

Signal Transduction in T Helper Cells: CD4 Coreceptors Exert Complex Regulatory Effects on T Cell Activation and Function

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Abstract

The immune system provides a highly sophisticated surveillance mechanism to detect diverse antigens and to protect the host organism from invading pathogens and altered cells (e.g., virus-infected and tumor cells). Adaptive immune responses depend on the recognition of antigen by specific antigen receptors that are expressed on the surface of T and B lymphocytes. Helper T cells provide regulatory functions and direct the adaptive immune system to respond appropriately to a particular antigen (i.e., cytotoxic T cell responses against viral infections and tumor cells, humoral responses against extracellular bacteria and parasitic worms). Helper T cells express CD4 coreceptors, which recognize conserved domains on proteins expressed by the class II major histocompatibility complex, the same proteins that present antigen to the T cell receptor. Recent progress in T cell biology has identified multiple regulatory functions of CD4 during thymocyte development and antigen stimulation of mature T helper cells. Signaling pathways induced by engagement of CD4 independently of T cell receptor signaling mediate these regulatory functions. In this review, we discuss the regulation of T cell signaling and emphasize the functional consequences of proper and improper CD4 coreceptor signaling.

Introduction

The immune system provides a highly sophisticated surveillance mechanism to detect diverse antigens and protect the host organism from invading pathogens and altered cells (e.g., virus-infected and tumor cells). Adaptive immune responses depend on the recognition of antigens by specific antigen receptors expressed on the surface of T and B lymphocytes. To initiate effector mechanisms, antigen recognition must induce intracellular signaling cascades that activate the lymphocyte and promote the differentiation to an effector cell appropriate for the

particular antigenic challenge. Importantly, regulatory mechanisms must also be present to safeguard against inadvertent self-reactivity, which could lead to autoimmunity, and to terminate immune responses, thus avoiding overexposure of the organism to toxic effectors (e.g., cytotoxic T cells, cytokines).

T lymphocytes are derived from the lymphoid lineage of hematopoietic stem cells. T cell progenitors enter the thymus where they develop into mature T lymphocytes (Res and Spits, 1999). During thymic development, the immature thymocytes undergo rearrangement of first the β and then the α T cell receptor (TCR) genes (Khor and Sleckman, 2002; Raulet *et al.*, 1985). This process increases the diversity of available TCRs and assures that each T cell expresses only a single type of TCR. Only those thymocytes that have successfully completed TCR gene rearrangement will be allowed to survive. Unsuccessful rearrangement leads to programmed cell death by apoptosis.

Following rearrangement, the functionality of the maturing thymocytes is tested by interactions with thymic antigen-presenting cells (APCs). In order to be functional, the thymocytes' TCR must be able to engage epitopes formed by short peptides bound to molecules encoded by the major histocompatibility complex (MHC). Because of the large isotypic and allelic variability of MHC molecules within each species, not all recombination events of TCR genes lead to matches with a peptide/MHC complex potentially present in the individual organism. Therefore, a positive selection event is required. Two classes of MHC molecules select T cells with different functions. MHC class I molecules bind peptides derived from proteins synthesized by the presenting cell and proteolytically processed by the cell's proteasome (Niedermann, 2002). These peptides can combine with nascent MHC class I molecules in the endoplasmic reticulum (Norbury *et al.*, 2001; Yewdell, 2001). MHC class II molecules bind peptides derived from extracellular, endocytosed proteins that are processed in lysosomes. Nascent and recycling MHC class II molecules bind these peptides while trafficking through lysosomes (Germain, 1994).

Positive selection of thymocytes requires that the interactions between the TCR and peptide/MHC complexes induce signals of sufficient strength and duration (Hogquist, 2001). Thymocytes that do not receive a TCR-mediated signal during positive selection die. Positive selection occurs on thymic stromal epithelial cells (Capone *et al.*, 2001; Chidgey and Boyd, 2001), which do not present a full complement of all possible antigens that the T cell might encounter during its lifetime. Thus, selected T cells have partial self-reactivity and the potential to recognize antigens

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that will only be encountered later in life. The mechanisms and models of positive selection have been recently reviewed (Germain, 2002; Singer, 2002).

Maturing thymocytes express both CD4 and CD8 coreceptors. Following positive selection, one coreceptor gene is silenced (Leung *et al.*, 2001; Taniuchi *et al.*, 2002; Zou *et al.*, 2001). Recent experimental results suggest that the duration of the selection signal determines lineage commitment with a short duration leading to differentiation to CD8⁺ and a long duration favoring differentiation to CD4⁺ thymocytes (Brugnera *et al.*, 2000; Yasutomo *et al.*, 2000).

A negative selection step eliminates thymocytes that overtly respond to self-antigens. Estimates suggest that of all thymocytes that mature up to the double-positive (CD4⁺CD8⁺) stage, only about 3% complete maturation and emigrate from the thymus to peripheral lymphoid tissues (e.g., blood, lymph nodes, and spleen) (Surh and Sprent, 1994). Negative selection is mediated by thymic stromal cells or APCs (mainly dendritic cells) that migrate from peripheral organs into the thymus (Brocker, 1999). These cells provide a large battery, but not a complete complement, of different peptides derived from self and foreign proteins. Thymocytes that vigorously respond to the stimuli presented during negative selection will undergo apoptosis (Bommhardt *et al.*, 2000; Robey and Fowlkes, 1994; Williams *et al.*, 1999).

In this review, we will discuss signal transduction in CD4⁺ T helper (Th) cells, which regulate adaptive immune responses by promoting and directing the differentiation and activation of B cells and cytotoxic CD8⁺ T cells (Schoenberger *et al.*, 1998; Snow *et al.*, 1994). Activation of Th cells is critical for the clearance of pathogenic infections (Diepolder *et al.*, 1998; Taylor-Robinson and Phillips, 1992) and for the destruction of tumor cells (Kahn *et al.*, 1991). Th cells also participate in the pathogenesis of many autoimmune disorders (Datta *et al.*, 1997; Rizzo *et al.*, 1996) and in transplant rejection (Bushell *et al.*, 1995). Th cells recognize antigen in the context of MHC class II molecules and express the CD4 coreceptor on their cell surface, which interacts with non-polymorphic regions of MHC class II (König *et al.*, 1992; König *et al.*, 1995). The binding of the antigen-specific TCR to antigen/MHC class II complexes initiates Th cell activation, but the engagement of CD4 by MHC class II regulates Th cell signaling, promoting progression in the cell cycle and expression of immune response genes.

T helper cell activation

The antigen-specific T cell receptor

Following thymic selection, peripheral T lymphocytes express a TCR that is composed of two transmembrane proteins, the α and the β chains, linked by a disulfide bridge. This $\alpha\beta$ TCR is the antigen-recognition unit of the T cell, but it does not have intrinsic signaling capability. The ability to transduce intracellular signals is conveyed to the TCR via its mandatory and constitutive association with a multi-protein structure, termed the CD3- ζ complex. None of the individual components of the TCR/CD3- ζ signaling machine can be transported to the cell surface without full assembly of the complex (Sun *et al.*, 2001).

The CD3 complex is composed of four transmembrane polypeptide chains, a $\gamma\epsilon$ and a $\delta\epsilon$ heterodimer. These proteins have very short extracellular domains and each intracellular domain contains a conserved protein tyrosine kinase (PTK) recognition motif, termed “Immunoreceptor Tyrosine-based Activation Motif” (ITAM). The associated, disulfide-linked ζ -dimer, sometimes substituted by a ζ - η heterodimer, contains three ITAMs per protein chain (De Aos *et al.*, 1997). ITAMs are substrates for *src* family PTKs (Iwashima *et al.*, 1994; Weil *et al.*, 1995), and their phosphorylation is a determining initiation event for T cell signaling (Qian *et al.*, 1993).

The binding of ligands to the TCR triggers the activation of receptor-associated *src* family PTKs, such as p56^{lck} and p59^{fyn}, leading to the rapid tyrosine phosphorylation of numerous proteins. The phosphorylation of ITAMs located in the cytoplasmic tails of CD3 and ζ_2 generates binding sites for proteins bearing Src homology 2 (SH2) domains such as the cytosolic *syk* family PTK “ ζ -associated protein of 70 kDa” (ZAP-70) (Chan *et al.*, 1991). Recruitment of ZAP-70 allows enhanced activation of that kinase (Lograsso *et al.*, 1996). ZAP-70 in turn phosphorylates components of distinct downstream signaling pathways (Elder *et al.*, 2001; Gong *et al.*, 2001; Magnan *et al.*, 2001). Thus, T cell activation depends on the activation of both *src* family kinases and ZAP-70 (Figure 1).

The formation of signalosomes

The engagement of TCRs by antigen/MHC ligands induces a complex temporal and spatial arrangement of signaling complexes and networks. Slightly different compositions of these signaling machines – also termed “signalosomes” (Werlen *et al.*, 2000) - using essentially the same components can induce different second messenger signals and lead to drastically diverse cellular responses. In addition, the dynamic assembly and disassembly of signalosomes is likely a major factor in regulating signal transduction networks. TCR signalosomes consist of transmembrane receptors, protein kinases, phosphatases and their substrates, all of which are organized into signaling machines by anchoring, adapter, and scaffolding proteins. Signalosomes connect events on the plasma membrane to distal signaling cascades, which ultimately modulate T cell biology. Several protein adapters, in particular “linker of activated T cells” (LAT), act as central switches that translate the quality, quantity, and duration of signals into the correct activation of specific downstream pathways (Zhang *et al.*, 1998). Although the precise parameters that regulate the formation of signalosomes still await further clarification, recent work in this area has identified some characteristics of the signalosomes.

Formation of signalosomes is aided by compartmentalization of the plasma membrane into detergent-insoluble, sphingolipid/cholesterol-enriched micro-domains, which promote the recruitment of signal transduction molecules to the TCR signaling machine upon TCR engagement (Harder and Kuhn, 2001; Leitenberg *et al.*, 2001). These areas of the T cell surface are also known as lipid “rafts”. Palmitoylation constitutively embeds several components such as Lck, Fyn, and LAT into these lipid micro-domains, whereas others such as ZAP-70 relocalize into rafts upon TCR engagement (Harder and Kuhn, 2001; Werlen *et al.*, 2000).

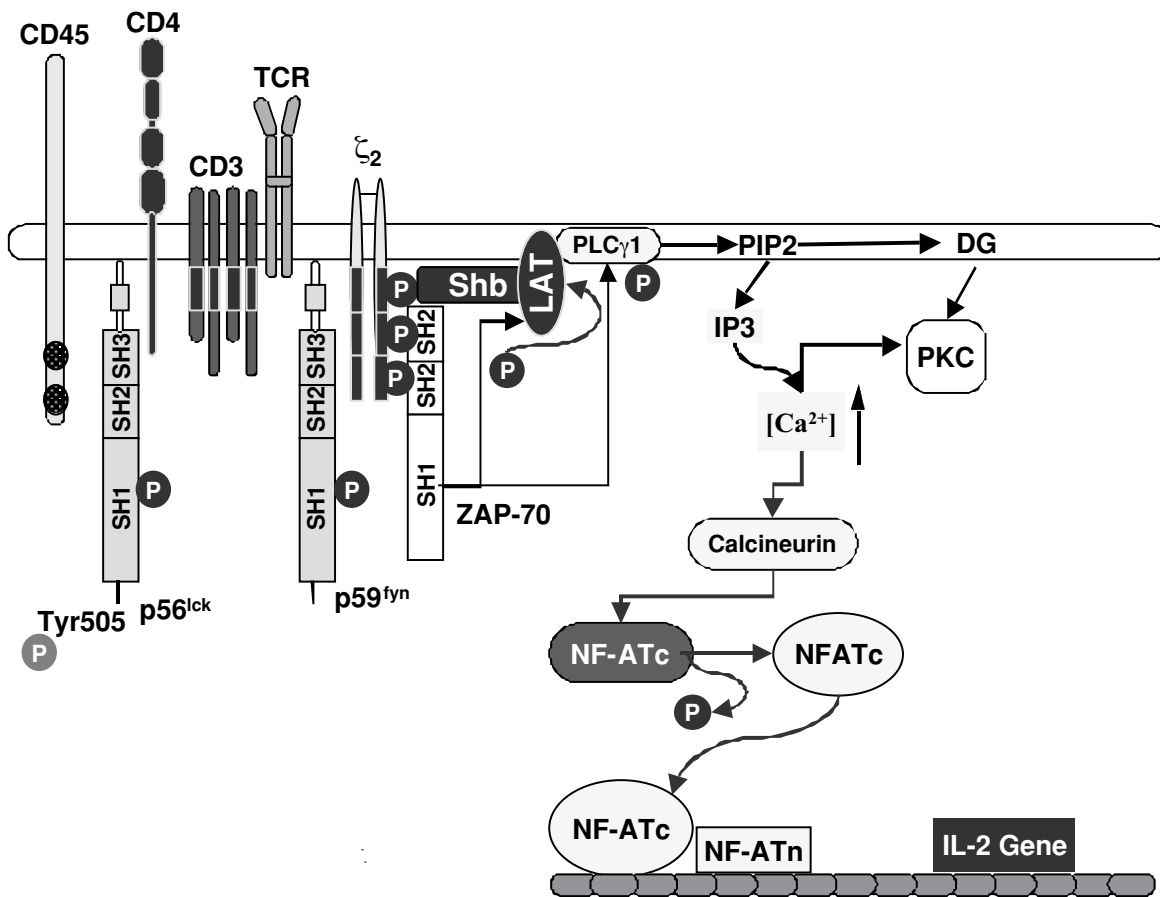


Figure 1. The TCR signaling machinery and activation of PLC γ 1. The initial signalosome consists of the TCR, the associated CD3/ ζ_2 complex, the co-receptor CD4, the phosphatase CD45, and the *src* kinases p56^{lck} and p59^{fyn}. The *syk* kinase ZAP-70 is recruited following phosphorylation of tyrosines located in ITAMs of CD3 and ζ_2 by *src* kinases. See text for details.

The relocation of signalosomes to receptor-associated scaffolds is crucial for effective signal transduction (Delgado *et al.*, 2000; Harder and Kuhn, 2000; Werlen *et al.*, 2000). Adapter proteins with SH2 domains bind to the phosphorylated ζ chain. Among these proteins is the “Src homology 2 protein of beta-cells” (Shb), which recruits LAT (Welsh *et al.*, 1998; Zhang *et al.*, 1998), a substrate for ZAP-70 (Figure 1). Tyrosine phosphorylation of LAT leads to the recruitment of additional signaling molecules with SH2 motifs (Figures 1 and 2), including the adapter “growth factor receptor-bound protein 2” (Grb2), the phospholipase C γ 1 (PLC γ 1), and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (Paz *et al.*, 2001; Sommers *et al.*, 2001; Yablonski and Weiss, 2001; Zhang *et al.*, 1998).

Active PLC γ 1 hydrolyzes phosphatidylinositol biphosphate (PIP₂), producing diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP₃). DAG in turn activates the serine/threonine kinase family of protein kinase C (PKC), while IP₃ induces calcium ion (Ca²⁺) mobilization in the

cytosol (Corado *et al.*, 1990; May *et al.*, 1986). Thus, ZAP-70 amplifies the TCR signal by specifically phosphorylating downstream components such as LAT and PLC γ 1 (Figures 1 and 2).

In this way, the signalosome expands in molecular complexity and amplifies the TCR initiated signal. Importantly, LAT can also bind proteins that negatively regulate TCR signaling. The “SH2 domain-containing hematopoietic phosphotyrosine phosphatase”, SHP-1, associates with LAT upon TCR stimulation (Kosugi *et al.*, 2001; Su *et al.*, 2001) and prevents further phosphorylation of the adapter by ZAP-70, suggesting a potential conversion from an “activating” to an “inhibiting” signalosome. Similarly, the “C-terminal *src* kinase” (Csk) relocates to rafts by docking to the transmembrane adapter, Csk-binding protein (Cbp), also known as “phosphoprotein associated with glycosphingolipid-enriched microdomains” (PAG) (Brdicka *et al.*, 2000). In rafts, Csk inhibits *src* family PTKs by phosphorylating their regulatory tyrosines, and thus blocks TCR-mediated signal transduction (Vang *et al.*, 2001).

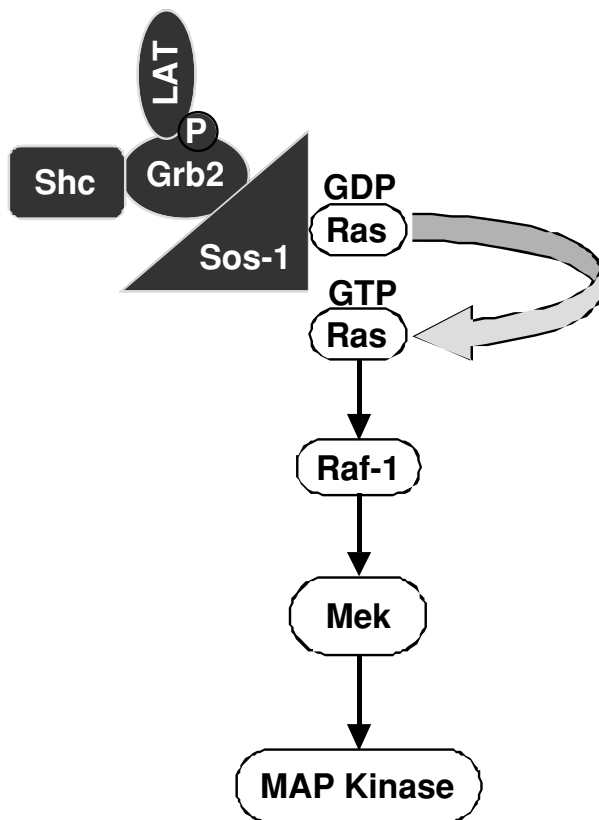


Figure 2. LAT and the MAP kinase signaling pathway. Phosphorylation of LAT by ZAP-70 induces recruitment of multiple adapter proteins and the bifunctional guanine nucleotide exchange factor, Son of sevenless (Sos-1), leading to the activation of the MAP kinase signaling pathway. See text for details.

The CD4 coreceptor

The best-characterized coreceptors, and most important for the discussions presented in this review, are the membrane glycoproteins CD4 and CD8. In mature T cells, the expression of CD4 and CD8 is mutually exclusive. In general, CD4⁺ T cells respond to antigen presented by MHC class II molecules, and CD8⁺ T cells respond to MHC class I-presented antigens (Bierer *et al.*, 1989; Swain, 1983). Therefore, CD4⁺ and CD8⁺ T cells are considered to be restricted by MHC class II and MHC class I, respectively. Importantly, MHC class restriction is independent of the Th or cytotoxic functions displayed by mature T cells (Krensky *et al.*, 1982; Meuer *et al.*, 1982; Swain, 1981). The correlation between CD4 or CD8 expression and MHC recognition results from direct interactions between the coreceptors and relatively monomorphic regions of the membrane-proximal, immunoglobulin-like domains of the MHC molecules (Connolly *et al.*, 1990; Gao *et al.*, 1997; Kern *et al.*, 1998; König *et al.*, 1992; König *et al.*, 1995; Salter *et al.*, 1990).

Coreceptors are associated with the TCR/CD3- ζ complex upon T cell activation. Their presence in the TCR multi-component signaling machine amplifies or modulates the activation signal. Often, their presence is absolutely

required, but not sufficient, for productive signaling, i.e., signaling that results in cell cycle progression and effector functions. Interestingly, three-dimensional live cell imaging of fluorescence resonance energy transfer between CD3- ζ and CD4 demonstrates recruitment of CD4 to the CD3- ζ complex dependent on antigen-stimulation of the TCR (Zal *et al.*, 2002). Importantly, the antigen threshold for T cell activation is affected by the presence of CD4 in the TCR/CD3 complex. In the presence of CD4, a single MHC class II molecule presenting antigen suffices to induce Ca²⁺ flux, and 10 class II MHC/antigen complexes cause the formation of an immunological synapse (Irvine *et al.*, 2002). Blocking of CD4 reduces the sensitivity to antigen stimulation by more than three-fold (Irvine *et al.*, 2002).

The structural basis of interactions between CD4 and MHC class II molecules

The proteins encoded by the class II MHC consist of two non-covalently associated integral membrane polypeptide chains, the α and the β chains (König *et al.*, 1996). Each polypeptide chain forms two protein domains (α 1, α 2, β 1, and β 2). The α 1 and β 1 domains are highly polymorphic and combine to form an antigen peptide-binding groove (Brown *et al.*, 1988; Brown *et al.*, 1993). Using a combination of mutational and functional analysis, we identified amino acid residues on MHC class II molecules that mediate the interaction with CD4 (König *et al.*, 1992; König *et al.*, 1995). A major CD4 binding site, forming a short loop exposed on the surface of the class II β 2 domain, consists of amino acids 134-148 (Cammarota *et al.*, 1992; König *et al.*, 1992). Specifically, glutamic acid 137 is mandatory for mediating the interactions with CD4, and an alanine substitution at this position alone will disrupt all T cell functions mediated by engagement of CD4 (König *et al.*, 1992). A second interaction site is located within the α 2 domain of MHC class II (Gaubin *et al.*, 1999; König *et al.*, 1995). Importantly, both sites are required for mediating interactions with CD4. We have recently reviewed the literature on interactions between MHC molecules and coreceptors of the TCR (König, 2002).

Functional consequences of CD4 engagement by monoclonal antibodies

The crystal structure of the exodomains of human CD4 suggests that the membrane proximal domains of CD4 may promote dimerization (Wu *et al.*, 1997). Extraction of CD4 oligomers from freshly isolated T lymphocytes and lymphoid cell lines also indicates that oligomerization may be an intrinsic property of CD4 molecules (Lynch *et al.*, 1999). Many cell surface receptors transduce signals following oligomerization (Papoff *et al.*, 1999; Rodriguez-Frade *et al.*, 1999; White and Tartaglia, 1999). Therefore, a common procedure to test whether CD4 can activate intracellular signaling pathways utilizes crosslinking anti-CD4 antibodies alone or in conjunction with anti-TCR antibodies (Luo and Sefton, 1990; Pallier *et al.*, 1998; Prasad *et al.*, 1993; Ravichandran *et al.*, 1993; Veillette *et al.*, 1989). However, antibody-mediated crosslinking prevents co-localization of CD4 with TCR molecules (Ratcliffe *et al.*, 1992), and does not adequately reflect CD4-mediated signaling induced by engaging MHC class II during antigen activation.

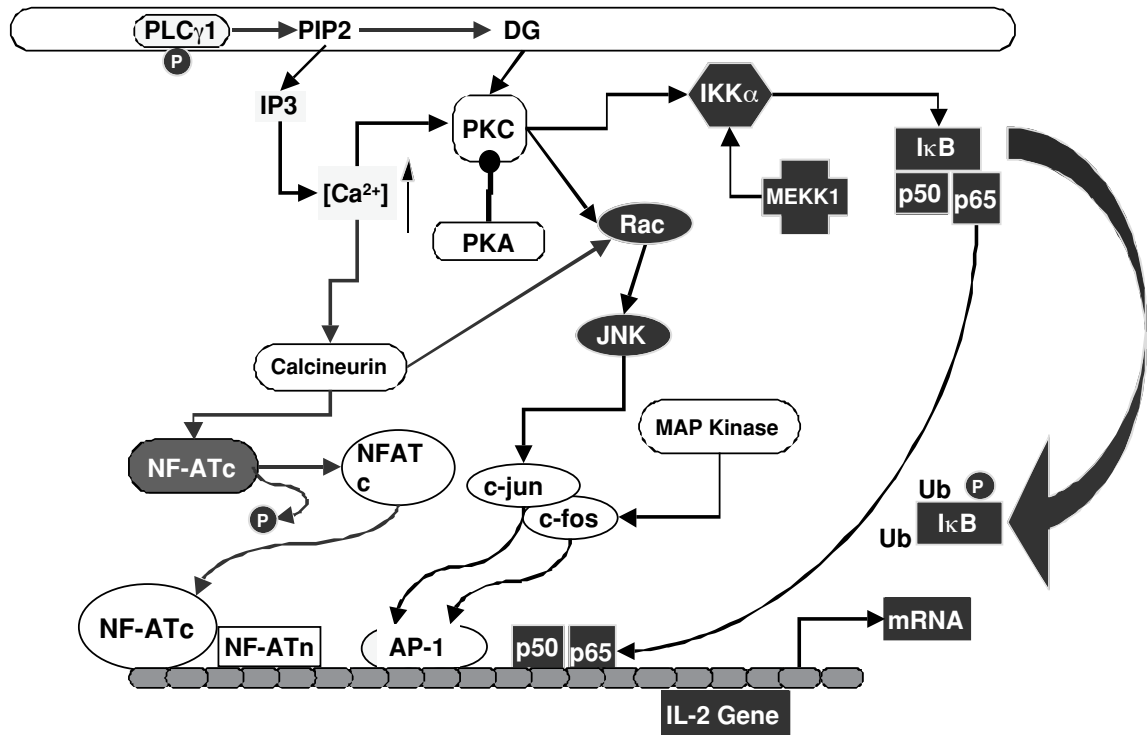


Figure 3. The signaling pathways induced by stimulation of the TCR/CD3/ ζ_2 complex mediate translocation of NF-AT, AP-1, and NF- κ B transcription factors to the nucleus, leading to IL-2 gene expression. See text for details.

Ligation of CD4 by anti-CD4 antibodies or the envelope glycoprotein of the human immunodeficiency virus (HIV), gp120, inhibits antigen-dependent and -independent T cell activation (Jabado *et al.*, 1994; Jauliac *et al.*, 1998; Marschner *et al.*, 2002). The inhibition of T cell activation induced by CD4 ligation depends on the cytoplasmic domain of CD4 (Marschner *et al.*, 2002). Binding of these cross-linking ligands decreases the DNA-binding activity of the nuclear transcription factors NF-AT, NF- κ B, and AP-1, thus preventing IL-2 synthesis (Jabado *et al.*, 1994). These results indicate that ligation of CD4 apart from the TCR/CD3 complex induces negative, regulatory signals that prevent the activation of nuclear factors necessary for IL-2 gene transcription and cell proliferation. Interestingly, memory CD4⁺ T cells appear to be more susceptible to negative signals transduced via CD4 as their activation can be prevented by both antibody-mediated CD4 ligation and MHC class II molecules on APCs in the absence of antigen (Farber *et al.*, 1995).

Experiments using various monoclonal anti-CD4 antibodies recognizing different epitopes suggested the existence of specific signaling epitopes on CD4 (Milia *et al.*, 1997). Depending on the epitope recognized by a specific monoclonal antibody, different signaling pathways are activated. Also, the simultaneous engagement of non-overlapping CD4 epitopes can modify the signals from individual epitopes (Milia *et al.*, 1997). These experiments and structure-function analysis of the CD4-MHC class II interaction suggest that MHC class II molecules bind to a

broad region on the membrane-distal domains of CD4 (König *et al.*, 1996), and induce signals via CD4 that differ from those induced by other natural CD4 ligands (e.g., gp120 and IL-16) or monoclonal antibodies. Therefore, we used a dual approach to identify CD4-mediated signals induced by MHC class II engagement. First, we separated TCR-mediated and CD4-mediated signals by restricting the ability of MHC class II to interact with CD4 during antigen stimulation (Zhou and König, 2003). Second, we avoided antibody-mediated crosslinking, but employed a peptide mimetic to engage the CD4 epitope recognized by MHC class II (Zhou and König, 2003). This peptide binds to CD4⁺ T cells (Shen *et al.*, 1996) and soluble CD4 (Cammarota *et al.*, 1992).

The formation of the immunological synapse

TCR-antigen/MHC interactions initiate the formation of a specialized junction between T cells and APCs, the immunological synapse (Monks *et al.*, 1998). Stimulation- and cytoskeleton-dependent processes cluster TCR/CD3 complexes in the center of the synapse, also termed the "central zone of the supramolecular activation cluster" (cSMAC), whereas adhesion molecules such as LFA-1 form a ring surrounding the central area called the peripheral SMAC (pSMAC) (Delon and Germain, 2000; Grakoui *et al.*, 1999). After stimulation of the T cell, CD4 coreceptors are rapidly recruited into the cSMAC, but migrate towards the periphery within a few minutes, while TCR/CD3 complexes stabilize within the central area

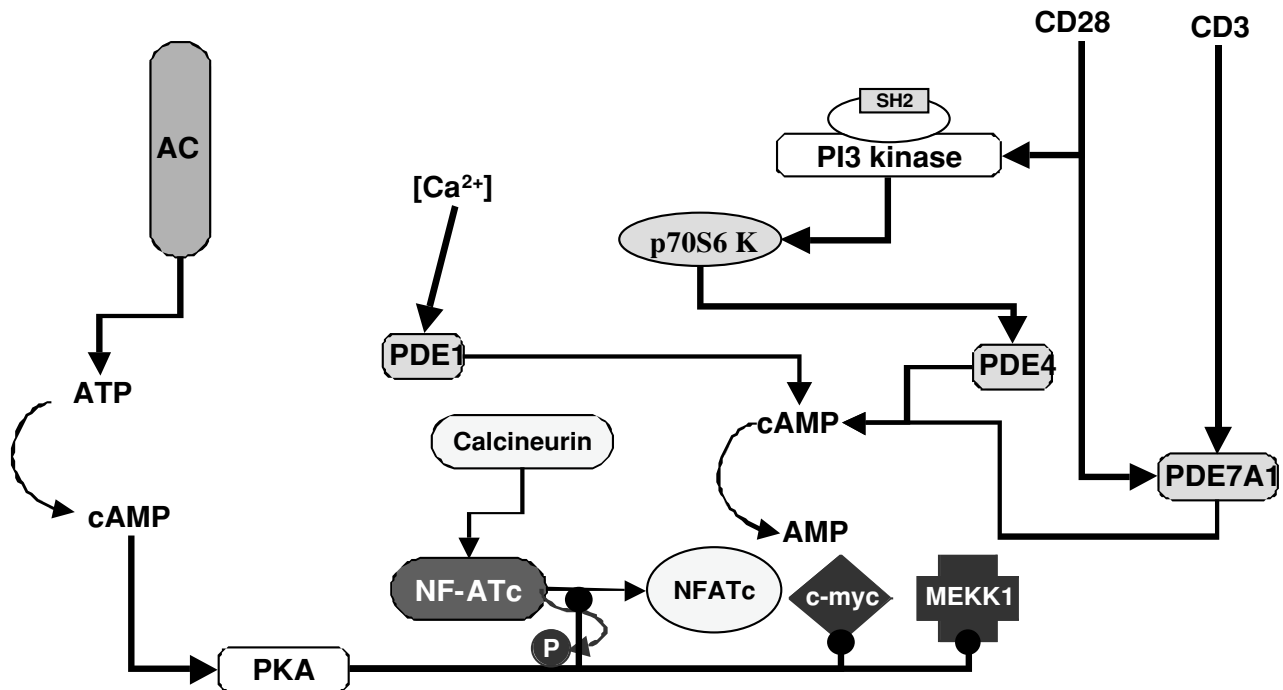


Figure 4. Cyclic AMP exerts multiple regulatory effects on T cell activation and is itself regulated by multiple signaling pathways that activate or block adenylyl cyclase and activate phosphodiesterases. Stimulatory effects are indicated by a line ending in an arrow, whereas inhibitory effects are depicted by a line ending in a filled circle. See also effects of PKA on Raf-1 (Figure 2) and PKC (Figure 3). The overall effect of PKA I activation is inhibition of IL-2 gene expression.

(Krummel *et al.*, 2000). Both CD4 and CD8 associate with the PTK p56^{lck}, and the efficient transport of p56^{lck} into the cSMAC is a major function of these coreceptors (Holdorf *et al.*, 2002).

T cell signaling pathways

One of the earliest antigen-induced signaling events is the mobilization of Ca^{2+} , which is essential for Th cell activation (Wülfing *et al.*, 1997). This second messenger activates several cytosolic enzymes, initiating downstream signaling cascades (Berridge, 1993a). However, antigen stimulation also generates signals through the TCR that antagonize Th cell activation. For example, TCR-mediated signals activate the 3',5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase, PKA (Laxminarayana and Kammer, 1996; Skalhegg *et al.*, 1992). PKA initiates a signaling pathway that inhibits antigen-induced T cell proliferation and cytokine production (Paliogianni *et al.*, 1993; Vang *et al.*, 2001). Therefore, to achieve full activation of Th cells, TCR-mediated signals must be modified. We have recently identified signaling pathways induced by MHC class II engagement of CD4. These TCR-independent signals participate in the regulation of intracellular concentrations of both Ca^{2+} and cAMP (Zhou and König, 2003).

Calcium mobilization

The TCR-induced signal transduction leads to the activation of PLC γ 1 (Figure 1). Binding of IP $_3$ to its receptor in the

membrane of the endoplasmic reticulum induces the release of Ca^{2+} into the cytosol (Corado *et al.*, 1990). The subsequent increase in intracellular free Ca^{2+} opens Ca^{2+} -regulated Ca^{2+} channels in the plasma membrane, inducing additional Ca^{2+} influx (Berridge, 1993b; Tsien *et al.*, 1982). Intracellular free Ca^{2+} acts as an essential second messenger for T cell activation (Weiss *et al.*, 1984). Its regulatory effects on T cell activation are mediated via calmodulin, a Ca^{2+} -binding protein expressed in all eukaryotic cells (Zhang *et al.*, 1998). Effective T cell activation leading to IL-2 secretion requires that intracellular Ca^{2+} levels be elevated for a period of 1-2 h (Karttunen and Shastri, 1991; Negulescu *et al.*, 1994). Sustained Ca^{2+} signaling is required for maintaining the transcription factor "Nuclear Factor of Activated T cells" (NFAT) in the nucleus in an active form (Loh *et al.*, 1996; Timmerman *et al.*, 1996). NFAT is a key transcriptional regulator of the IL-2 gene (Rooney *et al.*, 1995) (Figure 3).

Ca^{2+} signaling is required for various lymphocyte activities, for example cell motility is impeded by increases in Ca^{2+} (Donnadieu *et al.*, 1994; Negulescu *et al.*, 1996). Increases in Ca^{2+} cause changes in the cytoskeletal structure (Wülfing and Davis, 1998), induce cell death in immature thymocytes (Andjelic *et al.*, 1993), differentiation (Sloan-Lancaster *et al.*, 1997), and activation (Gearing *et al.*, 1985). Thus, a single second messenger can elicit multiple cellular responses. The type of response induced may depend on the amplitude, duration, and temporal fluctuations of Ca^{2+} mobilization. For example, activation of NF- κ B is induced by high levels of Ca^{2+} , because of this

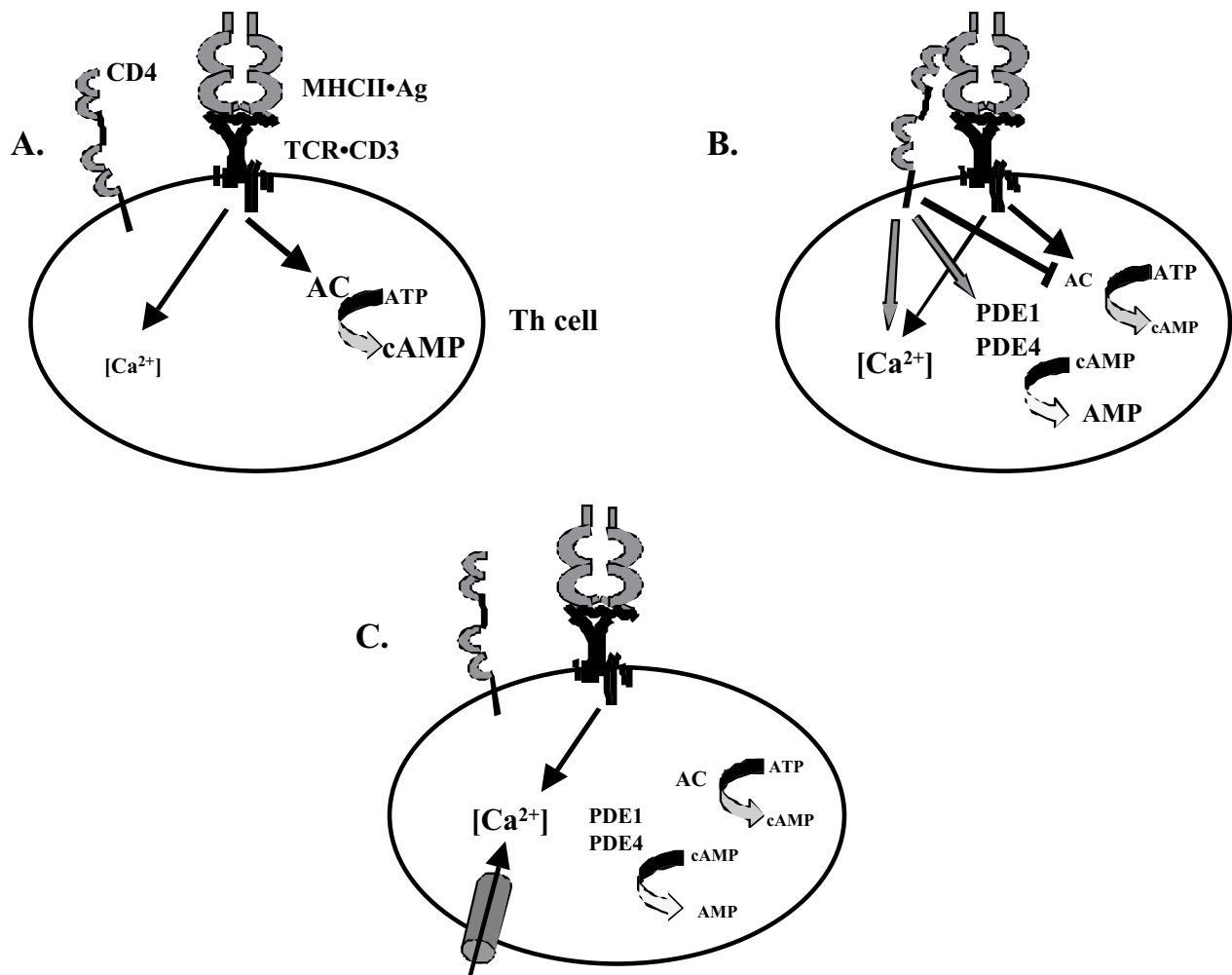


Figure 5. Regulation of intracellular Ca^{2+} and cAMP levels by TCR and CD4 signaling. A. Binding of the TCR to the antigen/MHC class II ligand induces Ca^{2+} mobilization and activates adenylyl cyclase, raising intracellular cAMP levels. B. CD4 is recruited to the signalosome and binds to MHC class II. CD4 signaling increases Ca^{2+} mobilization, activates cAMP phosphodiesterases, and inhibits adenylyl cyclase. The intracellular concentration of cAMP drops below resting levels. C. CD4 is released from the central area of the immunological synapse. Ca^{2+} -gated membrane Ca^{2+} channels maintain a sustained high level of intracellular Ca^{2+} .

transcription factor's low Ca^{2+} sensitivity. In contrast, even low elevations of Ca^{2+} levels, if maintained over a prolonged period of time, selectively activate NFAT, because NFAT is highly sensitive to Ca^{2+} but is rapidly inactivated after Ca^{2+} removal (Dolmetsch *et al.*, 1997).

Cyclic AMP, adenylyl cyclases, and cyclic nucleotide phosphodiesterases

Cyclic AMP has been defined as an intracellular second messenger to a wide variety of hormones and neurotransmitters. In T cells, numerous studies have shown that elevated cAMP levels antagonize T cell activation. In addition to inhibiting T cell proliferation (Lingk *et al.*, 1990), cAMP suppresses the production of IL-2 and IFN- γ in activated Th cells (Zidek, 1999). Opposite effects on IL-5 production from activated Th cells have been observed in different studies (Staples *et al.*, 2000). TCR signaling alone elevates adenylyl cyclase (AC) activity and intracellular cAMP (Feuerstein *et al.*, 1996). Importantly, TCR-independent signals induced by CD4 engagement regulate

cAMP levels (Zhou and König, 2003).

In T cells, the cAMP level is controlled by two types of enzymes: ACs and cyclic nucleotide phosphodiesterases (PDEs). ACs catalyze the production of cAMP from ATP, whereas PDEs control the rate of cAMP degradation to AMP. At least eleven families of PDEs have been classified by their primary sequences, substrate specificities, susceptibility to selective inhibitors, and tissue location (Manganiello *et al.*, 1995; Soderling and Beavo, 2000). Among them, PDE families 1,3,4, and 7 have been found in T cells (Ekholm *et al.*, 1997; Giembycz *et al.*, 1996; Hurwitz *et al.*, 1990). The activity of each of these four families is controlled by distinct mechanisms. The PDE1 family is stimulated by Ca^{2+} /calmodulin (Charbonneau *et al.*, 1991). The PDE3 family is inhibited by cyclic GMP (Tang *et al.*, 1997). Activation of the PDE4 family has been linked to different pathways. In 3T3-F442A fibroblasts, PDE4 activity is induced by stimulation of p70S6 kinase (Mackenzie *et al.*, 1998), whereas in FDCP2 myeloid cells, PDE4 is activated via MAP kinase-dependent pathways

(Ahmad *et al.*, 1999). In T cells, CD3 and CD28-mediated signals activate the PDE7 family (Li *et al.*, 1999).

Elevation of cAMP has long been regarded as inhibitory to T cell activation (Figures 3 and 4). However, inhibition of basal PKA I activity leads to decreased TCR-triggered IL-2 production (Sugiyama *et al.*, 1997; Zhou and König, 2003). Also, purified human T cells stimulated by mitogens transiently up-regulate AC and PDE activities with different kinetics for different PDE isozymes (Kanda and Watanabe, 2001). Both observations suggest a requirement of cAMP for T cell activation. We have recently demonstrated that cAMP is required for cell cycle progression and cytokine gene expression following antigenic activation of Th cells (Zhou and König, 2003). Thus, a precise kinetic regulation of the intracellular cAMP concentration is required for Th cell activation.

Cyclic AMP-dependent kinase

The second messenger, cAMP, activates a class of cyclic nucleotide-gated ion channels. It also directly activates the guanine-nucleotide-exchange factors Epac1 and Epac2, which are able to activate Rap1 by promoting its release of GDP and binding to GTP (De Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Rap is a small Ras-like GTPase that can suppress the oncogenic transformation of cells by Ras. It is also involved in other cellular activities, including cell differentiation, T cell anergy, and platelet activation (Boussiotis *et al.*, 1997; Franke *et al.*, 1997; York *et al.*, 1998).

The cAMP-dependent protein kinase, PKA, is the principal intracellular cAMP receptor (Beebe, 1994; Walsh and Van Patten, 1994). In the absence of cAMP, PKA is an enzymatically inactive, tetrameric holoenzyme, consisting of two catalytic subunits and two regulatory subunits. The cooperative binding of four cAMP molecules to two sites on each regulatory subunit drastically decreases the binding affinity between regulatory and catalytic subunits, and induces dissociation into dimeric regulatory and two monomers of catalytic subunits (Doskeland *et al.*, 1993). Once freed from the regulatory subunits, the catalytic subunits display serine/threonine kinase activity (Houge *et al.*, 1990).

PKA I, but not PKA II, mediates the inhibitory role of cAMP on T cell proliferation induced by TCR signaling (Aukrust *et al.*, 1999; Skalhegg *et al.*, 1992). PKA I antagonizes T cell activation at multiple levels (Chen and Rothenberg, 1994; Ramstad *et al.*, 2000; Van Oirschot *et al.*, 2001; Vang *et al.*, 2001), one of which is to activate the protein tyrosine kinase, Csk (Vang *et al.*, 2001). Csk inhibits Lck activity (Abraham and Veillette, 1990). By inhibiting Lck, PKA I can diminish T cell activation at the initiation stage. This suggests that at the early stage of T cell activation, PKA I activity needs to be restricted. PKA I also phosphorylates Raf-1 to block the MAP kinase pathway (Ramstad *et al.*, 2000) (Figures 2 and 3). In the nucleus, activation of PKA prevents stable protein-DNA interactions at the NF- κ B, NFAT, and AP1 binding sites of the IL-2 enhancer (Chen and Rothenberg, 1994) (Figures 3 and 4). In addition, PKA I activity also inhibits cyclin D3 expression and induces the cyclin-dependent kinase inhibitor p27^{kip1} (Van Oirschot *et al.*, 2001). For T cells to

enter the S phase of the cell cycle, D-type cyclins, including cyclin D3, are synthesized during the G₁ phase (Boonen *et al.*, 1999). These cyclins can bind to cyclin-dependent kinase (Cdk) and form an active kinase complex that phosphorylates and inactivates retinoblastoma protein, pRb (Weinberg, 1995). Inactivation of pRb then allows cells to pass through the late G₁ phase restriction point and enter the S phase. However, cyclin D/Cdk complexes can associate with the Cdk inhibitor p27^{kip1}, thus rendered inactive (Firpo *et al.*, 1994; Nourse *et al.*, 1994). Therefore, in addition to induction of cyclin D, downregulation of p27^{kip1} is also required for the initiation of T cell proliferation (Boonen *et al.*, 1999; Firpo *et al.*, 1994; Nourse *et al.*, 1994). Hence, inhibition of cyclin D3 expression and induction of p27^{kip1} by PKA I both block T cell cycle progression. Little is known about the distribution of PKA I in activated T cells. The only study reported so far demonstrated co-localization of the PKA I holoenzyme with the TCR/CD3 complex in human peripheral blood T cells after crosslinking with anti-CD3 mAb for 30 min (Skalhegg *et al.*, 1994).

Functional consequences of CD4 engagement by MHC class II

Interactions between CD4 and MHC class II increase antigen-induced Th cell proliferation and cytokine production. Initially, it was thought that CD4 functioned as an adhesion molecule, enhancing contact between Th cells and APCs (Gay *et al.*, 1987). However, CD4 and MHC class II interact with extremely low affinity (Weber and Karjalainen, 1993; Xiong *et al.*, 2001), and soluble CD4 does not affect the binding of soluble antigen/MHC class II complexes to immobilized TCR (Xiong *et al.*, 2001). Furthermore, CD4-MHC class II interactions do not increase the binding avidity between Th cells and APCs (Hamad *et al.*, 1998; Metz *et al.*, 1997; Zhou and König, 2003). Thus, an adhesive effect of CD4 is unlikely, and the major function of CD4 may be its active participation in Th cell signaling (Sleckman *et al.*, 1991; Zhou and König, 2003).

Role of CD4 in modulating T cell signal transduction

Experiments in our laboratory suggest that CD4-mediated signals facilitate Th cell activation by two mechanisms (Figure 5). First, engagement of CD4 by MHC class II induces increased Ca²⁺ mobilization from intracellular stores. This CD4-induced Ca²⁺ signal synergizes with the TCR-mediated Ca²⁺ signal, thereby allowing the sustained activation of Ca²⁺-regulated membrane Ca²⁺ channels and promoting a sustained rise in intracellular free Ca²⁺ (Zhou and König, 2003). A long-lasting elevation of Ca²⁺ is required for effective Th cell activation (Rabinowitz *et al.*, 1996; Wulfig *et al.*, 1997). Second, CD4 signaling counteracts the TCR-mediated increases in cAMP (Zhou and König, 2003). The CD4-mediated signals activate cAMP PDEs and inhibit AC. TCR signaling in the absence of additional, modifying signals results in the accumulation of cAMP in the cytosol and thus, in partial Th cell activation without the induction of efficient proliferation and cytokine production (Feuerstein *et al.*, 1996; Zhou and König, 2003). Therefore, signals induced by MHC class II engagement

of CD4 are critical for successful Th cell activation.

An important characteristic for effective regulation of intracellular cAMP by CD4 signaling is the temporal and spatial sequence of CD4 localization in the signaling synapse. Recruitment of CD4 depends on binding of the TCR to its specific antigen/MHC class II ligand (Krummel *et al.*, 2000). Thus, an initial TCR-mediated increase in AC activity may be countered by CD4-mediated AC inhibition and PDE activation to promote early T cell activation events. Exclusion of CD4 from the central TCR cluster to the periphery of the signaling synapse within a few min after the initial Ca²⁺ signal (Krummel *et al.*, 2000) may then remove the block on cAMP increases to promote proliferation and cytokine production.

The activities of ACs are controlled by hormones, neurotransmitters, chemotactic transducers, and other molecules. A common mechanism to inhibit AC activity is through the activation of G_i proteins (e.g., G_{i1}, G_{i2}, and G_{i3}), which are coupled to receptors for effector molecules. Members of the G_i family of proteins positively regulate T cell activation (Lippert *et al.*, 2000). A 32-kDa GTP-binding protein associates with CD4 and CD8 in human T cell lines (Telfer and Rudd, 1991). Therefore, inhibition of AC by CD4-MHC class II engagement may be mediated via G_i proteins. CD4 may serve as a docking protein, carrying G_i proteins to the signaling synapse in activated Th cells, similar to the mechanism by which CD4 promotes p56^{lck} function (Holdorf *et al.*, 2002).

Therefore, we propose that transduction of costimulatory signals induced by MHC class II engagement represent a major function of the CD4 coreceptor and that CD4-mediated signals participate in the precise regulation of intracellular levels of cAMP following T cell stimulation.

Role of CD4 in regulating T cell homeostasis

We have recently reported that coreceptor interactions with MHC molecules also regulate peripheral T cell homeostasis and the survival of naïve T cells in the absence of antigenic stimulation (König, 2002; König *et al.*, 2002). We discovered this function of CD4 in a transgenic mouse strain that we generated for the purpose of elucidating the role of CD4 engagement by MHC class II in thymic selection and peripheral activation of Th cells. These transgenic mice express mutant MHC class II molecules defective in their ability to engage CD4 (Gilfillan *et al.*, 1998). Despite the inability of the mutant MHC class II expressed on thymic epithelial cells to interact with CD4, positive thymic selection into the CD4 lineage proceeds, and CD4⁺ thymocytes mature. However, levels of single-positive CD4⁺ thymocytes in these mice are less than one third of those in normal mice. Furthermore, peripheral, naïve CD4⁺ T cells undergo apoptosis at an approximately three-fold higher rate in mutant MHC class II transgenic mice than do CD4 lineage cells in normal mice (Maroto *et al.*, 1999). The increased frequency of apoptotic cells is due to a lack of CD4-MHC class II interactions in peripheral lymphoid organs, because naïve CD4⁺ T cells from normal mice adoptively transferred into mutant MHC class II transgenic hosts meet the same fate as do endogenous Th cells (Shen and König, 2001). Similarly, CD4-deficient T helper lineage cells are apoptosis-prone, and fail to survive after adoptive

transfer into irradiated hosts (Strong *et al.*, 2001). Thus, CD4 lineage T cells require CD4-MHC class II interactions for efficient positive selection in the thymus and for maintenance of homeostasis in the periphery. Following the selection in the thymus for cells with TCRs that appropriately recognize self-MHC/peptide ligands, a peripheral recertification process enhances the stringency for proper matching between TCR restriction and coreceptor recognition of MHC (König *et al.*, 2002). This process depends on signals through CD4 (Shen and König, 2001; Strong *et al.*, 2001).

At the present time, we do not know whether the defect in peripheral Th cell homeostasis in the absence of CD4 engagement by MHC class II is related to an impaired regulation of intracellular cAMP levels. However, this is an attractive hypothesis and future research is targeted towards answering this question.

Outlook

The receptors that induce T cell signaling and the intracellular proteins mediating specific signal transduction pathways have been mostly identified. It is evident that signalosomes in T cells differ in composition depending on the extracellular signal received and the requirements of the stimulated cell. Depending on the level of participation of coreceptors in the induction and modulation of signaling pathways, the composition of established signalosomes may change over time in order to permit the fine-tuning of effector signaling pathways. Future research will focus on determining the kinetics of assembly, the dynamics of composition, and the compartmentalization of components of signalosomes induced during T cell activation. An important aspect will also be to define the interactions between different signaling pathways.

A clear understanding of Th cell signaling will provide a blueprint for the rationale intervention in multiple immune effector functions. Th cells promote adaptive immune responses against infective microorganisms and tumor cells, but also hamper tissue and organ transplantation. In addition, improper activation of Th cells can cause autoimmune diseases. Thus, targeting coreceptor-induced signaling pathways could improve vaccine development and therapeutic approaches to infectious diseases and cancer.

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