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EMBO WORKSHOP REPORT

Lymphocyte antigen receptor and coreceptor signaling Siena, Italy, November 6–10, 1999

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Introduction

On November 6–10, 1999, the EMBO workshop on 'Lymphocyte antigen receptor and coreceptor signaling' was held at the Certosa di Pontignano. Since the first EMBO workshop on the same theme held in 1997, exciting progress has been made towards further unraveling the molecular mechanisms of T and B cell activation. Novel signaling components have been identified, and their function characterized in cellular and animal models. Molecular adaptors that orchestrate the assembly of transducing complexes are dominating the scene for their role in signal initiation, integration, amplification and extinction. Insight has been been gained into the mechanisms regulating thresholds for receptor signaling and on the role of accessory membrane receptors in this process. Furthermore, new concepts have been brought to light, such as the requirement for antigen receptors to signal within specialized lipid microdomains and the highly ordered configuration of receptors and signaling effectors in the membrane area where T cell contacts the antigen-presenting cell. Antigen receptor signaling has become a paradigm of signal coordination by cell surface receptors in general. Here we shall present an overview of the topics discussed at the workshop in the context of the development of the field over the last 2 years.

Spatial and temporal orchestration of antigen receptor signaling

Ordered clustering of receptors and signaling components at the immunological synapse

When T cells interact with antigen-presenting cells (APCs), the antigen receptors and associated signaling molecules colocalize in a tight area of contact between the cells, which has been termed the immunological synapse. Concomitant with activation, this area becomes a highly ordered platform that favors signal delivery (van der Merwe *et al*., 2000). Receptors and intracellular proteins clustered at the contact area between T cell and APC segregate into spatially distinct domains, referred to as: (i) cSMAC (central supramolecular activation clusters), which includes the TCR, CD4, the protein tyrosine kinases (PTK) Fyn and Lck, and PKCθ; and (ii) pSMAC (peripheral SMAC), which includes the integrin LFA-1 and talin (Monks *et al*., 1998). The costimulatory receptors CD2 and CD28 are also part of the cSMAC (van der Merwe *et al*., 2000).

Real-time imaging of living T cells interacting with MHC/peptide and ICAM-1 in a lipid bilayer shows that within 5–10 min of contact, TCR/MHC–peptide interactions are clustered in the center and LFA-1/ICAM-1 interactions form a peripheral stabilization ring. This configuration (the mature synapse) is maintained for up to 1 h (Grakoui *et al*., 1999). The timing for the formation of the SMACs indicates that the initial activation steps of the TCR signaling cascade (ζ chain phosphorylation and ZAP-70 recruitment) do not necessitate but are rather required for SMAC formation. Weak peptide/MHC agonists, which fail to elicit full activation, are unable to promote the formation of an organized synapse (Grakoui *et al*., 1999), which therefore represents a checkpoint for full T cell activation, as well as a structure ensuring sustained TCR signaling. Mathematical models based on observations of chemotactic receptors in bacteria may explain how these stable clusters could result in greatly enhanced responses and sensitivity over a much wider range of ligand concentrations (Duke and Bray, 1999).

Both TCR and MHC extend above the membrane by only ~7 nM. However, T cells and APCs express much longer cognate pairs such as integrins (>25 nm), CD43 (>43 nm) or the membrane protein tyrosine phosphatase CD45 (23–50 nm). It has been proposed that other short and relatively more abundant receptors/counter-receptor pairs such as CD2/CD48 or CD2/LFA-3 and CD28/B7 seal the opposing membranes and facilitate TCR engagement, resulting in exclusion of proteins with long extracellular regions. Consistent with this model, A.van der Merwe (Oxford, UK) showed that increasing the length of CD48, while not affecting its interaction with CD2, inhibited TCR ligation by antigen/MHC and consequently T cell activation (Wild *et al*., 1999). O.Acuto (Paris, France) presented data indicating a key role of the extracellular domain of CD28 in facilitating T cell adhesion to the APC, which correlated with a dramatic enhancement of TCR-mediated tyrosine phosphorylation of signaling proteins. Moreover, S.Valitutti (Lausanne, Switzerland) showed by confocal microscopy that 5–10 min after conjugate formation, most CD45 was excluded from the center of the synapse, where the local concentration of tyrosine phosphorylated proteins was concomitantly increased, implying a physical and functional exclusion of CD45 (Leupin *et al*., 2000). Interestingly, since CD45 upregulates Lck

Table I. Molecules localized at the immunological synapse and in lipid rafts in T cells

		Immunological synapse	Lipid rafts	
			Resident	Recruited
Receptors	TCR	+		+
	CD4/CD8	+	+	
	CD2	+	+	
	CD5	+		+
	CD9	nd	+	
	CD28	+		+
	CD43	excluded	nd	nd
	CD44	+	+	
	CD45	excluded	+	excluded
	CD48	excluded		
	LFA-1	excluded	nd	nd
	L-selectin	+	nd	nd
	IP-3 R	+	nd	nd
Enzymes	Lck	+	+	
	Fyn	+	+	
	PKCθ	+	nd	nd
	cAK1	+	nd	nd
	ZAP-70	+		+
	PLCγ	+		+
	PI-3K	+	nd	nd
G-proteins	Ras	+	+	
and GEFs	Rac	+	nd	nd
	Sos	+	nd	nd
	Vav	+	nd	nd
Adaptors	LAT	+	+	
	Shc	+		+
	SLP-76	+		+
	Grb2	+		+
	TRIM	+	nd	nd
	SIT	+	nd	nd
	Cbl	+	nd	nd
	PAG85	nd	+	

nd = not determined.

activity by dephosphorylating its C-terminal regulatory tyrosine, this reaction must occur before transport to the center of the synapse. Together with a recent report on CD43 excluded from the cSMAC (Sperling *et al*., 1998), these are the first experimental data in support of the 'size discrimination model'.

Lipid rafts: flotsam and jetsam?

Lateral assemblies of glycosphingolipids and cholesterol in cell membranes, or lipid rafts, have been implicated in cellular processes such as membrane sorting, cell adhesion and signal transduction. Lipid rafts, also known as glycolipid-enriched membranes (GEMs) or detergent-insoluble glycolipid structures (DIGs), are enriched in GPI-anchored proteins, acylated Src PTKs and Ras. In T cells, antigen receptor triggering results in localization in the GEMs of tyrosine phosphorylated forms of the TCR/CD3 complex and a number of molecules that participate in the TCR signaling cascade (Table I). Using single particle tracking of the GPI-anchored protein Thy-1, Sheets *et al*. (1997) described confinement domains that correspond to lipid microdomains in both size and characteristics. Confinement for periods of between 6 and 8 s resulted in ~35% of these molecules being associated with the microdomains at any one time. From this it appears that the lipid microdomains are less than permanent raft structures, more like flotsam on the surface of the cell that accumulates and disperses, a process that might be slowed down when raft-associated molecules aggregate.

The Src PTK, Lck, requires palmitoylation for localization in the rafts and for participating in TCR-mediated signaling, indicating that raft localization plays a role in bringing Lck into the physical vicinity of the TCR complex (Kabouridis *et al*., 1997). Also, interestingly, the Lck-associated coreceptors CD8 and CD4 are palmitoylated, and partition in lipid rafts and raft localization is essential for coreceptor function (Arcaro *et al*., 2000). A.Lanzavecchia (Bellinzona, Switzerland) suggested that rafts might be stored intracellularly in naive T cells, since a large proportion of Lck is intracellular and only migrates to the cell surface upon TCR triggering. H.Tao-He (Marseille, France) showed that in T cell mutants defective in enzymes responsible for GPI attachment to proteins, GEMs have a normal composition of Src PTKs, but TCR signaling is profoundly impaired, suggesting that GPI-anchored proteins are essential for TCR activation while being dispensable for raft formation.

While the definition of a raft resident has been commonly based on the criterion of detergent (Triton X-100) insolubility, the contrasting results obtained in some instances by different groups underline the necessity to establish more precise operational criteria to address this issue. Using confocal microscopy of living cells expressing Lck–GFP or LAT–GFP fusion proteins and stained with cholera toxin B subunit, which binds GM1, S.Ley (London, UK) confirmed the resident localization of Lck and LAT in rafts (Janes *et al*., 1999). Using a similar approach, he showed that while not fitting the criteria for raft residency biochemically, the TCR colocalizes with GEMs in intact cells. Furthermore, H.Tao-He provided biochemical evidence that when the the milder detergent Brij-58 is used, the TCR can be co-isolated with GEMs also in resting cells, suggesting a state of proximity of the TCR with GEMs before TCR engagement.

The process of receptor aggregation in the proximity of the rafts appears to be sufficient for initiating signaling. S.Ley showed that GEM patching by antibody cross-linking of bound cholera toxin B triggers the TCR signaling cascade (Janes *et al*., 1999). A similar finding was reported for FcεRI, which is associated with GEMs on mast cells, by I.Pecht (Revohot, Israel), who showed that cross-linking of a monoclonal antibody that recognizes four different GEM-associated glycosphingolipids results in aggregation of FcεRI/IgE complexes and activation of the cell secretory response (Schwartz *et al*., 2000). Interestingly, the requirement for TCR engagement in the recruitment of the Shc adaptor to lipid rafts and subsequent activation of the Ras pathway can be bypassed by forced localization of Shc in the GEMs (Plyte *et al*., 2000). Collectively, these data suggest that lipid rafts are membrane platforms where the early TCR signaling machinery is pre-assembled.

The parallels between the process of GEM aggregation and recruitment to the immunological synapse are obvious. Many of the proteins that are recruited to the antigen receptor are also found to be recruited to the rafts following TCR engagement (Table I). TCR-mediated tyrosine phosphorylation is more stable in rafts, and its

duration seems to be a function of formation of large GEM aggregates (Viola *et al*., 1999), suggesting phosphatase exclusion and/or downregulation. Lipid rafts are thus likely to occupy the cSMAC.

During signaling the membrane beneath the engaged receptors becomes a hub for many proteins, some of which operate locally and are subsequently inactivated whereas others, still active, are delivered to another destination. C.T.Baldari (Siena, Italy) presented a kinetic analysis of the interactions among a subset of key signaling proteins occurring following TCR triggering and showed that a short-lived and dynamic multimolecular complex is assembled in a temporally regulated sequence at the activated TCR (Pacini *et al*., 2000). Furthermore, she presented a two-dimensional gel analysis of the proteins enriched in GEMs as a function of time following TCR crosslinking, showing that the raft-associated subproteome is a dynamic structure that includes, in addition to a subset of resident proteins, a further subset of ~50 major proteins inducibly recruited in a specific sequence in response to receptor engagement.

Another example of this dynamic behavior was discussed by D.Cantrell (London, UK). During BCR triggering, PCKμ, which is downstream from other PKCs, is recruited to the plasma membrane through its DAG binding site. Using GFP chimeras and video microscopy, PCKμ is seen as rapidly associating to and dissociating from the plasma membrane in 1–2 min. Interestingly, its catalytic activity remains high even 15–30 min after membrane dissociation (Matthews *et al*., 1999). These results exemplify the emerging requirement in the field of signal transduction of placing each signaling molecule in a spatial and temporal context to achieve a full understanding of its function.

The signaling machinery in lymphocytes

At the beginning were PTKs, PTPases and ITAMs

To signal their engagement with the ligands, immune receptors do not rely on intrinsic kinase activities but on phosphorylation by neighboring Src family PTKs of tyrosine-containing motifs (called ITAM for immune receptor tyrosine-based activation motif) in the cytosolic tails of their subunit components. In unstimulated cells, the TCR/CD3-ζ complex is in physical proximity with the GEMs where Src PTKs Lck and Fyn are confined (H.Tao-He; S.Ley). Ligand engagement brings the receptor into connection with the signaling machinery. What determines this change of state remains to be elucidated but protein tyrosine phosphatases (PTPases) appear to play a key role. In unstimulated cells, the ITAMs are mantained under a cycle of phosphorylation/dephosphorylation in which the latter prevails (Acuto and Cantrell, 2000). Src PTK activity in lymphocytes is thought to be controlled at the C-terminal negative regulatory tyrosine by the opposing action of the membrane PTPase, CD45 and the PTK, Csk. However, in CD45 knock-out mice, Lck and Fyn are more heavily phosphorylated and their basal catalytic activity is increased (Pingel *et al*., 1999). Therefore, CD45 may also have a negative regulatory role on Src PTKs, in agreement with data suggesting that CD45 dephosphorylates the tyrosine of their activation loop (D'Oro and Ashwell, 1999).

B.Schraven (Heidelberg, Germany) presented data on a novel GEM-resident adaptor protein named PAG (protein associated with GEMs) or Cbp, to which Csk associates (Brdicka *et al*., 2000; Kawabuchi *et al*., 2000). In unstimulated T cells, PAG is constitutively phosphorylated by Src PTKs and recruits Csk to the GEMs which, in turn, can directly control the activity of Src PTKs, thus creating an auto-regulatory loop. Upon receptor engagement, PAG is dephosphorylated and Csk association is lost, an event that should result in increased activity of Src PTKs (Brdicka *et al*., 2000). In lymphocytes, ITAM phosphorylation by Src PTKs is therefore controlled by at least four elements; CD45, Csk, PAG/Cbp and a PTPase whose activity or localization is modified by receptor engagement. According to Schraven, the PTPase that acts on PAG is not CD45, and PEST or PEP PTPases are suspected candidates.

CD45 activity is thought to be inhibited by homodimerization, resulting in the wedging of a loop of the catalytic site of one CD45 molecule into that of the other (Majeti *et al*., 1998). A.Weiss (San Francisco, CA) showed that knock-in mice harboring a mutation in CD45 wedge (E613R), and hence unable to be inhibited by dimerization, had a normal T cell development and response to *in vitro* TCR triggering. Therefore, the proposed inhibitory mechanism does not operate at the initiation of activation. Interestingly, however, with aging, all mice had a high proportion of activated T as well as B cells in their secondary lymphoid organs and developed overt autoimmune disease closely resembling human systemic lupus erythematosus. An attractive possibility is that once activated, memory T cells have to be reset to a higher activation threshold and that inhibition of CD45 may be necessary for this process.

From the ITAMs to the adaptors in the GEMs and to the nucleus: the connections to the major signaling pathways

ZAP-70 and Syk PTKs are essential for connecting triggered antigen receptors to all major signaling pathways leading to gene activation and cytoskeletal changes (Acuto and Cantrell, 2000). ZAP-70 and/or Syk bind, through two tandemly arranged SH2 domains, to the phosphorylated ITAMs and become fully active. Lymphocytes adapt their biological responses according to differences in the half-life of the antigen receptor–ligand interaction. In T cells, such quantitative differences might be translated into discrete patterns of TCR ζ chain ITAM phosphorylation and, supposedly, into qualitatively different signaling outcomes. This model was tested in the experiments described by L.Ardouin (Marseille, France) with mice expressing the ζ chain lacking all the ITAMs and therefore unable to bind ZAP-70/Syk. T cells expressing a particular TCR as a transgene could develop normally and only a small drop in T cell response was found. Also, antagonistic peptides did act in the absence of ζ ITAMs. Thus, transduction-competent ζ is not strictly essential for T cell development, survival and activation. In addition, when ε ITAMs were ablated, the (much fewer) developing T cells could still respond to high doses of superantigen (Ardouin *et al*., 1999). Whereas these data challenge the above

model, they support the idea of TCR ITAM redundancy allowing an extendable activation threshold range.

BCR ITAMs may change master in primed B cells. J.Cambier (Denver, CO) reported that in these cells, MHC class II crosslinking results in signaling (e.g. PTK activation/Ca^{2+} response) capacity as a result of Igα/Igβ association. BCR is required but this property is acquired upon mRNA *de novo* synthesis following BCR activation. A putative class II:Igα/Igβ 'linker' of 110 kDa was identified (Lang,P., Stolpa,J.C., Freiburg,B.A., Crawford,F., Kappler,J., Kupfer,A. and Cambier,J.C., manuscript submitted).

Once bound to the ITAM, ZAP-70 activity is up-regulated by phosphorylation on its activation loop by Lck (or Fyn). However, ZAP-70 is phosphorylated in the interdomain B between the SH2 domains and the catalytic domain. One of these sites, tyrosine 319 (Tyr^{319}), is critical for ZAP-70 function as it determines the capacity of ZAP-70 to phosphorylate the adaptors SLP-76 and LAT, and to activate the IL-2 gene (Di Bartolo *et al*., 1999; Williams *et al*., 1999). Biochemical data are consistent with Tyr^{319} being the site where Lck binds via its SH2 domain (Pelosi *et al*., 1999), suggesting that this interaction is important for activating ZAP-70. Consistent with this hypothesis, when Tyr^{319}-containing motif was modified to have higher affinity for Src PTK SH2 domains, ZAP-70 activity was increased (Pelosi *et al*., 1999). Perhaps a low-medium affinity of natural binding site for Lck SH2 on ZAP-70 (~400 nM) renders the activation of the latter PTK directly controllable by the level of TCR engagement.

Together with its partner SLP-76, the adaptor LAT, a GEM resident protein, is the central organizer of the antigen receptor signaling engine in T cells. The LAT/SLP-76 complex provides a scaffolding for recruiting signaling enzymes and for positioning them in an ordered spatial arrangement. Absence of LAT or SLP-76 independently blocks essential signaling pathways. In addition to binding sites for PLCγ1, LAT contains several sites capable of binding the Grb2 adaptor and related Gads, which juxtapose the Ras exchanger Sos and SLP-76 to LAT, respectively. L.Samelson (Bethesda, MD) reported that mutation of the two most distal tyrosines results in loss of Gad binding, loss of SLP-76 association and inhibition of Erk activation and Ca^{2+} increase. Indeed, there is a positive correlation between the levels of SLP-76 transgene expression in reconstituted SLP-$76^{-/-}$ mice and TCR-mediated Ca^{2+} entry and Erk activation (G.Koretzky, Philadelphia, PA). However, Ca^{2+} influx was not abolished but was very transient if the PLCγ1 site on LAT was mutated and, surprisingly, PLCγ1 phosphorylation was also reduced if Grb2 binding was ablated (Zhang *et al*., 2000). In B cells, where a LAT-like protein has not been found, an equivalent of SLP-76, called SLP-65 (or BLNK), exists. Since SLP-65 is able to bind PLCγ2, it is believed that SLP-65 fulfills the functions of both LAT and SLP-76 in B cells. However, in contrast to SLP-76 or LAT, lack of SLP-65 strongly reduces but does not completely abolish BCR-mediated Ca^{2+} increase. LAT is also required for FcεR signaling in mast cells leading to degranulation and histamine release (L.Samelson).

LAT phosphorylation in T cells or SLP-65 in B cells therefore generates an intricate network of adaptors including Grb2, Gads and SLP-76. This scaffolding is also the site where Vav, a hematopoietic cell-specific Rac-1 guanine nucleotide exchange factor, and the adaptor Nck/Pak are recuited through SLP-76 (or SLP-65), thus creating a close proximity between activated Rac-1 protein and its target, the serine/protein kinase Pak, and establishing a connection for activating cytoskeletal rearrangements (Bubeck-Wardenburg *et al*., 1998).

PLCγ activation promotes increased hydrolysis of PI-4,5 bisphosphate into DAG and IP3, which activate most PKCs and regulate channel Ca^{2+} opening in the ER, respectively. However, the identification of the plasma membrane channel(s) responsible for Ca^{2+} influx in lymphocytes has been elusive. A.Sharenberg (Boston, MA) approached this by screening the *Caenorhabditis elegans* proteome for proteins of >1000 amino acids with six transmembrane regions, then used these to screen lymphocyte ESTs. He identified a family of at least six members (the SOC family) having a 'pore signature' for Ca^{2+} selectivity. SOC1 overexpression proved to strongly increase weak tapsigargin-mediated FcεR-stimulated Ca^{2+} entry. SOC2 was required for B cell viability as shown by gene knock-out in the DT40 cell line. While this initial hunting might not have captured the right gene yet, efforts in this direction are promising.

Several isoenzymes of the PKC family are expressed in lymphocytes. Although their importance in many pathways is inferred, the specific role of each is unknown. The DAG-dependent, Ca^{2+}-independent PKCθ has attracted particular attention as it was found to localize to the T cell/APC interface (the cSMAC) and to be in a complex with Vav-1. PKCθ gene knock-out, discussed by D.Littman (New York, NY), revealed it not to be required for thymocyte development but to be essential for NF-κB activation in mature T cells (Sun *et al*., 2000).

Members of NF-AT family of transcription factors are also essential for cytokine gene expression. DNA binding of NF-AT is achieved in a cooperative fashion with AP-1-like factors. JNK is thought to regulate c-Jun and may therefore influence NF-AT activity. I.Graef (Stanford, CA) reported that knock-out of NF-ATc4 (also known as NF-AT3), which was thought to be central nervous system specific, resulted in upregulation of IL-4 expression in lymphocytes. Unexpectedly, the phenotype of c-Jun phosphorylation site mutants in lymphocytes did not mirror the phenotype of the JNK1 and -2 knock-out (E.Wagner, personal communication), leading to the conclusion that the target of JNK in lymphocyte activation must be different from c-Jun. JNK knock-out (especially JNK2) correlated with a dramatic decrease of NF-AT DNA binding in response to T cell activation. This was not due to a defect in nuclear translocation nor to a defect in AP-1.

Thresholds: a question of degree

M.Neuberger (Cambridge, UK) discussed the dependency of B cell responses on BCR affinity for antigen. For effective presentation to T cells, the affinity threshold and ceiling of Ag–BCR interaction were estimated to be within 10^5–10^{10} M^{-1}. Surface-bound (or particulate) antigen, however, triggers a response at very low affinity compared with soluble antigen (Batista and Neuberger, 2000). Interestingly, the BCR can literally wrench antigen from

a non-internalizable surface, a situation perhaps mimicking antigen tethered to a cell surface via complement or Fc. These data have interesting implications for understanding the onset and maturation of humoral immunity.

Duration of TCR triggering is a key parameter influencing the T cell response to polarizing cytokines. A.Lanzavecchia, using an *in vitro* Th1/Th2 polarization model of plastic-bound MHC/peptide and CD28 costimulation, indicated that a shorter time of TCR stimulation plus IL-12 were required for Th1 polarization (Iezzi *et al*., 1999), whereas a prolonged stimulation in the presence of IL-4 was required for Th2. This temporal coincidence of stimuli is probably critical for demethylation of the IL-4 and IL-13 genes.

The capacity of the TCR to recognize a poorly represented foreign peptide/MHC in a considerable background of weakly interacting self peptide/MHC is a real headache in terms of signaling. R.Germain (NIH, Bethesda, MD) addressed the problem using antagonistic peptides, a property likely to be shared by many self peptides. He showed that engagement by peptide antagonists inhibits the response by desensitizing the TCR through recruitment of the PTPase SHP-1 by Lck SH2 and that this desensitization spreads to non-engaged TCR. Moreover, Lck binding to SHP-1 might be downregulated by Erk phosphorylation of Lck. Thus, a positive feedback loop would exist in which upregulating Erk activity would couteract SHP-1 recruitement to the TCR (Germain and Stefanova, 1999; Stefanova *et al*., unpublished). A consequence of this model is that effective agonist triggering requires Erk-dependent prolongation of TCR signal transduction for a period long enough to ensure gene activation. One example of where TCR signal duration controls cell fate was shown for the CD4/CD8 lineage decision in the thymus, which in accordance with this new model has previously been shown to be regulated by Erk activity (Yasutomo *et al*., 2000).

Modulation of antigen receptor signaling: many hands make light (or heavy?) work

Costimulation in B cells can be achieved through CD19, which is likely to be brought into physical proximity to the BCR during B cell response to Ag. D.Fearon (Cambridge, UK) presented data identifying IgM as a ligand for the immunoglobulin-like domain 3 (D3) of CD19 in germinal centers. Staining with recombinant D3 co-localized with PNA^+ cells and with areas containing follicolar dendritic-M1 cells. He proposed a model in which IgM with bound antigen and covalently attached C3d complement component bridges CD19 to the BCR.

In T cells, the nature of CD28 costimulation is far from being clear. Vav-1 is tyrosine phosphorylated following CD28 ligation and may be a key effector of the signal. When overexpressed in a T cell line, Vav-1 strongly increased activation of NF-AT (Michel *et al*., 2000). Biochemical and functional data suggest also that CD28 reinforces, via Vav-1, the efficiency of the TCR-proximal signaling. CLTA-4 on the other hand is expressed on activated T cells and delivers negative signals upon activation. T.Saito (Chiba, Japan) showed that although CTLA-4 recruits SHP-2 to a tyrosine-based motif in its cytoplasmic tail, the capacity to suppress T cell activation is maintained by CTLA-4 mutants lacking this motif, suggesting that negative signaling by CTLA-4 does not require SHP-2 association (Nakaseko *et al*., 1999). In the absence of phosphorylation, the tyrosine-based motif is also responsible for downregulation of surface CTLA-4 by promoting its internalization by endocytosis through interaction with the adaptor complex AP-2. Internalized CTLA-4 accumulates in lysosomes that are inducibly secreted to the cell surface upon TCR triggering, resulting in high expression of surface CTLA-4 (Iida *et al*., 2000). T.Saito also showed that Gab2 (a member of the IRS family) assembles with CD3ζ, ZAP-70, SLP-76 and LAT upon TCR stimulation and in turn recruits SHP-2 into this complex, which results in signal extinction, suggesting a negative role for for Gab2 in TCR signaling (Yamasaki,S., Nishida,K., Hibi,N., Takeuchi,A., Sakuma,M., Ohnishi,H., Hirano,T. and Saito,T., manuscript submitted).

Co-aggregation of the BCR to the FcγRIIB by immune complexes results in negative feedback in B cells. On crosslinking, immunoreceptor tyrosine-based inhibition motifs (ITIM) in the cytoplasmic tail of the FcγRIIB are phosphorylated by Lyn and both SHP-1 and SHP-2 are recruited in an intricate play of site overlaps that require both phosphorylated tyrosines of the ITIM. The same ITIM, however, also binds the lipid phosphatase SHIP but requires only one of the ITIM tyrosines (Muraille *et al*., 2000). As these two types of phosphatase affect very distinct substrates, their recruitment as a function of ITIM phosphorylation may have qualitatively different effects on signaling pathways controlled by the BCR (M.Daeron, Paris, France). A similar mechanism may be involved in IgE receptor function.

Signaling paradigms for lymphocyte development

Positive and negative signals in B cell development

Signaling by the BCR requires membrane Ig-associated Igα and Igβ, each of which have a single ITAM. Contrary to expectations, M.Reth (Freiburg, Germany) provided biochemical evidence that the BCR stoichiometry is one Igα/Igβ dimer for one membrane immunoglobulin, which may help to explain why knock-in mice lacking the Igα cytoplasmic tail have severe defects in B cell development (Torres *et al*., 1996). An unexpected inhibitory role for Igα has emerged from data reported by K.Rajewsky (Cologne, Germany) in a mouse model expressing the tailless Igα and a hen egg lysozyme (HEL)-specific BCR. This mouse was engineered to express either soluble HEL (sHEL) or membrane-bound HEL (mHEL), which, in the context of a normal BCR, induce tolerance or clonal deletion, respectively. Clonal deletion of autoreactive B cells was fully operational in mice expressing mHEL. However, expression of the weak self-antigen sHEL unexpectedly also resulted in negative selection of most B cells. Moreover, the few mature B cells found had lost HEL specificity due to secondary rearrangements of endogenous κ chain genes. *In vitro*, BCR engagement on immature B cells from the tailless Igα mice induced exaggerated and prolonged calcium flux and protein tyrosine phosphorylation (Kraus *et al*., 1999). Hence the Igα cytoplasmic tail contains information for negative signaling operating at least at an early stage of B cell maturation.

In mice harboring a Tyr→Phe mutation of the Igα ITAM, conventional B cell maturation was largely normal (K.Rajewsky/R.Torres, Basel, Switzerland); however, B-1 cells were completely lacking. B-1 cells have a distinct surface phenotype and anatomical localization as compared with conventional B-2 cells and are thought to be responsible for natural immunity. Mice lacking other genes involved in BCR signaling or signal modulation, including the SLP-65 adaptor (Jumaa *et al*., 1999), also lack B-1 cells. Hence, the default pathway appears to favor development of B-2 cells, while B-1 cell development requires active signaling, a requirement possibly related to their unique self-renewal capacity.

In mature B cell models, Src PTKs are essential for phosphorylation of Igα and Igβ ITAMs, Syk recruitment and cellular activation. A.Tarakhovsky (Cologne, Germany) addressed the role in development of the three principal Src kinases expressed in B cells, Lyn, Fyn and Blk. Deletion of a single PTK has no impact on B cell development (Texido *et al*., 2000 and references therein). In mice lacking all three kinases ($LFB^{-/-}$ mice), the transition from pro-B to pre-B cells was unaffected, as was pro-BCR activation of Syk and phosphorylation of downstream effectors, suggesting a role for another PTK in ITAM phosphorylation in the pro-BCR. LFB mice had however highly reduced numbers of both immature and mature B cells. Surprisingly, the very few mature B cells were mostly plasma cells producing poly- and auto-antibodies, and Rag-1/-2 genes were reactivated, suggesting a negative function for Src family PTKs at a checkpoint for differentiation of mature B cells to plasma cells (Saijo,K., Schmedt,C., Su,I.-h., Texido,D., Desiderio,S., Lowell,C.A. and Tarakhovsky,A., manuscript submitted).

Mice lacking CD22, a B-cell specific α-2,6-sialoconjugate costimulatory receptor phosphorylated by Lyn and recruiting SHP-1, were discussed by M.Neuberger. Mutant mice show facilitated tyrosine phosphorylation upon BCR triggering but have high affinity auto-antibodies in their serum with no overt autoimmune desease. In mature B cells CD22 may be implicated in controlling autoimmunity, whereas in differentiating immature B cells it may serve to raise the BCR activation threshold (O'Keefe *et al*., 1999).

SLP-65/BLNK has recently been shown to be a positive regulator throughout B cell development. Its absence provokes a substantial decrease of pre-B, immature and mature B cells. The blockade begins at pro-BCR signaling but is not complete at any developmental stage, suggesting a partial redundance of SLP-65 (Jumaa *et al*., 1999). The phenotype of SLP-65 deficient mice is strikingly similar to that of mice carrying a mutation in Syk, PLCγ or Btk, a tyrosine kinase coupled to the BCR and involved in PLCγ recruitment. M.Reth showed that SLP-65 directly recruits Btk by interacting with its SH2 domain (Su *et al*., 1999). Collectively, the data are consistent with a role for SLP-65 in promoting a Ca^{2+} response by sequential recruitment of Btk and PLCγ.

T cell development: the emergence of adaptors

Pro-thymocytes express a pro-TCR, formed from a calnexin–CD3 complex. Following a productive TCRβ gene rearrangement, the TCRβ pairs with the invariant pre-Tα subunit to form the pre-TCR and further β-chain rearrangement ceases. Four distinct stages of differentiation have been defined in these double-negative thymocytes (DN1–4), based on the expression of the cell surface markers CD25 and CD44 and the rearrangement state of the β locus. K.Eichmann (Freiburg, Germany) presented data on the requirement for Lck and ζ/η in pro-TCR signaling using single and double knock-out mice. Lck and CD3ζ/η are required for β gene expression and pro- to pre-TCR transition, but not for β-chain rearrangement (Würch *et al*., 1998; Biro *et al*., 1999). The DN3 ($CD25^{+}CD44^{-}$) stage, at which VDJ rearrangements occur, marks a critical developmental step. Wild-type cells expressing the pre-TCR survive, proliferate and differentiate to DN4 ($CD25^{-}CD44^{-}$) and then DP ($CD8^{+}CD4^{+}$) expressing a mature αβTCR. DN3 cells defective in TCRβ expression proceed to the DN4 stage before being deleted by apoptosis. Thymocytes lacking CD3ζ/η or Lck survived to DN3/β^{+} but their entry into the cell cycle was severely impaired, suggesting different requirements for pre-TCR signaling in survival and proliferation (Würch *et al*., 1999).

Mice lacking Rag-1 or -2 have a block in thymocyte development at the DN3 stage, in which only the pro-TCR is present. Triggering the pro-TCR with anti-CD3 Abs results in thymocyte differentiation to DP. D.Alexander (Cambridge, UK) reported that in DN1 cells from mice doubly defective for CD45 and Rag-1, this treatment did not rescue DP thymocyte development. The defect was however overcome by expression of a constitutively active Lck transgene (Pingel *et al*., 1999; Seavitt *et al*., 1999). Hence, activation of Src PTKs by CD45 is essential for driving the differentiation program leading to DP thymocytes.

L.Samelson and G.Koretsky discussed the function of adaptors in thymocyte development. $LAT^{-/-}$ or $SLP\text{-}76^{-/-}$ mice have a developmental arrest at the DN stage (Clements *et al*., 1998; Pivniouk *et al*., 1998; Zhang *et al*., 1999). $LAT^{-/-}$ DN thymocytes rearrange the TCRβ chain and express low levels of pre-TCR. However, pre-TCR engagement fails to induce either proliferation or differentiation to DP. Both LAT- and SLP-76-deficient mice have a normal NK cell development, however a deficiency in SLP-76 results in defective platelet aggregation and granule release leading to severe fetal hemorrhage (Clements *et al*., 1999). Furthermore, SLP-76 deficiency was shown to impair signaling via the high-affinity IgE receptor in mast cells (Pivniouk *et al*., 1999). A.Weiss showed that mice lacking the adaptor SLAP, composed essentially of an SH3 and SH2 domain, have normal T cell numbers, but TCR/CD3 and the activation markers CD69 and CD5 were upregulated in DP thymocytes (Sosinowski *et al*., 2000). Interestingly, when expressed in HeLa cells, SLAP localizes in endosomes, suggesting that it may be part of a protein retention system.

B.Shraven reported upregulation of TCR surface expression in T cell lines overexpressing TRIM, a transmembrane adaptor with several phosphorylatable tyrosine residues that is associated with the TCR–CD3 complex (Bruyns *et al*., 1998). In mice deficient for the adaptor Cbl, which targets proteins to the ubiquitin-dependent degradation pathway, TRIM and TCR surface

expression are strongly upregulated. B.Schraven and K.Eichmann showed that TRIM is expressed during thymus development as two isoforms. One, which lacks the transmembrane domain, is present early in fetal thymus and is substituted at day 14 by the membrane-bound form. This suggests that the changing levels of TCR expression during thymocyte development may be regulated by differential expression of TRIM isoforms.

D.Littman described the effect of ablating a thymus-specific isoform of the the hormone receptor/transcription factor RORγT/TOR. RORγT is upregulated by signaling through the pre-TCR (Villey *et al*., 1999) and its expression ceases in DP thymocytes. RORγT$^{-/-}$ mice are viable, however both the DP and SP thymocyte subpopulations are severely reduced due to enhanced apoptosis at the DP stage. In RORγT$^{-/-}$ DP thymocytes the anti-apoptotic protein bcl-x_L is not expressed, while $p27^{kip}$ is reduced and cdk2 upregulated. Thymocyte development is fully restored by a bcl-x_L transgene. RORγT may prevent death of positively selected thymocytes by counteracting apoptosis by thymic glucocorticoids.

D.Kioussis (Cambridge, UK) addressed the question of how signals triggered by positively and negatively selecting peptides are translated in the thymocyte. He showed *in vitro* data obtained on thymocytes expressing a class I-restricted transgenic TCR indicating that the differences in ZAP-70, PLCγ, Cbl and p38 MAP kinase phosphorylation elicited by agonists, weak agonists or antagonists are only quantitative (Smyth *et al*., 1998). Since weak agonists did not promote accumulation of phosphoproteins at the thymocyte/APC contact zone then very weak biochemical alterations induced by TCR triggering suffice for positive selection. Interestingly, weak agonists lose potency for DP thymocytes during development, so that mature T cells are protected against activation by self-ligands (Lucas *et al*., 1999).

In apparent contradiction with the above data, E.Palmer (Basel, Switzerland) presented data suggesting a qualitative difference in signaling in thymocyte selection. Mutation of a conserved motif in the membrane-proximal extracellular portion of the TCR α chain (α-CPM) results in impaired TCR signaling and in a severe block in positive selection (Bäckström *et al*., 1998). In thymocytes expressing a mutant α-CPM TCR, a positively selecting peptide selectively failed to activate Erk, while not affecting the activation of other MAP kinases. The defect was associated with an impaired recruitment of activated Lck and ZAP-70, as well as phosphorylated forms of CD3ζ and LAT, to the GEMs (Werlen *et al*., 2000). Interestingly, the CD3δ chain associates only loosely with the mutant TCR (Bäckström *et al*., 1998), suggesting that CD3δ may be selectively involved in signaling to the Ras/Erk pathway and in positive selection.

Conclusions

The complexity of antigen receptor is beginning to resolve into structural and functional modules capable of controlling distinct cellular processes at different stages of development. Individual modules are used as multifunctional components to build highly specific and 'personalized' pathways leading to control of calcium homeostasis, cytoskeleton rearrangement, activation of specific gene expression and entry into the cell cycle. Key decisions such as whether to proliferate or to enter apoptosis and Th1/Th2 differentiation are based on the interbalance of the pathways activated. Hence, antigen receptors and their coreceptors are not simple switches that trigger signaling cascades leading to the nucleus, but complex systems capable of sensing their context and of directly controlling cellular processes, only one of which is specific gene activation. These 'smart' receptor systems rely on a series of kinases and phosphatases to modulate the signals and a number of versatile adaptors to deliver them to the appropriate effectors. The pace of current research should lead, in the near future, to a better understanding of the process of signal initiation and extinction and the relationship between lipid rafts and the structures involved in the immunological synapse.

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