrast, participation of the naive CD4⁺ T cell's cytoskeleton in formation of the I-Synapse with DCs was antigen-independent. Antigen-dependent T cell binding and T cell activation is critically dependent on DC cytoskeletal rearrangement. Importantly, DC cytoskeletal rearrangement and T cell activation occurred at the same threshold of peptide-MHC complexes. The signal that results in DC cytoskeletal reorganization is mediated in part via directional ligation of MHC class II molecules. These findings suggest that the high antigen specificity required for DC actin cytoskeletal rearrangement leading to I-Synapse formation permits prolongation of DC-T cell interactions, thereby creating a microenvironment that facilitates the activation of antigen-specific T cells.

4.2 Results

4.2.1 DC actin cytoskeletal involvement in formation of the I-Synapse is highly antigen-dependent

I have previously demonstrated that the DC's cytoskeleton participates in forming an I-Synapse with resting allogeneic CD4⁺ T cells (Al Alwan et al., 2001b). This was the first report to demonstrate the dynamic involvement of the APC's cytoskeleton in the formation of the I-Synapse with T cells. To determine the specificity of DC participation during formation of the I-Synapse I evaluated the polarization of DC actin cytoskeletal proteins during DC-T cell interactions using the DO11.10 TCR transgenic model. Naive transgenic CD4⁺ T cells from this

model recognize OVA₃₂₃₋₃₃₉ agonist peptide in the context of MHC class II (I-A^d) (Murphy et al., 1990). A control peptide (OVA₃₂₄₋₃₃₅) binds with similar affinity to I-A^d but does not result in T cell activation (Sette et al., 1987). In the presence of agonist peptide, DC forming conjugates with CD4⁺ T cells demonstrated polarized expression of F-actin towards the T cell interface (Fig 1C). In contrast, there was no polarization of F-actin in DCs bound to T cells in the absence of peptide or in the presence of similar doses of control peptide (Fig 1A and B). The percentage of agonist peptide pulsed-DCs that polarized their F-actin toward the interface with the T cells was 91% compared with 15% and 18% polarization in control DCs and DCs pulsed with control peptide, respectively (Fig 1D). I also evaluated the distribution of fascin, an actin-bundling protein expressed only in mature DCs (Al Alwan et al., 2001a), in DC-T cell conjugates. I have previously shown that fascin is involved in DC dendrite formation and inhibition of its expression leads to a marked reduction in the ability of DCs to activate resting T cells (Al Alwan et al., 2001a). Similar to F-actin, fascin also demonstrated a polarized expression in DC towards the T cell interface with agonist but not with control peptide or in the absence of peptide (Fig 1A-D). This demonstrates the specificity of DC cytoskeletal participation during formation of the I-Synapse.

The microtubules and microtubule organizing center (MTOC) are tightly associated with the Golgi apparatus and are involved in the directional release of mediators by T cells (Kupfer and Singer, 1989b). Directional secretion of the cytokine, IL-1 β , by DCs during interaction with allogeneic T cells has recently been described (Gardella et al., 2001). I evaluated the specificity of microtubules (β -

tubulin) and MTOC (γ -tubulin) polarization in DCs during binding to T cells.

Similar to F-actin and fascin, the DC's γ - and β -tubulin also polarized towards the T cell in an antigen-dependent manner (Fig 2A and B). However, there was a significant increase in β -tubulin polarization with control peptide compared with the no peptide group (data not shown), indicating that β -tubulin polarization in DCs was not as specific as the polarization seen with F-actin or fascin. Talin is a cytoskeletal protein which parallels LFA-1 surface distribution (Kupfer and Singer, 1989b). Polarization of talin in T cells during conjugation with B-cells has been reported to be less antigen-specific than MTOC or TCR polarization. Polarization of talin in DCs was not antigen-dependent (Fig 2E), in contrast to the results seen with F-actin, fascin and MTOC. Together these data indicate that actin cytoskeletal rearrangement in DCs leading to I-Synapse formation is highly antigen-dependent and specific.

4.2.2 Naive CD4⁺ T cell actin cytoskeletal participation in the I-Synapse with DCs is antigen-independent

T cell actin cytoskeletal rearrangement required for formation of the I-Synapse is thought to occur in a highly antigen-dependent fashion. However, a recent study has demonstrated that naive T cells may form antigen-independent synapses with DCs (Revy et al., 2001). To allow direct comparison with our findings in DCs, I examined whether T cell cytoskeletal rearrangement was antigen-dependent. Polarization of F-actin, talin, β -tubulin and tyrosine kinase

activity was not significantly different in T cells binding control DCs as compared with the T cells binding control or agonist peptide-pulsed DCs (Fig 2F). These findings indicate that, in contrast to DC cytoskeletal rearrangement, T cell cytoskeletal participation in the I-Synapse occurs in an antigen-independent manner.

4.2.3 DC actin cytoskeletal reorganization is critical for antigen-dependent T cell binding

DCs have a unique ability to bind T cells in both an antigen-dependant and -independent fashion (Inaba and Steinman, 1986). DC-T cell binding increased significantly over time and reached a maximum by 30 min (Fig 3A). In spite of the low levels of T cell binding by DCs at the earlier time points, the majority of DCs polarized their F-actin and fascin as early as 5 min although there was also an increase over time (Fig 3B). This demonstrates a correlation between DC actin cytoskeletal polarization and T cell binding. I then evaluated whether DC actin cytoskeletal rearrangement was necessary for either antigen-dependent or -independent DC-T cell binding. The overall number of T cells binding DCs increased by 3.6 fold in the presence of peptide (Fig 3C). The increase in the number of T cells binding to DCs in the presence of antigen occurred in a dose dependent manner (Appendix Fig. 7). Importantly, there was a very strong correlation between the increases in DC-T cell binding seen with antigen and DC actin cytoskeletal polarization (correlation coefficient 0.96) suggesting that antigen-dependant T cell binding requires DC actin cytoskeletal polarization. To directly

test this association, DCs were pretreated with CytD, Jasp or LatA (Spector et al., 1999) and their ability to bind naive CD4⁺ T cells was evaluated. Disruption of DC's actin with these drugs significantly reduced the number of T cells binding to DCs and the percentage of DCs bound by T cells but only in the presence of peptide (Fig. 3C and D). This indicates that the cytoskeletal changes in DCs are critical for the antigen-dependent binding of naive CD4⁺ T cells.

4.2.4 DC actin cytoskeletal polarization and T cell activation occur at the same dose of peptide-MHC complexes

The number of peptide-MHC class II complexes required to trigger antigen-dependent T cell cytoskeletal changes are below that required to induce T cell activation (Delon et al., 1998a). To evaluate the concentration of peptide required for T cell activation and its relationship to DC actin cytoskeletal rearrangement, DCs were pulsed with different doses of peptide and the sample was divided in two. One half of the DC sample was used to determine T cell proliferation and the remainder was used to evaluate DC polarization. This allowed a direct comparison between the peptide doses required for T cell proliferation and those required for DC cytoskeletal rearrangement. DCs pulsed with agonist peptide induced T cell activation in a dose-dependent manner with 50pM being the lowest concentration at which significant proliferation of T cells was observed (Fig 4A). Control DCs and DC pulsed with control peptide did not result in T cell activation. Polarization of DC's F-actin and fascin toward the T cell interface also occurred only with agonist peptide doses of 50pM or greater (Fig 4B). In none of our

independent experiments (n=5) was there significant polarization of the DC's cytoskeleton in the absence of significant T cell proliferation or vice versa. There was also a clear dose response in the percentage of DC polarizing their F-actin and fascin towards the T cell. Less than 24% of the DC polarized their F-actin and fascin in the control-, 300nM control peptide pulsed- and 10pM agonist peptide-pulsed groups, consistent with the lack of T cell activation in these groups. In contrast, 39%, 50% and 85% of the DC pulsed with 50pM, 1nM and 300nM polarized their F-actin at the contact point with T cells, respectively (Fig 4B). DC polarization and T cell activation were highly correlated with a correlation coefficient of 0.98.

T cell activation may have different requirements for costimulation and duration of stimulation depending on the antigen dose (Viola and Lanzavecchia, 1996). I evaluated whether the importance of the DC's cytoskeleton for T cell activation was dependent on the peptide-MHC concentration. Pretreatment of DCs with CytD or LatA significantly reduced their ability to induce T cell proliferation at all agonist peptide doses (Fig 4C). Similar results were obtained when DCs were pretreated with Jasp (data not shown). This indicates that the DC's cytoskeleton is critical for naive T cell activation at low as well as at high peptide doses. Altogether, our data suggests a direct relationship between DC cytoskeletal participation in the I-Synapse and T cell activation. It also indicates that polarization of the DC's cytoskeleton occurs at the same threshold of peptide-MHC complexes that results in T cell activation.

4.2.5 DC actin cytoskeletal rearrangement is required for T cell activation and is mediated via MHC class II ligation

Productive naive CD4⁺ T cell activation induces IL-2 mRNA expression and secretion leading to T cell proliferation. Other early activation events such as Ca⁺² flux are necessary for but not indicative of T cell activation since Ca⁺² flux was also observed in naive CD4⁺ T cells binding DCs in the absence of antigen (Revy et al., 2001). To evaluate the functional significance of DC participation in the I-Synapse, I determined the effects of inhibiting DC actin cytoskeletal rearrangement on naive T cell activation. I measured IL-2 mRNA expression after 4 h as an early marker of T cell activation. DCs were pulsed with agonist peptide (300nM) and then pretreated with CytD or Jasp prior to mixing with naive CD4⁺ T cells. Total RNA was isolated from cultures after 0, 2 and 4 h and IL-2 mRNA expression was assessed. The earliest time point at which IL-2 mRNA was detected in T cells mixed with DCs and peptide was 4 h (Fig. 5A). Pretreatment of DCs with actin cytoskeletal inhibitors abolished the expression of IL-2 mRNA. To demonstrate that T cell IL-2 protein expression was also dependent on the DC involvement in the synapse, I evaluated IL-2 bioactivity in our cultures using the IL-2 dependent T cell line CTLL-2. The level of IL-2 bioactivity in cultures containing DC pretreated with CytD or Jasp was significantly reduced compared with the untreated groups (Fig. 5B). This demonstrates that DC participation in the I-Synapse is critical for IL-2 mRNA expression and release by naive CD4⁺ T cells.

The fact that DCs form the I-Synapse only in the presence of peptide suggests that DC involvement in this process may be triggered through signaling

via MHC class II. To explore the role of MHC class II molecules in DC participation in the I-Synapse, I made surrogate T cells using polystyrene beads to directionally crosslink specific proteins on the surface of the DCs. Our data demonstrated that >50% of DCs polarize their F-actin following MHC class II crosslinking (Fig 5C-E). In contrast, <20% F-actin polarization was observed in DCs when MHC class I, B7-2, or LFA-1 were crosslinked. This suggests an important role for MHC class II signaling in antigen-dependent DC participation in the I-Synapse.

Figure 1. A-D, DC actin cytoskeletal rearrangement is highly antigen-dependent. DC-T cell conjugates were stained for F-actin and fascin and examined by confocal microscopy. In A-C, the upper panel (a-c) represents the true color, the lower panel (d-e) represents the staining intensity (blue to red = low to high), and (f) shows the Nomarski image. (A) No polarization of F-actin (a and d) or fascin (b and e) in DC binding T cells in the absence of peptide. (B) No polarization of F-actin (a and d) or fascin (b and e) in control peptide (300nM)-pulsed DC binding T cells. (C) Polarization of both F-actin (a and d) and fascin (b and e) in agonist peptide (300nM) pulsed-DC binding T cells. (D) Percentage polarization of F-actin and fascin in DC binding T cells expressed as mean polarization \pm SD of 50 conjugates and is representative of three independent experiments.

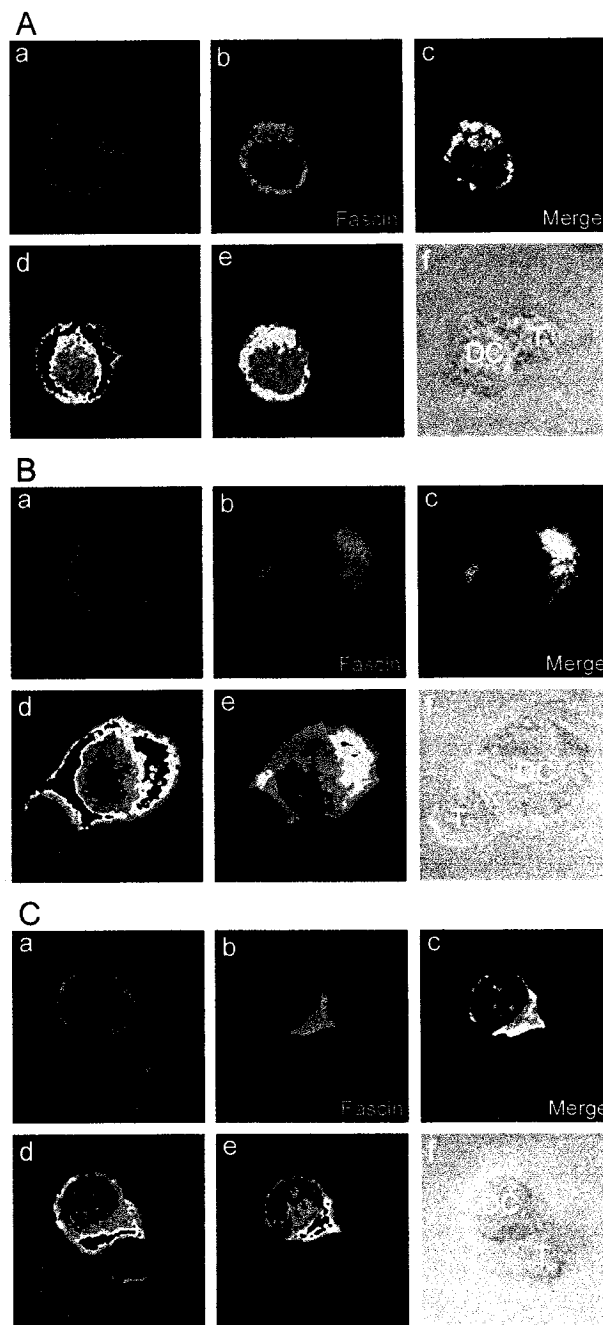


Fig. 1 (A-C)

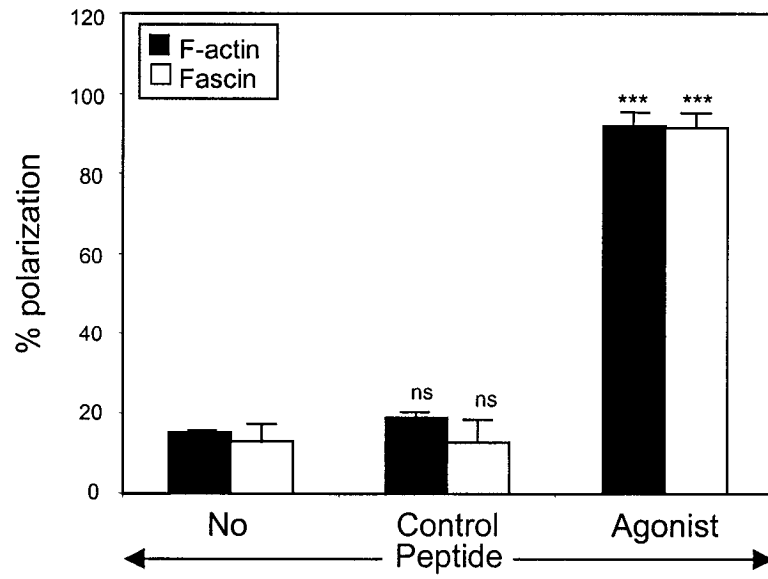


Fig. 1D

Figure 2. A-F, Naive CD4⁺ T cell actin cytoskeletal rearrangement is antigen-independent. DC-T cell conjugates were stained for different proteins and examined by confocal microscopy. In A-D, (a) shows the true color, (b) shows the staining intensity and (c) shows the Nomarski image. (A) Polarization of MTOC in the T cell but not in the DC in the absence of peptide. (B) Polarization of MTOC in the T cell and the DC in the presence of agonist peptide (300nM). (C) Polarization of talin in the T cell and the DC in the absence of peptide. (D) Polarization of talin in the T cell and the DC in the presence of agonist peptide (300nM). (E) Percentage polarization of microtubules, MTOC or talin in DC binding to T cells. (F) Percentage polarization of F-actin, microtubules, talin or phospho-Y in T cells binding to DC. The results are expressed as mean polarization \pm SD of 50 conjugates and are representative of three independent experiments.

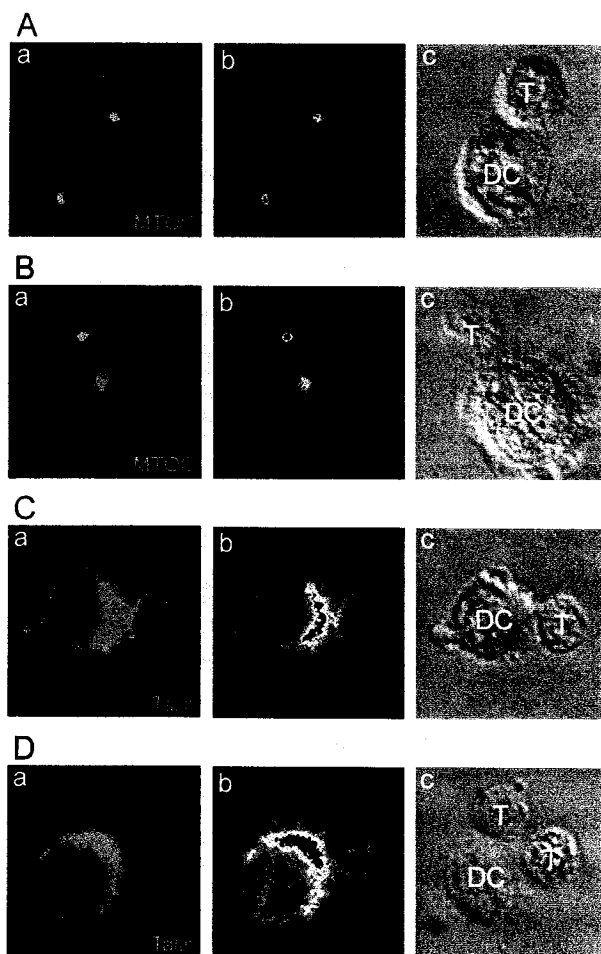


Fig. 2 (A-D)

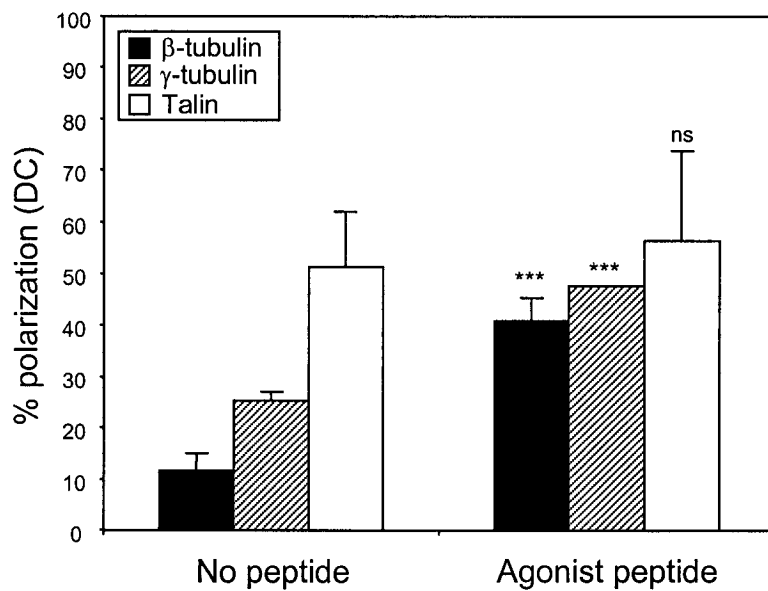


Fig. 2E

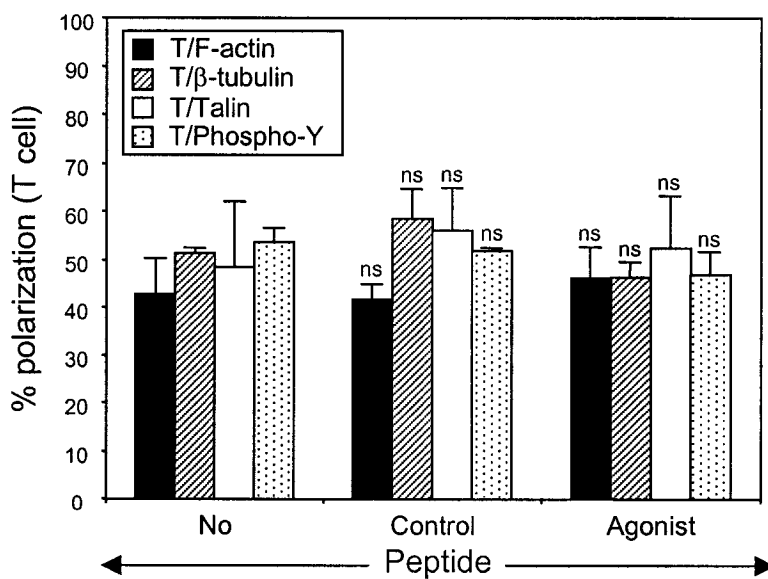


Fig. 2F

Figure 3. A-D, DC actin cytoskeletal rearrangement is required for stable DC-T cell binding. (A) DC pulsed with agonist peptide (300nM) were labeled with CFMDA dye and mixed with T cells labeled with CM-Dil dye. Conjugates were counted using an inverted fluorescence microscope. The number of T cells bound to 100 DC at different time points is expressed as the mean \pm SD. (B) Percentage polarization of F-actin and fascin in agonist peptide (300nM) pulsed DC binding T cells at different time points is expressed as mean polarization \pm SD of 50 conjugates. (C-D) Control DC (no peptide) or DC prepulsed with 300nM of agonist peptide were pretreated with DMSO (control), CytD (20 μ M), Jasp (10 μ M) or LatA (5 μ M) washed extensively and mixed with T cells. (C) The number of T cells bound to 100 DC expressed as the mean \pm SD. (D) The percentage of DC binding to T cells expressed as the mean \pm SD. The results are representative of three independent experiments.

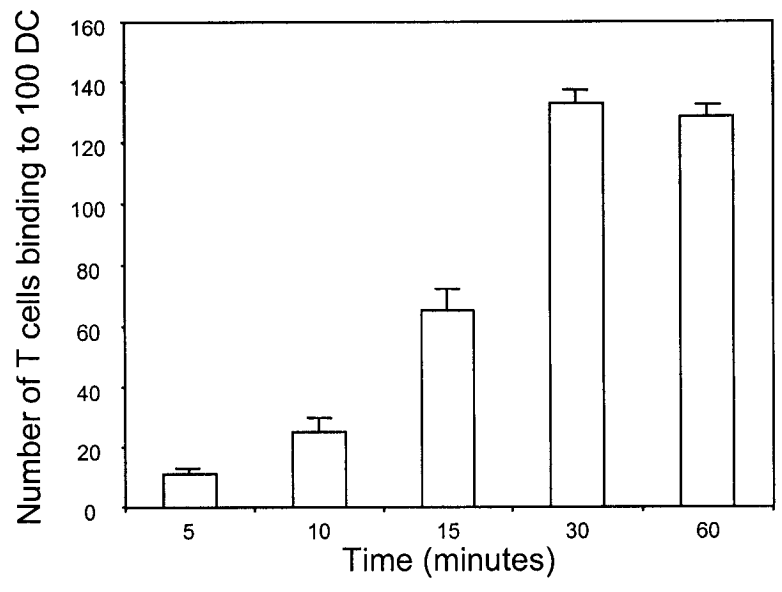


Fig. 3A

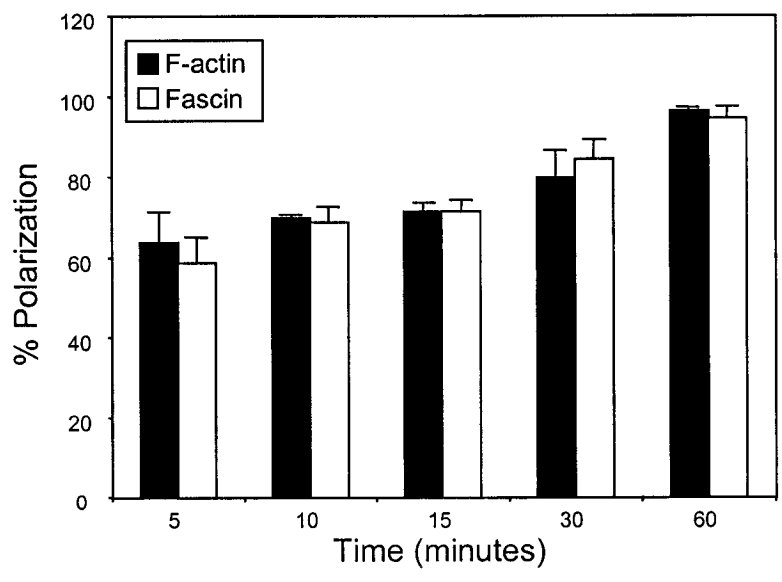


Fig. 3B

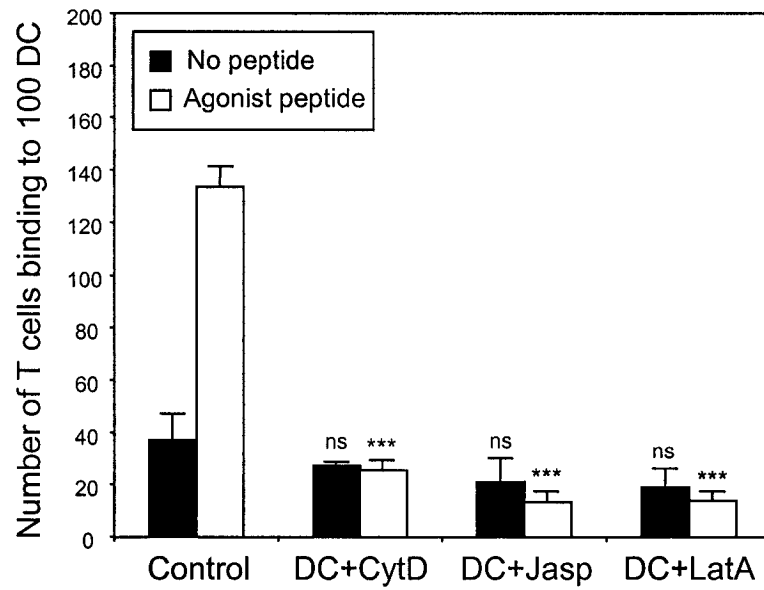


Fig. 3C

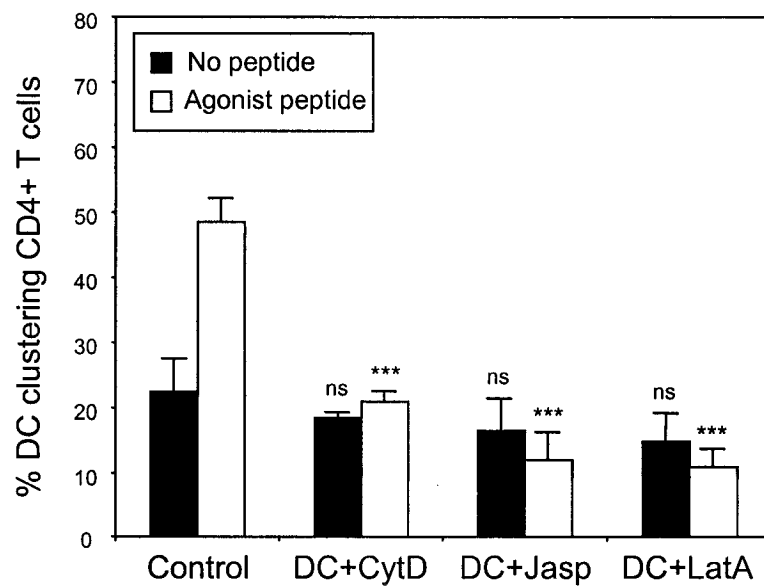


Fig. 3D

Figure 4. A-C, DC cytoskeletal polarization occurs at the same peptide threshold as does T cell activation. (A) Proliferation of T cells mixed with media, control or agonist peptide prepulsed-DC was assessed by measuring thymidine uptake. The results are expressed as mean dpm \pm SD and are representative of five independent experiments. (B) Percentage polarization of F-actin and fascin in DC binding T cells in the presence of different doses of peptide. The results are expressed as mean polarization \pm SD of 50 clusters and are representative of five independent experiments. (C) T cell proliferation was assessed as in A. Control, CytD (20 μ M), or LatA (5 μ M)-pretreated DC were mixed with T cells \pm peptide. The results are expressed as mean dpm \pm SD and are representative of five independent experiments.

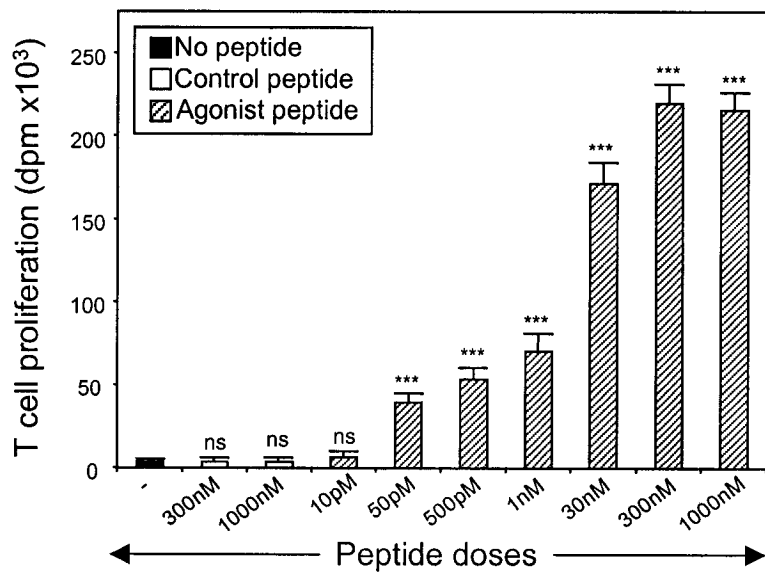


Fig. 4A

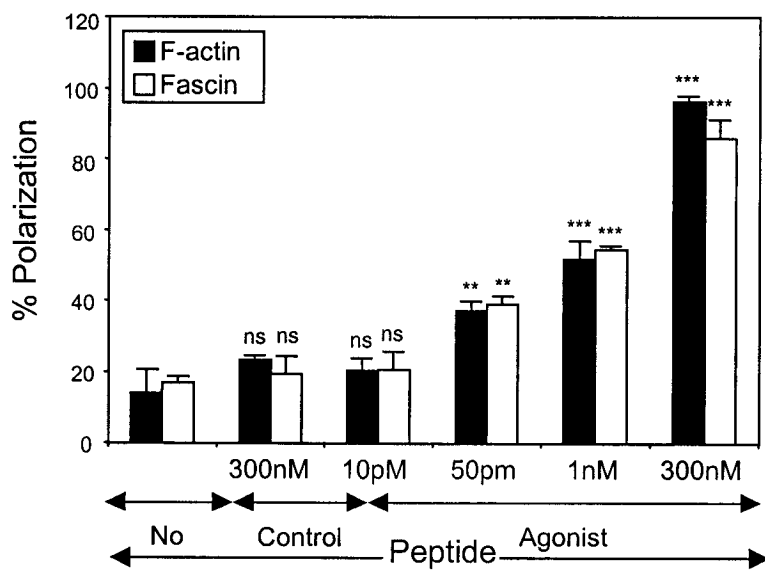


Fig. 4B

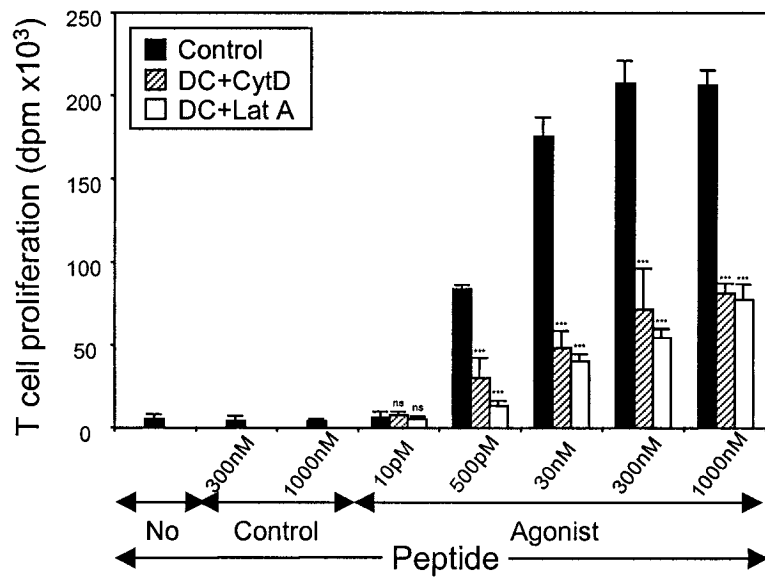


Fig. 4C

Figure 5. A-E, Actin cytoskeletal reorganization in DC is critical for naive CD4⁺ T cell activation and is mediated via MHC class II crosslinking. (A) Semiquantitative IL-2 RT-PCR: control (DMSO), CytD (20 μ M) or Jasp (10 μ M)-pretreated DC were incubated with T cells in the absence or presence (300nM) of agonist peptide and IL-2 and GAPDH mRNA expression was determined from total RNA. (B) IL-2 bioactivity: control, CytD or Jasp-pretreated DC were mixed with T cells and agonist peptide (300nM). Supernatants were added to CTLL-2 cells and proliferation was determined as a measure of IL-2 bioactivity. DC labeled with anti-MHC class II (C) or anti-LFA-1 (D) antibodies were mixed with beads coated with secondary antibodies, stained for F-actin and evaluated by confocal microscopy. In C and D, a-c represents the true color, the staining intensity (blue to red = low to high) and the Nomarski image, respectively. (E) F-actin in DC labeled with different primary antibodies and mixed with secondary antibody-coated beads. The results are expressed as mean percentage polarization \pm SD of 50 conjugates and are representative of three independent experiments.

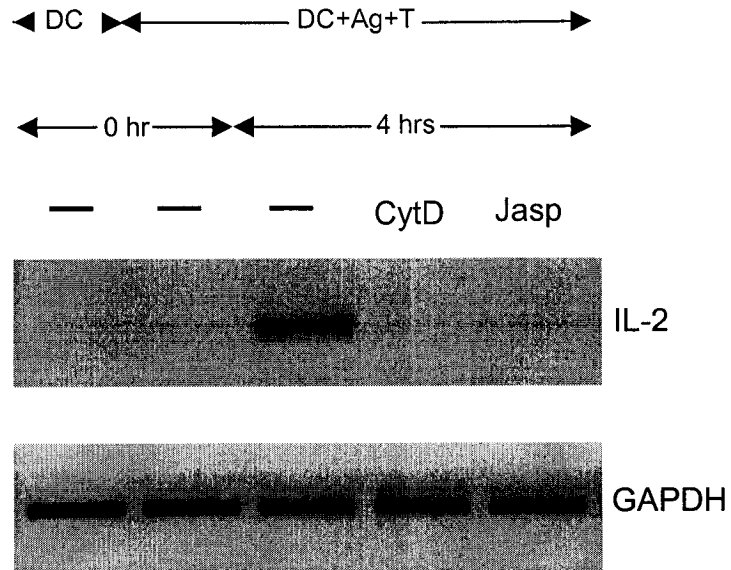


Fig. 5A

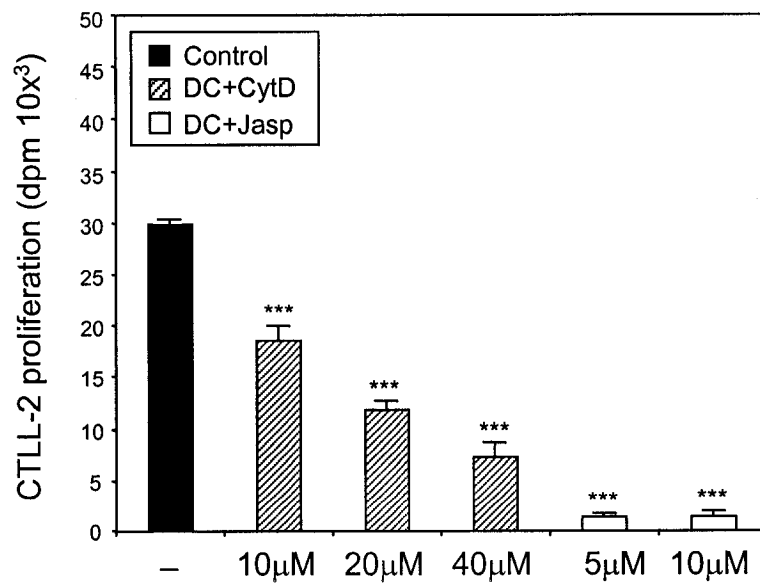


Fig. 5B

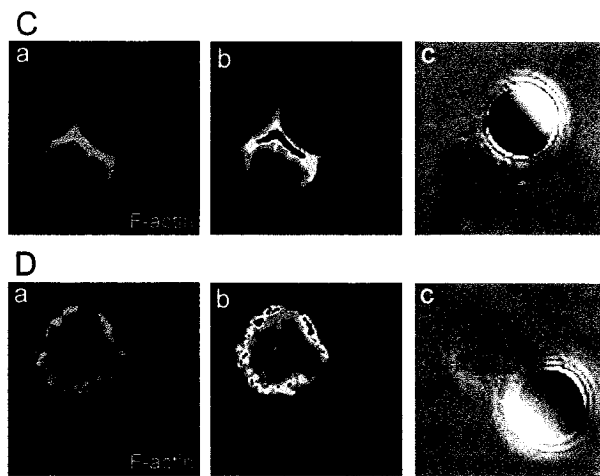


Fig. 5 (C-D)

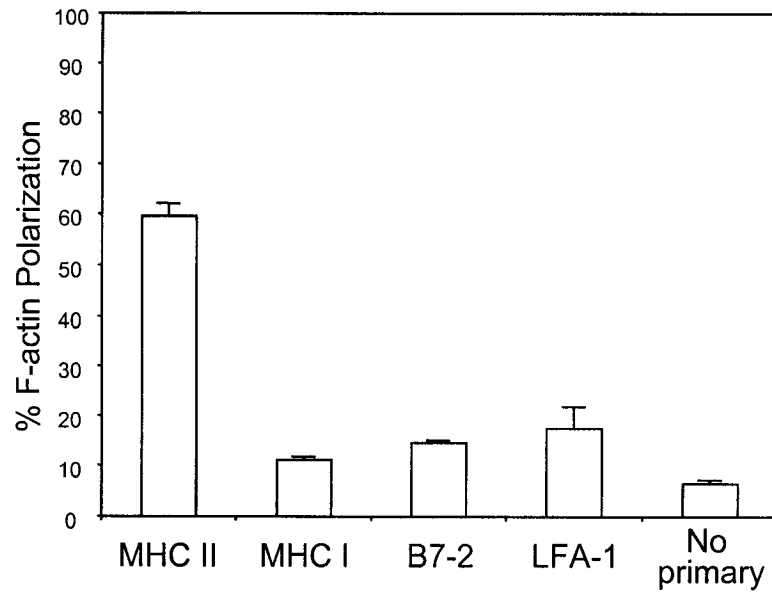


Fig. 5E

4.3 Discussion

The results presented in this chapter demonstrate a fundamental difference between DCs and naïve T cells with respect to their actin cytoskeletal participation in the I-Synapse formation. While the DC's actin cytoskeleton involvement in formation of the I-Synapse was antigen-dependent, naïve CD4⁺ T cell participation in this process occurred in the absence of antigen. Participation of DC's cytoskeleton in the I-Synapse was mediated in part through MHC class II ligation. Most importantly, these changes were critical for the antigen-dependent T cell binding and occurred at the same peptide-MHC threshold required for T cell activation. This indicates that DC's cytoskeletal rearrangement is an essential step that defines the threshold for activation of naïve T cells.

4.3.1 Polarization of DC's and not naïve T cell's cytoskeleton during I-Synapse formation is highly antigen-dependent

The observation of DC actin cytoskeletal polarization at the interface with allogeneic but not syngeneic T cells is an indicator of but not a definitive proof for the concept that DCs active participation in the I-Synapse formation is an antigen-dependent event. In addition, allogeneic T cells may recognize processed allogeneic antigen presented by self-MHC like any other foreign antigens (indirect recognition) or the foreign MHC itself (direct recognition) (Sherman and Chattopadhyay, 1993). In contrast to foreign antigen, the membrane density of alloantigen displayed on a DC is high and thus the magnitude of activation is likely to be elevated. Therefore, the antigen dose in an allogeneic model is very high

and the observed cytoskeletal reorganization may not reflect changes that would normally take place under conditions seen in most immune responses. In this study, I used the DO11.10 TCR transgenic model to further explore the antigen-dependence and specificity of DC's actin cytoskeletal participation during formation of the I-Synapse with naïve CD4⁺ T cells at lower and possibly more physiological doses of antigen. The DO11.10 transgenic CD4⁺ T cells recognize OVA₃₂₃₋₃₃₉ agonist peptide presented in the context of MHC class II (I-A^d) (Murphy et al., 1990). The agonist peptide is known to induce a strong T cell activation and is thus useful for evaluation of the antigen-dependent DC's cytoskeletal involvement in the I-Synapse. The ability to control the peptide doses was valuable in determining the lowest peptide dose that induced DC participation in the I-Synapse and how it correlates with T cell activation. In addition, the availability of a control peptide (OVA₃₂₄₋₃₃₅), which may also be recognized by the transgenic CD4⁺ T cells and yet not result in activation, was useful for the evaluation of the specificity of the DC actin cytoskeletal participation in formation of the I-Synapse.

I observed a significant polarization of F-actin and fascin in DCs at the contact point with T cells in the presence of agonist peptide. However, polarization of these proteins in the presence of a similar dose of control peptide was not increased above the background levels observed in the absence of peptide. These data confirmed the previous findings with the allogeneic system and they showed that these active changes in DC leading to I-Synapse formation with T cells are highly antigen-specific. It should be noted that both agonist and

control peptides were shown to bind to I-A^d with similar affinity (Sette et al., 1987; Sette et al., 1989). Furthermore, the structure of a given TCR is essentially the same whether bound to agonist, superagonist, or antagonist peptide-MHC (Degano et al., 2000; Ding et al., 1999). Therefore, the observed cytoskeletal changes were likely to be due to the different signals transmitted by the different peptides rather than difference in the binding affinity to the MHC class II or conformational changes in the TCR structure. Previous studies reported that although the control and agonist peptides bind to the MHC class II similarly, their binding kinetics to the TCR may be different. Indeed, altered peptide ligands have been shown to reduce the half-life of the TCR-peptide-MHC interactions (Alam et al., 1996; Kersh et al., 1998b; Lyons et al., 1996), thereby interfering with the signal transduction via sustained TCR triggering. Furthermore, there is evidence that altered peptides prevent TCR oligomerization and functional TCR triggering (Bachmann et al., 1998), which again affects TCR-mediated signal transduction. Finally, it is well established that alteration of a single amino acid in an antigenic peptide may have significant and distinct biological effects on a T cell that can range from optimal activation to tolerance induction (Allen et al., 1987). Polarization of DC's cytoskeleton during interactions with T cell in the presence of agonist but not control peptide may determine the fate of the T cells at the DC level. The highly antigen-specific DC cytoskeletal rearrangement may result in the activation of naïve T cells into effector cells, whereas the lack of DC cytoskeletal involvement in the absence of specific antigen may prevent inappropriate activation. In summary, the observed

polarization of F-actin and fascin demonstrates the specificity of DC's cytoskeletal involvement in formation of the I-Synapse with naïve T cells.

In addition, I have also evaluated the specificity of MTOC and talin participation in the I-Synapse. While F-actin accumulation at APC-T cell interface was suggested to stabilize and favor sustained TCR-peptide-MHC interactions, MTOC reorientation is thought to position the T cell secretory apparatus in close proximity with the APC. Indeed, several studies showed that positioning of the MTOC was accompanied by the polarized concentration of cytokines and cytotoxic mediators at the T cell-B cell/target interface (Brickell et al., 1998; Kupfer and Dennert, 1984; Kupfer et al., 1991; Kupfer et al., 1994). MTOC polarization correlated with polarized secretion of cytokines by T cells toward the site of the stimulus. Recent studies have also reported a directional secretion of IL-1 β by DC during interaction with allogeneic T cells, suggesting MTOC involvement in this process in DC (Gardella et al., 2001).

Talin is another cytoskeletal protein that binds to the cytoplasmic tail of the LFA-1 (Kupfer and Singer, 1989b), which is known to be involved in mediating the antigen-independent binding. Polarization of the DC's MTOC towards the T cell occurred in presence but not in the absence of peptide. This is consistent with an earlier study that demonstrated that MTOC reorientation in T cell-B cell conjugates occurs in an antigen-dependent fashion (Kupfer et al., 1987). However, I observed a significant increase in DC's MTOC polarization with control peptide compared with the no peptide group. This suggested that MTOC polarization in DC is less stringent as compared with F-actin or fascin.

Alternatively, the control peptide has not been fully characterized and thus it may function as a weak agonist. Contrary to the results seen with F-actin, fascin and MTOC, talin polarization in DC occurred in an antigen-independent fashion. This is consistent with Kupfer's finding of antigen-independent polarization of talin in T cell-B cell conjugates (Kupfer and Singer, 1989b).

Contrary to that seen in DCs, polarization of F-actin, MTOC and talin in T cells occurs in an antigen-independent fashion. On the one hand, Grakoui *et al* reported that T cells were able to stimulate peptide-MHC accumulation on planar membranes even in the presence of antagonist peptide (Grakoui *et al.*, 1999), indicating that T cell participation in forming the I-Synapse with surrogate APC is not an antigen-specific event. My findings are in direct contrast with previous studies showing T cell's F-actin and MTOC polarization to occur in an antigen-dependent manner (Grakoui *et al.*, 1999; Kupfer *et al.*, 1986; Kupfer *et al.*, 1987; Kupfer and Singer, 1989b; Valitutti *et al.*, 1995a). However, these studies used preactivated T cells with B cells or surrogate APCs, while my studies used primary cultures of naïve T cells and DC. This emphasizes once more that the APC's actin cytoskeletal participation is different when the I-Synapse is formed between DC and naïve T cell as compared with one formed between B cells or surrogate APC and preactivated T cells.

Most interestingly, there was an antigen-independent polarization of tyrosine kinase activity in T cells at the interface with the DC. This indicates that beside the antigen-independent polarization of actin cytoskeletal proteins, T cells also trigger one of the earliest signaling events in an antigen-independent

fashion. Consistence with my findings, Revy *et al* have recently demonstrated that naive T cells may form antigen-independent synapses with DC (Revy et al., 2001). It is not surprising to see polarization of DC's and T cell's talin in the absence of antigen since its movement has been assumed to parallel that of LFA-1 polarization (Kupfer and Singer, 1989b; Peter and O'Toole, 1995). Although LFA-1 mediates DC-T cell antigen-dependent binding (Geijtenbeek et al., 2000), talin may induce polarization of low affinity LFA-1 in the absence of antigen that only becomes activated (high affinity) upon the recognition of the proper peptide. In addition, it has been reported that talin redistribution is not dependent on F-actin rearrangement (Kupfer et al., 1990; Yan and Berton, 1998). It is also possible that distinct signals trigger participation of different cytoskeletal elements in the I-Synapse. For instance, talin polarization was observed to occur with altered peptide ligands (Monks et al., 1998), consistent with the polarization I saw with my control peptide. However, MTOC and F-actin polarization is dependent on TCR triggering (Kupfer and Singer, 1989b) and enhanced by integrin engagement (Sedwick et al., 1999).

Together these data indicated that actin cytoskeletal rearrangement in DC leading to I-Synapse formation is highly antigen-dependent, whereas T cell actin cytoskeletal participation in this process is antigen-independent. This antigen-independent rearrangement of the naïve T cell's cytoskeleton may allow them to form "presynapses", which promote antigen sampling by T cells on the surface of DC. DC-SIGN/ICAM-3 interactions mediating the antigen-independent binding between DC and resting T cells (Geijtenbeek et al., 2000; Montoya et al., 2002)

are likely to be responsible for the observed “presynapse” formed by my naïve T cells. Once naïve T cells forming “presynapse” recognize the proper antigen, the DC’s cytoskeleton then becomes involved to establish and stabilize a fully mature synapse.

4.3.2 Increased antigen-dependent T cell binding is related to DC actin cytoskeletal rearrangement

The unique ability of DC to bind T cells in both an antigen-dependant and -independent fashion has been well established (Inaba and Steinman, 1986; Nussenzweig and Steinman, 1980). T cell binding by DC precedes and is critical for T cell activation (Austyn et al., 1988b). Therefore, binding by DC and maintenance of the binding stability are key issues for the ultimate T cell activation. Consistent with previous studies, my results show that DCs bind T cells in the presence as well as in the absence of peptide. However, increased DC-T cell binding over time was only observed in an antigen-dependent situation. This is in line with my previous findings using the allogeneic system (Al Alwan et al., 2001b) and together with the polarization results strongly suggests that the DC actin cytoskeletal participation in the I-Synapse is responsible for increased antigen-dependent T cell binding. Further support for this suggestion comes from the fact that at an earlier time points (5-15 min), the majority of the DCs polarized their actin cytoskeleton in spite of the low levels of clustering. However, it is important to note that DC-T cell clusters were resuspended 3 times prior to evaluation for clustering or polarization. Therefore, the high levels of polarization at 5 min (67%)

indicate that DCs rearranged their actin cytoskeleton in most of the remaining DC-T cell clusters following resuspension. Since most of the DC-T cell binding at earlier time points is likely to be antigen-independent and very weak, one would expect to see low levels of polarization at these time points if the clusters were not resuspended. This suggests a direct correlation between DC actin cytoskeletal reorganization and T cell binding.

To examine the link between DC cytoskeletal rearrangement and antigen-dependent T cell binding, I evaluated the importance of the DC's cytoskeleton for DC-T cell binding in the presence or absence of antigen. Both the percentage of DCs binding T cells and the overall number of T cells binding to DCs were increased significantly in the presence of peptide as compared with the control (no peptide). These results are consistent with previous studies indicating increased T cell binding by APC in the presence of the peptide (Bromley and Dustin, 2002; Krawczyk et al., 2002). This increase in T cell binding to DCs is antigen-dependent, because the number of T cell binding to DCs increased in an antigen dose-dependent manner. Most importantly, I noted a strong correlation between the increases in antigen-dependent DC-T cell binding and DC actin cytoskeletal polarization. This data suggests but does not prove that DC's cytoskeletal reorganization was responsible for the increased T cell binding seen with antigen.

To further explore the link between DC cytoskeletal reorganization and antigen-dependent T cell binding, I evaluated the effect of disrupting DC's cytoskeleton on their ability to bind T cells in the presence or absence of peptide. Consistent with the stated hypothesis, perturbation of the DC cytoskeletal

rearrangement using CytD reduced the antigen-dependent DC-T cell binding to the baseline levels observed in the absence of antigen. However, pretreatment of DC with CytD did not affect the baseline antigen-independent DC-T cell binding. The inhibition in antigen-dependent binding was cytoskeletal-dependent, because similar results were observed when I used Jasp and LatA. Altogether, increased polarization of DC's cytoskeleton as well as enhanced binding in the presence of antigen, which was inhibited to the antigen-independent baseline when DC's cytoskeleton was disrupted, indicates that DC cytoskeletal reorganization was responsible for the increased levels of T cell antigen-dependent binding. This data is in direct agreement with that of Krawczyk's and Bromley's findings, which showed increased APC-T cell binding in the presence of antigen as compared with antigen-independent binding (Bromley and Dustin, 2002; Krawczyk et al., 2002). Krawczyk *et al* claimed that disruption of the T cell cytoskeletal rearrangement reduced only the antigen-dependent but not the baseline antigen-independent binding (Krawczyk et al., 2002). However, they added the actin inhibitors throughout the assay and thus their findings may result from the effect of the drug on the APC's rather than the T cell's cytoskeleton. Moreover, studies employing mice deficient in Vav-1, a protein known to regulate the cytoskeletal reorganization following TCR engagement (Fischer et al., 1998), evaluated the importance of cytoskeletal rearrangement for antigen-dependent binding. In the presence of the specific antigen, binding of Vav-1^{-/-} T cells to APCs was not increased above the baseline antigen-independent binding when compared with Vav-1^{+/+} T cells (Krawczyk et al., 2002). This study, which used a physiological approach as

compared to that with actin cytoskeletal disrupting drugs, leads to the conclusion that the T cell's cytoskeleton was responsible for the antigen-dependent binding. However, treatment of Vav-1^{-/-} T cells with Mg²⁺, which activates integrins, allowed them to bind to APCs like their wild-type counterparts regardless of the peptide specificity.

The role of the Vav family of GEF in APC function has only been evaluated in B cells (Tedford et al., 2001). In contrast to T cells, only combined disruption of Vav-1 and Vav-2, which are closely related members of the GEF family, resulted in a significant reduction of B cell antigen receptor-mediated proliferation and intracellular calcium mobilization. These findings demonstrated a role for Vav in B cell development and function. However, none of the previous studies have actually evaluated the ability of APCs from Vav-1^{-/-} to bind or activate T cells. Since this is the first demonstration of the unique ability of APC/DC's cytoskeleton for naïve T cell antigen-dependent binding, one may speculate that Vav-1^{-/-}/Vav-2^{-/-} DC might have reduced ability to bind naïve T cells. Further support for this speculation is the observation that inhibition of actin polymerization by drugs such as CytD mimics the defect in Vav-1 (Penninger and Crabtree, 1999). Using a combination of Vav-1^{-/-} and drug approaches, the study reports that disruption of the cytoskeletal rearrangement reduced the antigen-dependent binding of Vav-1^{+/+} T cells to the baseline levels observed with Vav-1^{-/-} T cells in the presence of antigen or with Vav-1^{+/+} T cells in the absence of antigen (Krawczyk et al., 2002). Consistent with these findings, I have also used the approaches of CytD treatment as well as other actin cytoskeletal inhibitors to demonstrate that pretreatment of

DC with these drugs reduces T cell binding only in the presence of antigen without affecting the baseline antigen-independent binding. This indicates that the DC's cytoskeletal reorganization leading to formation of the I-Synapse is an essential step for naïve T cell antigen-dependent binding.

The exact mechanism by which DC's cytoskeletal changes may increase T cell antigen-dependent binding is not known. However, there is some evidence in the literature demonstrating the involvement of integrins in this process. For instance, when *Vav-1^{-/-}* T cells were treated with Mg^{2+} , which activates integrins, they bind to APC like their wild-type counterparts regardless of the peptide specificity (Krawczyk et al., 2002). Furthermore, disruption of cytoskeletal reorganization, which is known to regulate integrin function (Dustin and Springer, 1989; Kucik et al., 1996; van Kooyk and Figdor, 2000), blocks antigen-dependent but not baseline binding. In line with the Krawczyk *et al* finding, addition of Mg^{2+} to my CytD pretreated-DCs restores their binding ability to nearly the control level only in the presence of the antigen (Appendix Fig. 8). In addition, blockage of all integrins ($\beta 1$, $\beta 2$ and $\beta 3$) reduced binding to nearly the baseline antigen-independent binding (Krawczyk et al., 2002). It appears that one integrin may compensate for the other, because when $\beta 2$ (LFA-1) was blocked only partial inhibition was observed. This is also consistent with my finding that the blockage of DC LFA-1 had only partial effects on T cell antigen-dependent binding (Appendix Fig. 9). In addition, the involvement of DC LFA-1 in T cell mediated binding occurs after DC-SIGN mediated antigen-independent binding (Geijtenbeek et al., 2000). This may explain why there was only partial inhibition of binding

when the DC LFA-1 was blocked during the initial DC-T cell interactions.

Altogether, my findings suggest that DC cytoskeletal reorganization may activate integrins leading to formation of the I-Synapse, which in turn is critical for T cell antigen-dependent binding.

4.3.3 Direct correlation between DC cytoskeletal rearrangement and T cell proliferation

Previous studies showed that T cell activation is critically dependent on their clustering by DCs (Austyn et al., 1988b; Inaba et al., 1985; Nussenzweig and Steinman, 1980). I have demonstrated above that DC cytoskeletal rearrangement leading to formation of the I-Synapse plays a critical role in the antigen-dependent T cell binding. In order to directly evaluate the relationship between DC cytoskeletal changes and T cell activation, DCs were prepulsed with wide ranges of peptide and their cytoskeletal changes during binding with T cells as well as their ability to stimulate T cell proliferation were assessed. Consistent with my previous polarization data, DCs pulsed with control peptide did not result in T cell activation above the baseline observed when DCs bind T cells in the absence of peptide. However, DCs pulsed with agonist peptide induced a very strong T cell activation consistent with the high levels of polarization I observed in this DC group. These findings linked DC cytoskeletal changes, which lead to antigen-dependent binding, with increased T cell activation. However, the peptide dose that regulates DC cytoskeletal changes may be different than that induces T cell activation. Indeed, there is evidence in the literature suggesting

that the peptide dose required to induce T cell cytoskeletal changes are lower than that needed for activation (Delon et al., 1998a). In my system, T cell activation increased in a dose-dependent fashion and plateaued at 300nM. The lowest concentration of agonist peptide that consistently induced T cell activation above the baseline levels was 50pM. Interestingly, this was also the lowest dose that consistently induced polarization of the DC's cytoskeleton at the interface with the T cells. Furthermore, when lower doses (10pM) of agonist peptide were used, neither DC cytoskeletal polarization nor T cell activation was increased above the baseline levels observed in the absence peptide. The dose-dependent increase in DC cytoskeletal rearrangement and T cell activation suggests a direct link between the two processes. In line with this suggestion is the high level of correlation between DC polarization and T cell activation I observed. Furthermore, disruption of the DC's cytoskeleton resulted in a significant reduction of T cell activation, demonstrating that the increased in T cell activation was a DC cytoskeletal-dependent event. Finally, my data demonstrates that DC cytoskeletal reorganization and T cell activation occurs at the same threshold of peptide-MHC complexes.

Previous studies reported that the strength of antigen dose inducing TCR-triggering determines the requirements for costimulation and duration of signaling (Bachmann et al., 1997; Viola and Lanzavecchia, 1996), which may be a cytoskeletal-dependent event. Therefore, one might expect APC/DC cytoskeletal reorganization to be more essential for T cell activation at lower antigen doses, where sustained TCR triggering is likely to be more critical for optimal T cell

activation. This speculation is further supported by the finding that activation of T cells mixed with CytD pretreated-DCs was greater when the peptide dose was increased. However, this increase was not as pronounced when I used CytD maybe owing to the reversibility feature of this drug (Cooper, 1987), especially after 3 day of a proliferation assay. Similar doses of LatA, which is a more potent inhibitor (Spector et al., 1989), resulted in a more significant effect at lower peptide doses compared with higher doses.

Consistent with my study, Valitutti *et al* reported that when T cells were stimulated with APCs pulsed with low dose of peptide (25nM), CytD had a marked and time-dependent inhibitory effect on IFN- γ production compared with little inhibitory effect at high peptide dose (50uM) (Valitutti et al., 1995a). Although I used similar doses of CytD, the discrepancy between my findings and Valitutti's may stem from the fact that their CytD was added to culture throughout their assay compared to only 1 h treatment with CytD followed by extensive washing in my experiments. In addition, their high dose peptide was more than 165 times greater than my high dose (300nM) that induced optimal T cell activation. Finally, I measured T cell proliferation as compared with the measurement of IFN- γ production by Valitutti's group and in studies using the DO11.10 transgenic model reported the differential development of Th1-like or Th2-like cells depending on the peptide dose used (Hosken et al., 1995). For instance, IFN- γ production requires less TCR occupancy than IL-2, indicating that the peptide threshold may dictate the profile of cytokine produced by activated T cells (Itoh and Germain, 1997). It is possible that at a low peptide concentration,

the APC cytoskeletal rearrangement may facilitate the accumulation of activation signals up to a threshold required for the optimal activation of T cells. Therefore, it will be interesting to evaluate the fate of T cells that interact with DCs that can not rearrange their cytoskeleton to form an I-Synapse. Lack of DC cytoskeletal reorganization may give rise to distinct functional outputs such as tolerized or regulatory T cells. Alternatively, it may influence the duration of T cell binding to DCs, thereby interfering with development of Th1- or Th2-like cells (Langenkamp et al., 2000). In summary, the data presented herein demonstrate that DC actin cytoskeletal reorganization is critical for optimal naïve T cell activation.

4.3.4 DC cytoskeletal rearrangement is essential for the mRNA expression and bioactivity of IL-2 leading to T cell activation

The findings described above looked at the effect of disrupting the DC's cytoskeleton on T cell activation in a three-day proliferation assay. This is considered a late event following antigen recognition, particularly since DCs rearrange their cytoskeleton within minutes. In order to isolate the role of DC cytoskeletal changes in T cell activation, I looked at early markers of activation. While Ca^{+2} flux and tyrosine kinase activity are thought to be among the first consequences of antigen recognition in T cells, a recent study demonstrated that both events occurred in naïve CD4^{+} T cell binding DC in the absence of antigen (Revy et al., 2001). I have also observed antigen-independent tyrosine kinase activity in naïve T cells binding DC. Furthermore, B cells trigger Ca^{+2} flux in naïve

T cells at a comparable level to DCs, but result in poor proliferation (Delon et al., 1998b). Since B cells do not rearrange their cytoskeleton, Ca^{+2} flux in T cells may be an APC cytoskeletal-independent event. Therefore, neither Ca^{+2} flux nor tyrosine kinase activity may be used as an early marker of T cell activation. Furthermore, earlier studies demonstrated Ca^{+2} flux in T cells binding MHC class II⁻ DCs, indicating that this event occurs in an antigen-independent fashion (Delon et al., 1998b). Upregulation of IL-2 mRNA expression and secretion is considered one of the earliest non-controversial events that is essential for T cell activation (Crabtree, 1989; Taniguchi and Minami, 1993). Time course analysis showed that 4 h was the earliest time point at which IL-2 mRNA was detected in T cells mixed with DC prepulsed with agonist peptide (300nM). Although previous studies reported the expression of IL-2 mRNA by DCs (Granucci et al., 2001), in my hands DCs did not express IL-2 mRNA ruling out the possibility that the source of IL-2 in my experiments was DC rather than T cells. The discrepancy may be due to the fact that I activated DCs with a low dose of LPS followed by extensive washing before addition to cultures, whereas Granucci's group activated their DCs with whole bacteria. In my experiments, IL-2 mRNA expression is strongly dependent on DC cytoskeletal integrity, because disruption of the DC's cytoskeleton with CytD, Jasp or LatA completely abolished IL-2 mRNA expression. Since the interactions between the TCR and the peptide-MHC complexes are of relatively weak avidity, high avidity interactions between receptors and their ligand are required to maintain the APC-T cell conjugates. For instance, costimulation via CD28 has been shown to be essential for the synthesis and stability of IL-2 mRNA

(Lindstein et al., 1989) as well as for IL-2 receptors (Wells et al., 1997). A recent study demonstrated that although all the TCRs were internalized by 15-30 min there was no proliferation if the APC-T cell conjugates were disrupted prior to 2 h (Lee et al., 2002). It is thus possible that the DC cytoskeletal reorganization stabilizes the TCR-peptide-MHC interactions, which results in sustained TCR triggering leading to IL-2 mRNA upregulation and ultimately T cell activation. One could further look at the correlation between the APC cytoskeletal reorganization and persistence of TCR triggering and internalization.

To further explore the significance of DC cytoskeletal reorganization for optimal T cell activation, I extended the IL-2 mRNA findings to evaluate IL-2 at the protein levels. Consistent with the poor proliferation of T cells mixed with DCs prepulsed with media or control peptide (300nM), there was no detectable IL-2 in these cocultures (date not shown). However, cocultures containing DCs pulsed with agonist peptide expressed a high level of IL-2, which was significantly reduced if DCs were pretreated with CytD consistent with the IL-2 mRNA results. The reduction in IL-2 bioactivity levels when DCs were pretreated with CytD was dose-dependent (Appendix Fig. 10A). Most importantly, the dose-dependent reduction of IL-2 bioactivity was also paralleled by a dose-dependent decrease in T cell proliferation (Appendix Fig. 10B), further linking early activation events to ultimate T cell stimulation. Moreover, the decrease in IL-2 levels was also observed when DCs were pretreated with other drugs such as Jasp or LatA (data not shown), indicating that the observed effect was a cytoskeletal-dependent event. Although the disruption of the DC's cytoskeleton abolished IL-2 mRNA expression, it only

inhibited the IL-2 bioactivity. It is possible that these discrepancies resulted from the reversibility effect of these drugs on IL-2 bioactivity after 12-24 h as compared with only 4 h for IL-2 mRNA expression. The effect of these drugs on F-actin is reversible and varies between drugs (Bubb et al., 1994; Cooper, 1987; Spector et al., 1989). This is consistent with the observed discrepancy between the extent of effect of these drugs on IL-2 bioactivity after 12-24 h as well as inhibition of proliferation after 3 days. In summary, the data demonstrate that DC actin cytoskeletal participation in the I-Synapse is critical for naive CD4⁺ T cell's IL-2 mRNA expression and release, leading to optimal T cell activation.

4.3.5 MHC class II ligation on DCs induced their actin cytoskeletal rearrangement

The fact that DC actin cytoskeletal participation in the I-Synapse with naïve CD4⁺ T cells occurs quickly suggests that this is mediated via a pre-existing surface molecule rather than via upregulation of new molecules during the APC-T cell interactions. In addition, these changes occur in DCs only in the presence of the correct antigen, suggesting that the signal that triggered this process may be mediated through MHC class II molecules. The best documented function of MHC class II molecules is their role in peptide binding and presentation to T cells (Weiss et al., 1986). A second described function of MHC class II molecules however, is as transmembrane signal transducers (Cambier et al., 1987). In mast cells, direct ligation of MHC class II using beads induced IL-4 mRNA transcription (Frandsen et al., 1998). There are other examples

of compelling evidence in the literature supporting the role of MHC class II signaling in APCs, but the phenomenon has mostly been studied in B cells. Stimulation of B cells with anti-MHC class II antibodies leads to proliferation (Cambier and Lehmann, 1989), immunoglobulin secretion and enhanced humoral immune responses, respectively (Bishop and Haughton, 1986; Palacios et al., 1983). Elevation of intracellular cAMP and translocation of protein kinase C to the nuclear compartment has been shown in B cells following treatment with anti-MHC class II antibodies (Cambier et al., 1987; Chen et al., 1987). Thus, while formation of a complex of peptide, MHC class II and TCR initiates T cell activation, MHC class II engagement through this complex appears to initiate signaling in APCs as well.

Importantly, signaling through MHC class II molecules has been shown to enhance cell-cell adhesion. For instance, ligation of MHC class II on rat DCs or B cells induces their clustering, which is cytoskeletal- and partially LFA-1-dependent (Kushnir et al., 1998). In APCs such as B cells or monocytes, crosslinking of MHC class II results in homotypic clustering (Fuleihan et al., 1992; Kansas et al., 1992; Lang et al., 2001). Furthermore, treatment of DCs with anti-MHC class II antibodies has been reported to induce homotypic aggregation (McLellan et al., 2000). In line with these findings, I observed homotypic clustering of DCs following treatment with anti-MHC class II antibodies. It is possible that the triggering of MHC class II activates adhesion molecules thereby leading to this homotypic clustering. Indeed, previous studies demonstrated that signaling through MHC class II activates LFA-1, thereby providing a mechanism

for enhanced adhesion between T cells and MHC class II⁺ APCs (Mourad et al., 1990). However, other studies reported LFA-1-independent adhesion triggered by anti-MHC class II signaling (Fuleihan et al., 1992; Kansas et al., 1992). This LFA-1-independent binding may be mediated via other adhesion molecules but not by DC-SIGN since both studies used B cells, which lack DC-SIGN expression. In summary, triggering of MHC class II molecules enhances homotypic as well as APC-T cell adhesion.

DC cytoskeletal rearrangement is critical for their ability to bind naïve CD4⁺ T cells in antigen-dependent fashion. Since triggering of MHC class II enhances cell-cell adhesion, I examined whether DC cytoskeletal reorganization is the result of signaling through the DC's MHC class II molecules. The use of beads to directionally crosslink MHC class II on the surface of the DC resulted in DC's F-actin polarization toward the bead's interface. However, crosslinking of other DC's surface molecules did not trigger cytoskeletal changes above the background levels. This demonstrates that the involvement of DC actin cytoskeletal rearrangement in the I-Synapse is specifically mediated in part through MHC class II ligation. When compared to CD4⁺ T cells, crosslinking of MHC class II on DCs using beads was less efficient, indicating a potential role for additional interactions between receptor-ligand pairs in this process. Signaling through MHC class II has been shown to induce B7 expression (Nabavi et al., 1992), a key step for optimal T cell activation. In addition, the engagement of MHC class II by recombinant CD4 or monoclonal antibody results in homotypic adhesion of B cells (Kansas et al., 1992). However, it may be noted that the

percentage of DCs reorganizing their cytoskeleton using beads was increased if IgM anti-MHC class II antibodies were used (data not shown), indicating that these changes are crosslinking-dependent. A previous study reported that MHC class II molecules with truncated intracytoplasmic tails are much less immunogenic than normal MHC class II molecules (Nabavi et al., 1989; Wade et al., 1994). While the decreased immunogenicity may reflect a failure in the signal transmitted to the TCR on T cells, the truncated MHC class II presumably have a defect in transducing signal into the APC that in turn affects T cell activation. Alternatively, truncation of MHC class II molecules may reduce their lateral movement in the cell membrane, which is believed to be mediated by the accumulation of these molecules in lipid rafts to facilitate antigen presentation (Anderson et al., 2000).

In line with the observed role of MHC class II signaling in DC cytoskeletal reorganization using the bead system, I observed an antigen-dependent polarization of MHC class II on DCs during formation of the I-Synapse with naïve CD4⁺ T cells (data not shown). Providing further support for this work, Boes *et al* recently used time-lapse imaging to visualize the intracellular movement of the GFP-tagged MHC class II molecules in DCs that encountered T cells in the presence or absence of antigen (Boes et al., 2002). Their findings clearly demonstrate the delivery of MHC class II-positive compartments toward the plasma membrane of DC that point directly to the area of contact with the T cells in an antigen-dependent manner. More recent evidence on the role of MHC class II signaling in DCs is provided by Andreae *et al* who demonstrated human

DC maturation following treatment with lymphocyte activation gene-3 (CD223), which is an MHC class II ligand (Andreae et al., 2002). These findings further support my studies that showed functional changes in DC following MHC class II triggering. Most importantly, the finding that triggering of MHC class II on DCs results in morphological changes (Andreae et al., 2002), which are known to be dependent on the cytoskeleton, provides another piece of evidence that links MHC class II signaling and cytoskeletal changes in DC.

Based on my findings, I propose that upon the recognition of specific peptide, the T cell sends a signal through MHC class II to initiate DC cytoskeletal participation in the I-Synapse. This process will in turn promote the T cells to seek more help from DCs to allow optimal activation. This may be accomplished by tightening the interaction between the two cells as well as involving other surface and signaling molecules at the I-Synapse. Thus, this process may be tightly regulated and occurs only to promote productive activation of T cells that have recognized specific antigen on the DC. In summary, my findings suggests that signaling through MHC class II leading to DC's cytoskeletal participation in the I-Synapse may enhance their antigen-dependent binding as well as their ability to deliver efficient activation signal to naïve CD4⁺ T cells.

SUMMARY

The original paradigm was that formation of an I-Synapse with the APC requires participation of the T cell's but not the APC's actin cytoskeleton. My studies provide the first evidence that the APC/DC cytoskeleton also plays a dynamic role in establishing the I-Synapse with resting allogeneic CD4⁺ or naïve transgenic CD4⁺ T cells in the presence of the specific peptide. These findings thus shift the original I-Synapse paradigm especially with respect to the design of potential mechanism(s) of regulating the immune responses that target the DC rather than the T cells. The results of my studies suggest that actin cytoskeletal rearrangement by DCs leading to formation of the I-Synapse is probably one of the mechanisms that allows them to be superior APCs in clustering and in activating resting/naive T cells.

The exact mechanism by which DC actin cytoskeletal rearrangement enhances their ability to cluster and activate T cells remains unknown. However, it is likely that actin polymerization will promote the correct orientation of surface molecules such as integrins, thereby facilitating the interaction between T cells and APCs (Appendix Fig. 11). DCs may reorganize their cytoskeleton to promote polarized secretion of mediators, effector molecules and delivery of signals to a confined region of the T cell, which are ready to be triggered. This process will result in a microenvironment that not only boosts T cell activation, but it will also minimize bystander effects on other T cells in close proximity. DC actin cytoskeletal rearrangement may also enhance their binding ability by

activating molecules like LFA-1, which then may participate in clustering and activation of resting T cells (Geijtenbeek et al., 2000).

A previous study by the Gunzer group using video microscopy demonstrated that the interaction of naïve CD4⁺ T cells with DCs is short-lived (6-12 min) and dynamic, where a T cell jumps from one DC to another (Gunzer et al., 2000). Although, in my experiments, DCs rearrange their cytoskeleton as early as 5 min, the upregulation of other DC accessory molecules as well as cytokine release may take more than 12 min. Therefore, a significant problem with the Gunzer model is that by the time DCs release their cytokines into the milieu, the T cell that induced this has already left to make contact with another DC. This is likely to result in the activation of bystander T cells, leading to undesirable immune responses. The Gunzer group used a collage matrix to mimic the T cell activation process in a lymphatic tissue environment, whereas other studies evaluate the APC-T cell interactions using liquid culture models. However, a recent study by Stoll *et al* visualized the antigen-specific T cells interacting with DCs within intact lymph nodes (Stoll et al., 2002). This study demonstrated that the interaction lasts for hours (>15 h) thus questioning Gunzer's findings. Nonetheless, I observed DC cytoskeletal polarization very quickly (at less than 5 min) and this could allow participation of the DCs in the temporary synapses seen by Gunzer. There is also a low frequency of contacts between TCRs and rare specific peptide-MHC complexes that are small in size and have low affinity, which may be enhanced by cytoskeletal-dependent changes in DCs and T cells. Recognition of the specific peptide-MHC complexes

by the TCR will activate integrins (Negulescu et al., 1996), which eventually leads to the formation of stable DC-T cell conjugates. Alternatively, actin rearrangement might be required for setting up a signaling scaffold in the DCs much as it does in the T cells. This might facilitate important signaling into the DCs through accessory molecules and receptors at the DC-T cell contact areas. Such changes might further increase conjugate stability and result in the formation of signaling complexes downstream of the TCR. The significance of the engaged TCR stability is further supported by the observation that the peptide-MHC complexes in the center of the I-Synapse were sequestered from the pool of free peptide-MHC complexes and did not exchange or are released from the clusters (Grakoui et al., 1999). Furthermore, polarization of the cytoskeleton may be more critical in an in vivo system to ensure the delivery of a polarized communication between DCs and T cells in a crowded environment such as in secondary lymphoid organs.

Finally, while DC actin cytoskeletal participation in the I-Synapse occurs in an antigen-dependent fashion, naïve T cell actin involvement in this process is antigen-independent. It appears that participation of T cell's actin cytoskeleton in the I-Synapse takes place in three steps. 1) T cells make initial contact with the DCs. 2) An immature synapse or "Presynapse" forms that allows a T cell to sample various antigens on the surface of a DC and is expected to result in a low affinity binding. 3) A mature synapse forms leading to a high affinity binding which is formed only when a T cell recognizes the specific antigen and which requires participation of the DC's actin cytoskeleton. It is likely that the high

antigen-dependent participation of the DC's actin cytoskeleton is to create a microenvironment that sends an activation signal to only antigen-specific T cell and thus avoids the activation of antigen-nonspecific T cells that bound to DC in an antigen-independent manner. Altogether, MHC class II-mediated DC actin cytoskeletal reorganization leading to I-Synapse formation is likely to regulate the overall specificity of the T cell activation process at the DC levels.

Disruption of the I-Synapse that formed between DCs and naïve CD4⁺ T cells, which leads to inhibition of the immune response, might be useful in the treatment of transplant rejection. However, enhancement of formation of the I-synapse between these two cells, which leads to the initiation of immunity, might be useful in the treatment of cancer and infection. A better understanding of the mechanism(s) that regulate formation of the I-Synapse between DCs and T cells will certainly provide a valuable knowledge that may be used for the development of new therapeutic strategies to prevent undesired immune responses and yet boost beneficial immune responses.

**APPENDIX
(ADDITIONAL FIGURES)**

Figure 1. Treatment of DCs with CytD inhibits F-actin. Control or CytD (20 μ M) treated DCs were washed 3 times and reseeded for 1 h in GM-CSF containing media. F-actin was analyzed by Western blot essentially as described in the Materials and Methods. Data indicates the relative band intensity as assessed by densitometry. Inset shows the actual Western blot of F-actin in control and CytD-treated DCs.

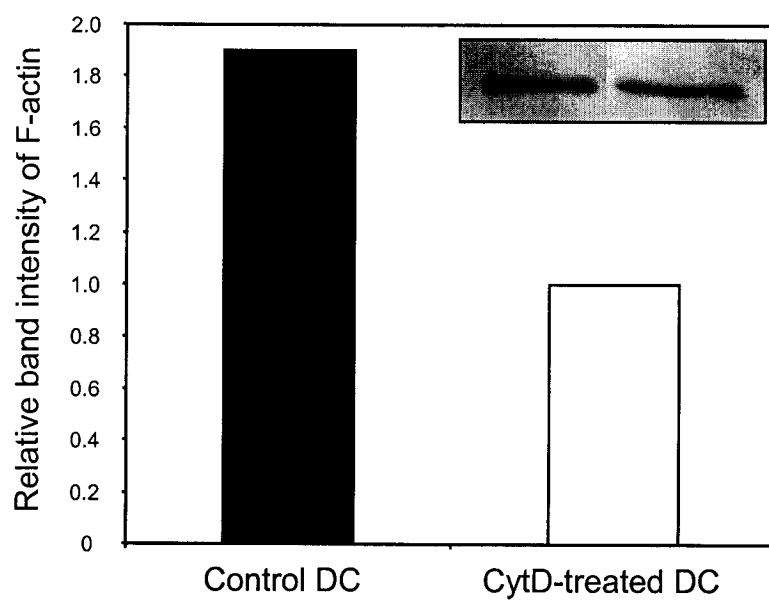


Fig. 1

Figure 2. Clustering of syngeneic T cells is not affected by pretreatment of DCs with CytD. DCs were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. Control or CytD (20 μM)-pretreated DCs were mixed with resting syngeneic CD4⁺ T cells. Clusters were formed by low-speed centrifugation of DCs and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean ± SD of the percentage of the DCs clustering CD4⁺ T cells.

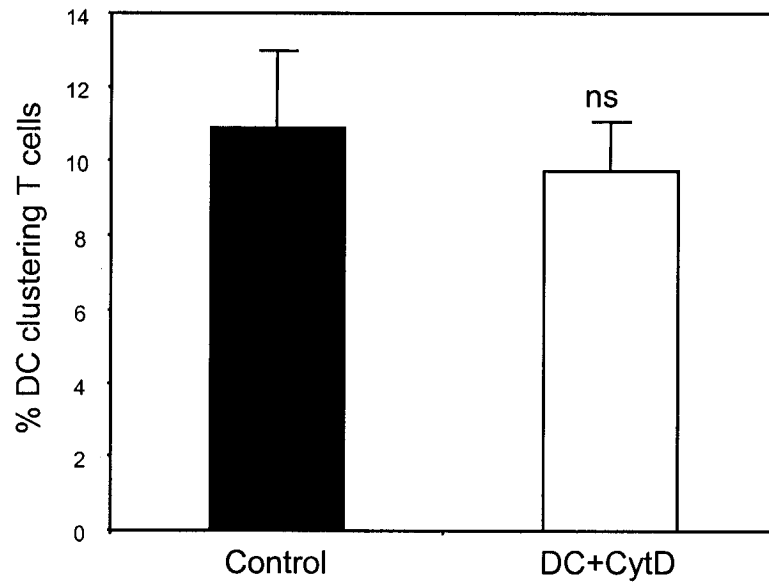


Fig. 2

Figure 3. Pretreatment of DCs with LatA inhibits their ability to cluster resting allogeneic CD4⁺ T cells. DCs were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. Control or LatA (20 μM)-pretreated DCs were mixed with control or CytD (20 μM)-pretreated resting allogeneic CD4⁺ T cells. Clusters were formed by low-speed centrifugation of DCs and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean ± SD of the percentage of the DCs clustering CD4⁺ T cells.

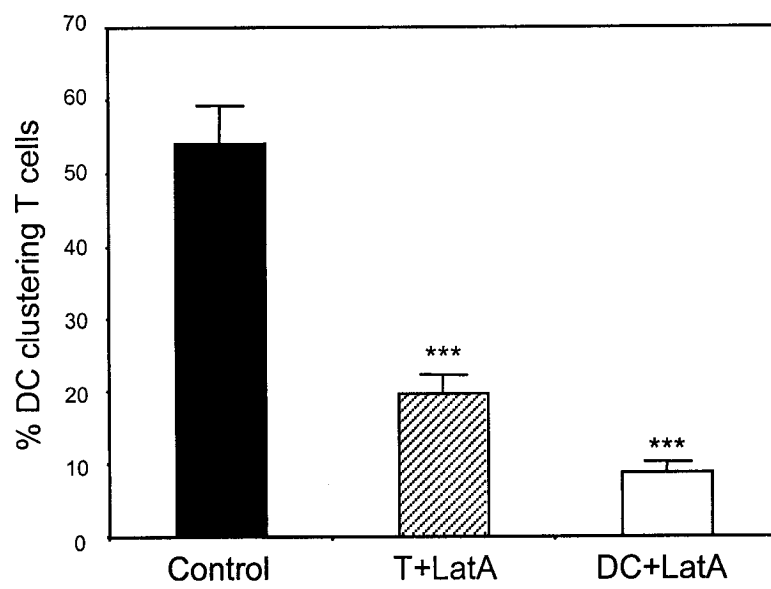


Fig. 3

Figure 4. *a–c*, Treatment of DCs with CytD does not affect their expression of MHC class II, B7-2 or fascin. Control or CytD (20 μ M)-pretreated DCs were stained for MHC class II (*a*) or B7-2 (*b*). Cells from both groups were permeabilized prior to fascin (*c*) staining. The expression of these proteins was evaluated by FACS, where the dark and light lines represent control and CytD-pretreated DCs, respectively.

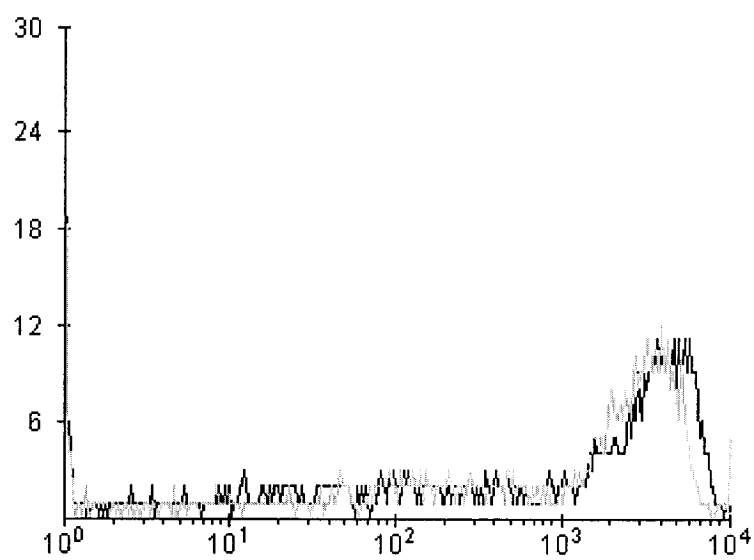


Fig. 4A

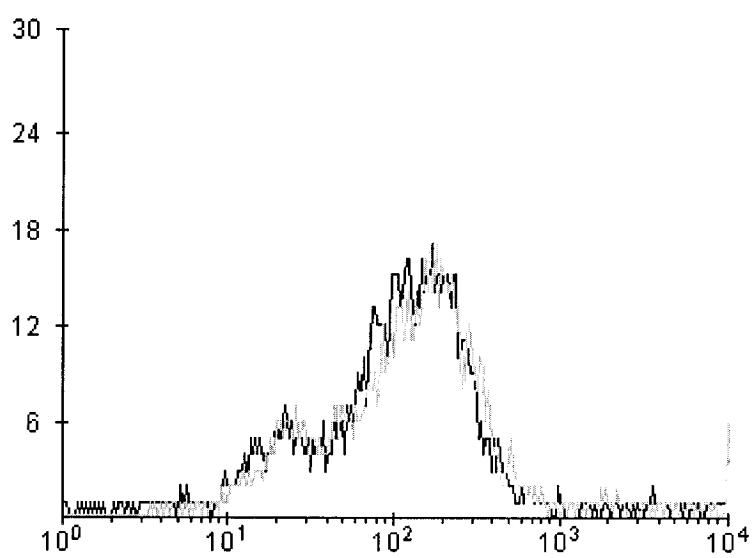


Fig. 4B

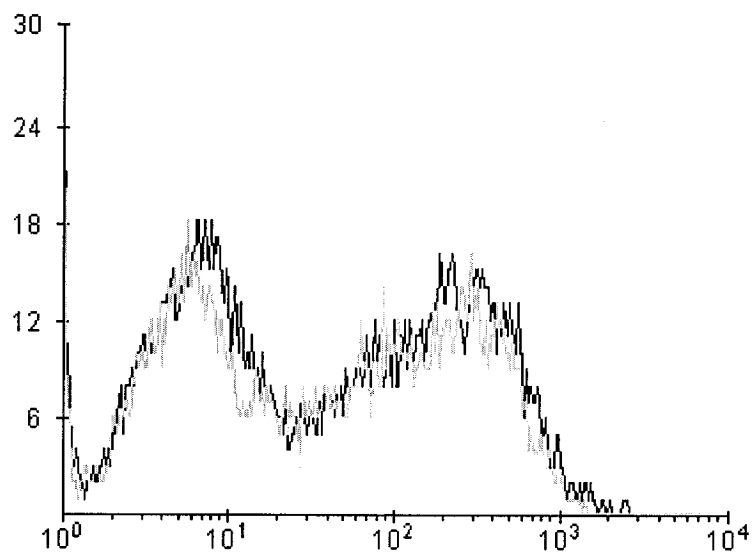


Fig. 4C

Figure 5. *a* and *b*, Pretreatment of DCs with LatA inhibits their ability to activate resting allogeneic CD4⁺ T cells. Control or LatA (20 μM)- pretreated DCs were mixed with control or LatA (20 μM)-pretreated CD4⁺ T cells. *b*, Control or LatA (20 μM)-pretreated DCs were mixed with CD4⁺ T cells at different DC:T cell ratios. The cells were pulsed with [³H] thymidine in the last 18 h of the 4-day MLR. T cell proliferation was assessed by measuring the [³H] thymidine uptake in a liquid scintillation counter. The results are expressed as mean dpm ± SD.

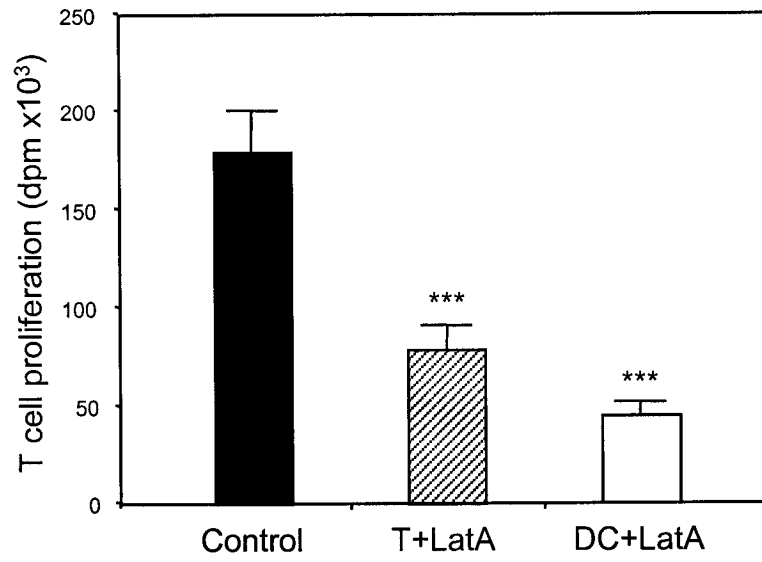


Fig. 5A

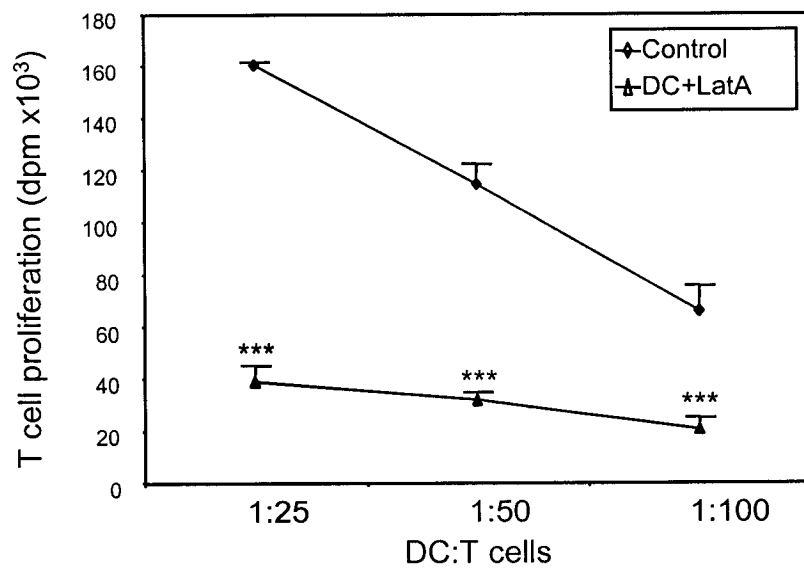


Fig. 5B

Figure 6. *a* and *b*, No inhibition of resting allogeneic CD4⁺ T cells clustering or activation when mixed with CytD-pretreatment B cells. *a*, Control or CytD (20 mM) pretreated B cells were mixed with resting CD4⁺ T cells. B cells were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. Clusters were formed by low speed centrifugation of B cells and CD4⁺ T cells and then transferred to a 96-well flat bottom plat for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean \pm SD of the percentage of the DC clustering CD4⁺ T cell. *b*, Control or CytD (20 mM) pretreated B cells were mixed with resting CD4⁺ T cells. The cells were pulsed with [3H] thymidine in the last 18 h of the 4 day MLR. T cell proliferation was assessed by measuring the [3H] thymidine uptake in a liquid scintillation counter. The results are expressed as mean dpm \pm SD.

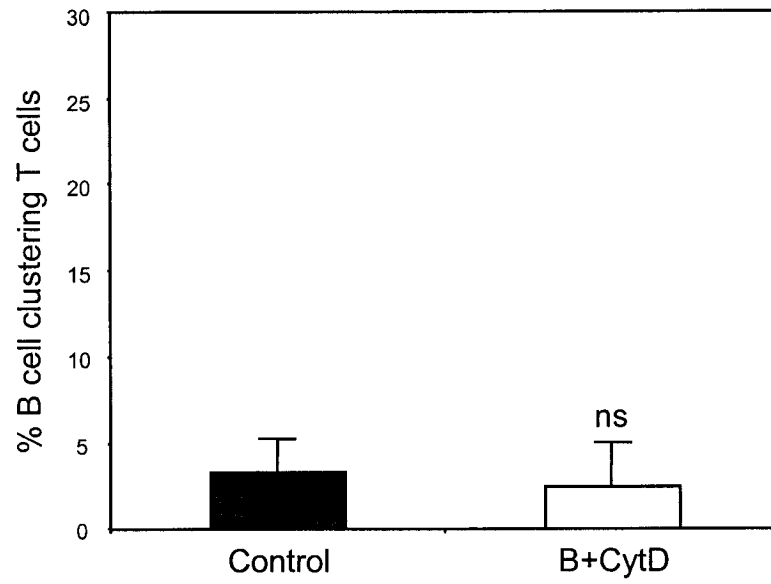


Fig. 6A

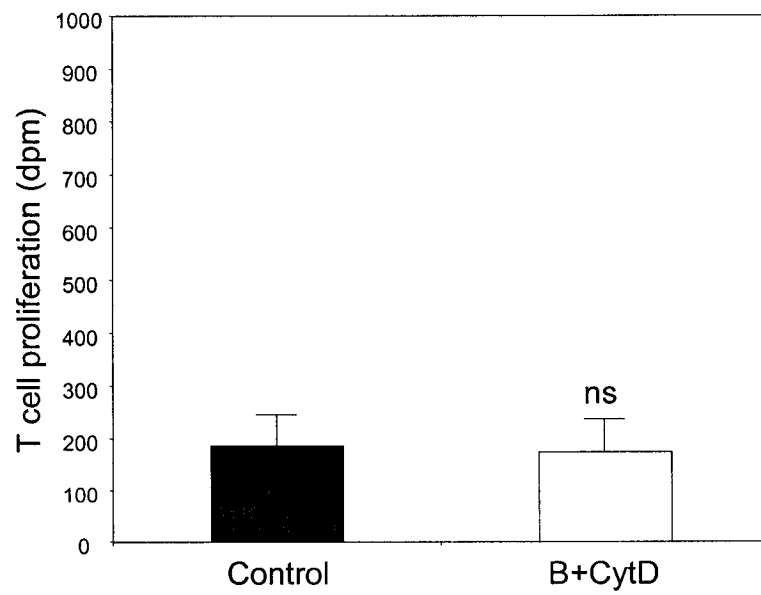


Fig. 6B

Figure 7. T cell binding to DCs in the presence of antigen occurs in a dose-dependent fashion. DCs were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. DCs prepulsed with media (control) or various doses of agonist peptide were mixed with naïve CD4⁺ T cells. Clusters were formed by low-speed centrifugation of DCs and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean \pm SD of the number of T cells bound to 100 DCs.

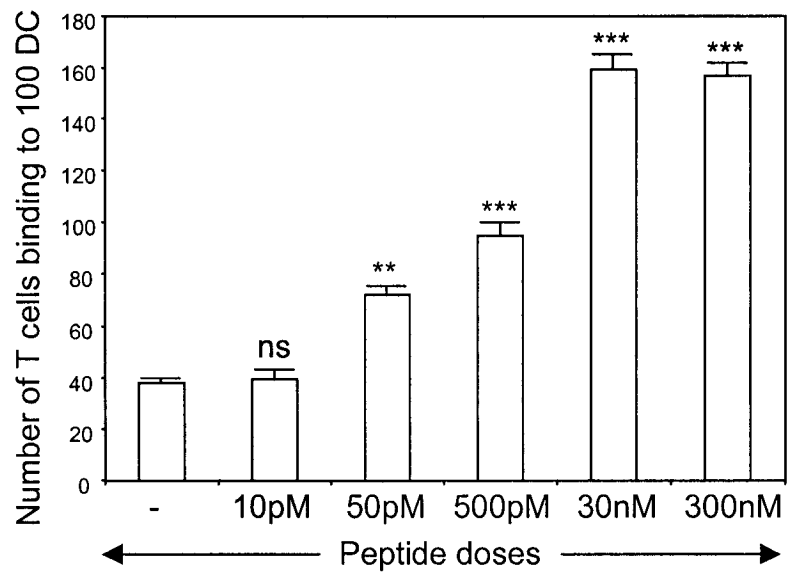


Fig. 7

Figure 8. The inhibition of antigen-dependent T cell binding to CytD pretreated-DCs is restored by Mg^{2+} treatment. DCs were labeled with green CFMDA dye and $CD4^+$ T cells with red CM-Dil dye. DCs prepulsed with media or agonist peptide (300nM) were treated with CytD prior to mixing with naïve $CD4^+$ T cells $\pm Mg^{2+}$ (5mM $MgCl_2$ + 1mM EGTA). Clusters were formed by low-speed centrifugation of DCs and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean \pm SD of the number of T cells bound to 100 DCs.

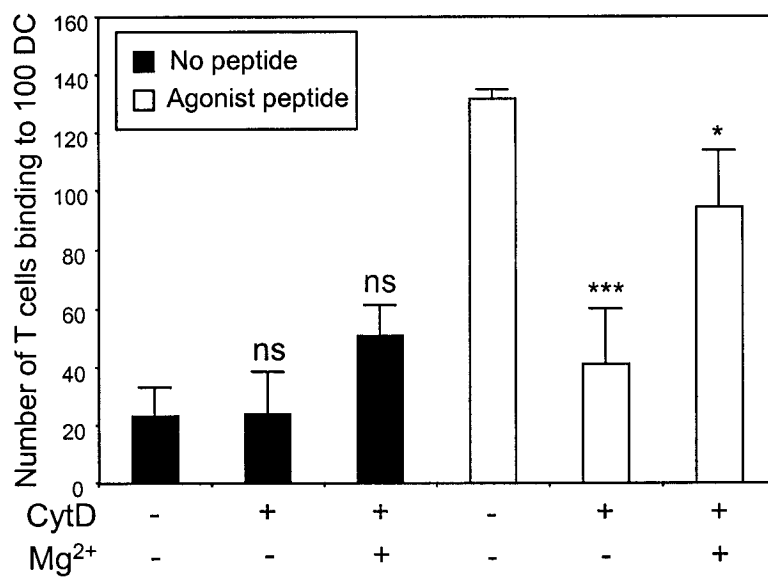


Fig. 8

Figure 9. T cell binding is partially inhibited by blocking DC adhesion molecules. DCs were labeled with green CFMDA dye and CD4⁺ T cells with red CM-Dil dye. DCs prepulsed with media or agonist peptide (300nM) were treated with anti-LFA-1, anti-ICAM-1 or anti-LFA-3 blocking antibodies prior to mixing with naïve CD4⁺ T cells. Clusters were formed by low-speed centrifugation of DCs and T cells as described in Materials and Methods and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean \pm SD of the number of T cells bound to 100 DCs.

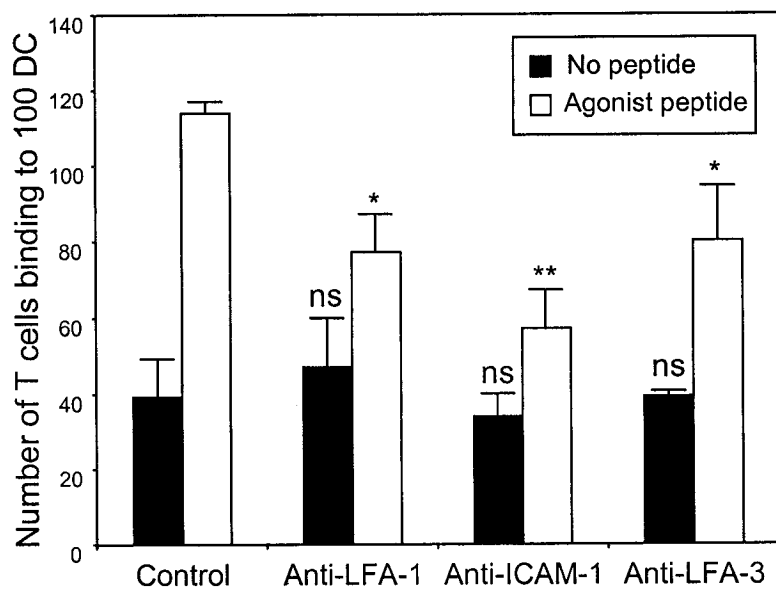


Fig. 9

Figure 10. *a* and *b*, Pretreatment of DCs with CytD inhibits IL-2 bioactivity and T cell activation in a dose-dependent manner. DCs pretreated with DMSO (control) or various doses of CytD and mixed with T cells and agonist peptide (300nM). *a*, Supernatants were collected (12h), added to CTLL-2 cells and proliferation was determined as a measure of IL-2 bioactivity. *b*, Proliferation of T cells mixed with control or CytD pretreated-DCs was assessed by measuring [³H] thymidine uptake. The results are expressed as mean dpm \pm SD.

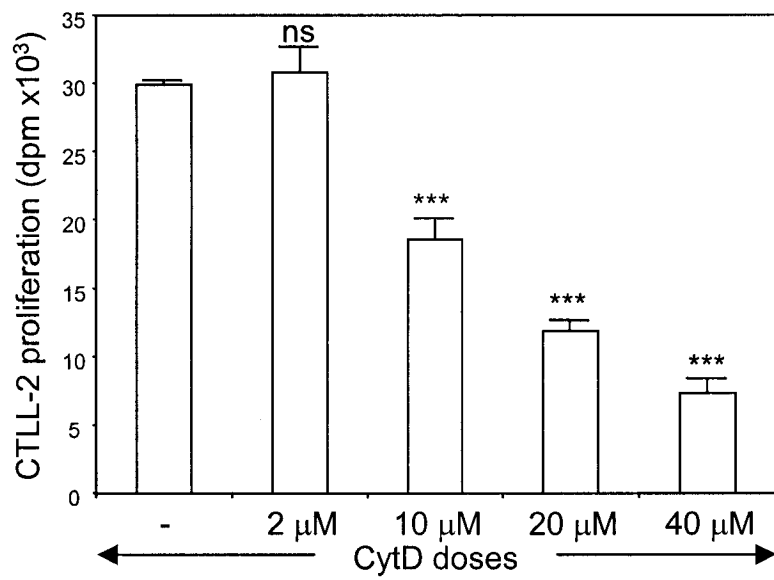


Fig. 10A

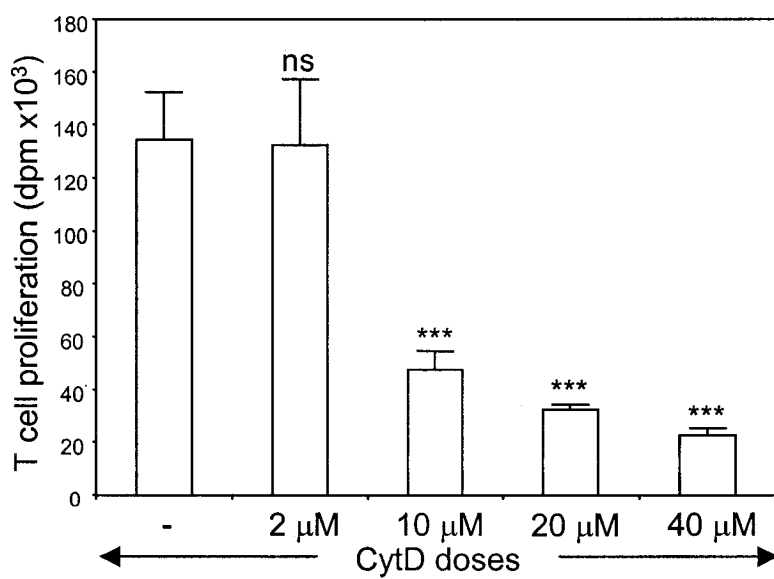
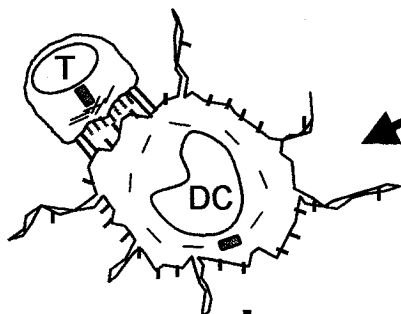
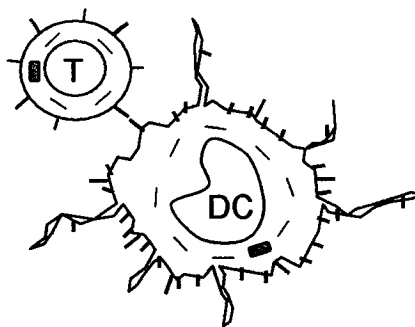


Fig. 10B

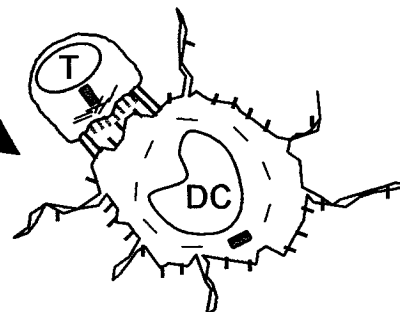
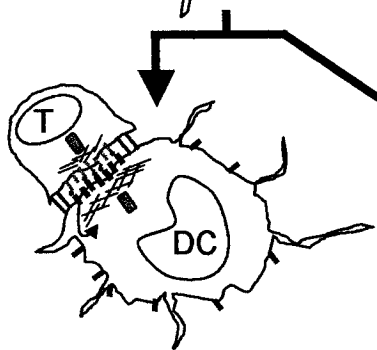
Figure 11. A proposed model illustrating the different steps in DC-naïve CD4⁺ T cell synapse formation, and the consequences on the fate of T cell development.

- | TCR
- | MHC
- | Adhesion molecules
- | F-actin
- ▣ MTOC
- Cytokines

1) T cell-DC binding. The T cell makes initial contact with the DC through adhesion molecules.



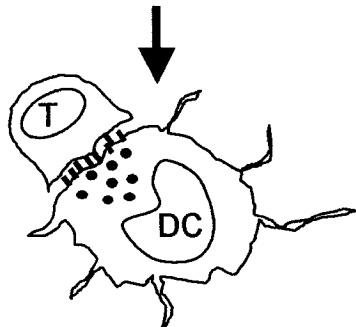
2) T cell "presynapse" formation. The T cell and not the DC forms a synapse in the absence of antigen. This allows the T cell to sample peptide-MHC complexes on the DC.



3A) Mature synapse formation.

Specific antigen encounter by TCRs on the T cell sends a signal through MHC class II on the DC. This signal triggers the DC's participation in the synapse, which enhances membrane alignment and cell-cell adhesion.

3B) Lack of DC involvement in the synapse. The T cell does not recognize specific peptide-MHC complexes on the DC. Consequently, the DC does not participate in the synapse. This may lead to the development of tolerized or regulatory T cells.



4) Consequence of DC participation in the synapse. DC cytoskeletal polarization may create a microenvironment of polarized cytokines as well as surface molecules such as MHC class II. This process leads to productive T cell activation in a highly antigen-dependent manner.



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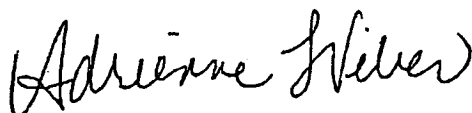
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