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Recent Insights in T Cell Activation and Differentiation

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INTRODUCTION

T cells are a heterogeneous group of cells in the immune system that have in common the surface expression of the T cell receptor complex (TCR), composed of two variable chains (α−β or γ−δ), two dimmers of CD3 proteins and a ζ chain homo-dimmer. They are classified according to the presence or absence of other surface molecules, such as CD4 and CD8 or the alternative γ and δ chains (instead of the classical ones α and β). The classical lymphocytes ($\alpha\beta^+$ and either $CD4^+$ or $CD8^+$), on which this chapter will center, recognize antigenic peptides, presented by MHC molecules on the surface of Antigen Presenting Cells. They are in charge of organizing adaptive immune response and of the clearance of infected or dysfunctional cells. Additional receptors, both intracellular and embedded on the cell membrane, further add to the information received by the T cells and altogether lead to the T cell response. The nature of T cell response depends on the maturation and differentiation status of the cell and the signaling events started by the engaged receptors. Typical responses are activation, anergy or apoptosis. The initial activation event gives rise to the reorganization of the cells, preparing them for further information sensing, which finally leads to proliferation and differentiation into either memory, regulatory or effector T cell populations.

T cell activation and differentiation has been a major research topic for over 3 decades and good comprehensive reviews are available [1-6]. These recent years, however, have seen the

arrival of a number of new technologies that have contributed to significant advances in the field. Among these, we can name "*omics*" technologies and single cell analysis, particularly multi-parametric flow cytometry, high-resolution microscopy and single cell sequencing. The picture is becoming increasingly complex, not only for the increasing number of interacting proteins, but also for the complexity of gene expression regulation. It does not only depend on the activation of a set of transcription factors, but also on the condensation status of the chromatin at the gene promoter and enhancer elements. Controlling elements include DNA methylation, posttranslational modifications of histone tails, the expression of none coding RNAs and other post-transcriptional modifications that alter the stability of mRNA itself. Changes in metabolism due to cell responses (metabolome) and protein interactions (interactome) also help to decipher the cells dynamic nature and the complexity of T cell signaling and responses.

In this chapter, we will address data obtained in recent years and the perspectives for the comprehensive understanding of this fascinating subject.

T CELL ACTIVATION

T cell activation start upon TCR recognition of a peptide-MHCII complex, initiating a series of signaling cascades that trigger rearrangement of actin cytoskeleton and centrosome re-localization. This leads to the formation of TCR micro-clusters, that initiate the signaling events and later on to the formation of the immunological synapse, which is necessary for maintaining, controlling and terminating TCR-mediated signaling [7]. TCR signaling network is a complex process involving posttranslational changes and re-localization of signaling proteins, a series of transient proteins interactions that finally lead to the T cell response. This process is coupled to the metabolic reprogramming of the cells, from an aerobic, mainly fatty acid-dependent mechanism to a glycolisis dependent mechanism, necessary to fulfill the energetic requirements of the cell and to provide metabolites, necessary for T cell activation. The first step in this signaling network is TCR triggering, ultimately leading to changes in cell shape and migration, gene expression, through the activation of several members of the AP-1, NFAT, NF-κB and CREB families of transcription factors, and proliferation. For better understanding this process, we will divide the process into discrete events.

Early Signaling

Early TCR signaling is characterized by the Src family of protein kinases (Lck and Fyn) -driven phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) present in CD3 and ζ chains of the TCR. These motifs are characterized by the presence of two tyrosines in a particular sequence that, upon phosphorylation, recruit Src homology 2 (SH2) domain containing proteins. In TCR signaling, these phospho-ITAMs (pITAMs) function as anchors for the recruitment of the ζ chain associated protein kinase 70 (ZAP70), which is also phosphorylated and activated by Lck and possibly Fyn [8]. Then ZAP70 and Lck phosphorylate linker for activation of T cells

(LAT), a transmembrane protein that function as a scaffold for the generation of multi-protein complexes [9,10].

Phosphorylation of ITAMs by Lck and Fyn is a key point in early TCR signaling and the activation and localization of these kinases is highly regulated. Phosphatase CD45 and kinase Csk are key regulators of Lck activation. CD45 de-phosphorylates the K505 inhibitory residue of Lck, therefore promoting its activation. However it also mediates de-phosphorylation of the activatory K394 residue. On the other hand, Csk phosphorylates K505, promoting the inhibition of Lck. Residue K394 is thought to be a trans-phosphorylation site for Lck. Fyn regulation is similar to that of Lck [11].

Four isoforms of Lck activation stages are identified, according to the combination of phosphorylation in its 2 principal regulator residues. This leads to 3 conformational states, the closed, the primed and the open state. The open state, characterized by K394p whether or not K505p is present, is the active kinase, which forms clusters and co-localize with its target proteins. The closed state correspond to the un-phosphorylated K394 and phosphorylated K505p in Lck and is the inactive kinase. Primed Lck is un-phosphorylated, in the open conformation and has a basal kinase activity [12,13].

In resting T cells, high-resolution microscopy allowed to determine that 40% of Lck is already active forming nano-clusters in the plasma membrane. This proportion has been reported to remain invariable in response to T cell activation or to only slightly augment. Instead, T cell activation allows the recruitment of active Lck to micro-clusters in the plasma membrane, co-localizing with TCR and LAT, due to its association with CD4 or CD8 intracellular domains [10,14,15]. Thus, recruitment of active Lck to TCR rather than Lck activation, which occurs even in unstimulated cells, is the key event driving phosphorylation of CD3 and ζ chains' ITAMs and downstream TCR signaling.

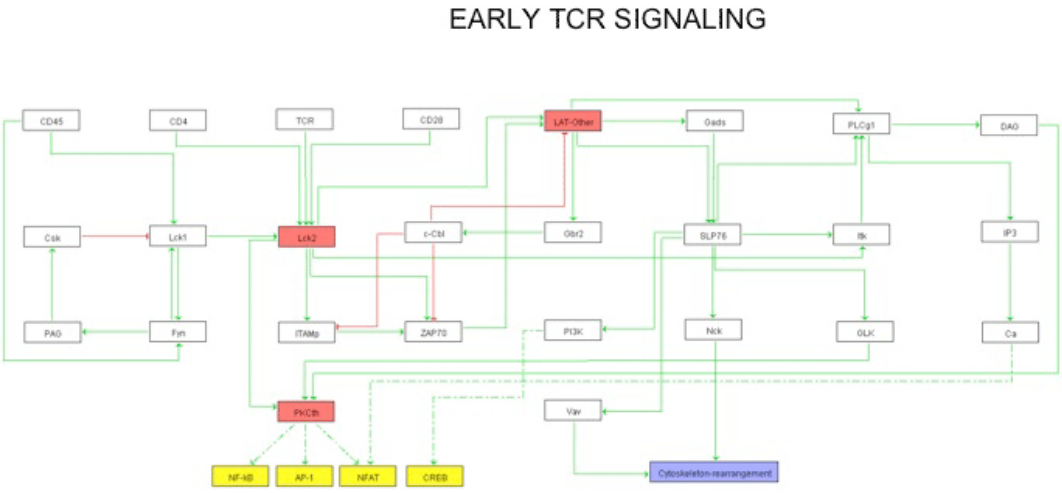

Figure 1: Early TCR signaling. TCR recognition of its ligand induces the recruitment of Lck to its intracellular chains and the phosphorylation of ITAMs. Generation of phospho-ITAMs is needed to induce ZAP70 recruitment and activation as well as the formation of LAT signalosome, and other protein LAT independent complexes in the plasma membrane. Among others, two major players in early TCR signaling are activated, PLCγ and PKCθ. Together, these two proteins mediate the activation of the signaling cascades leading to IKK, MAP kinases and Calcium-NFAT signaling pathways, finally leading to the activation of NF-κB, AP-1 and NFAT families of transcription factors. LAT signalosomes also allows the recruitment and activation of PI3K, a process that can lead to CREB activation. Negative regulators such as c-Cbl are also recruited to LAT signalosome to control and terminate signaling. CD4 co-receptor and CD28 co-stimulatory receptor both contribute to the signaling network enhancing the strength and promoting the ramification of the signals.

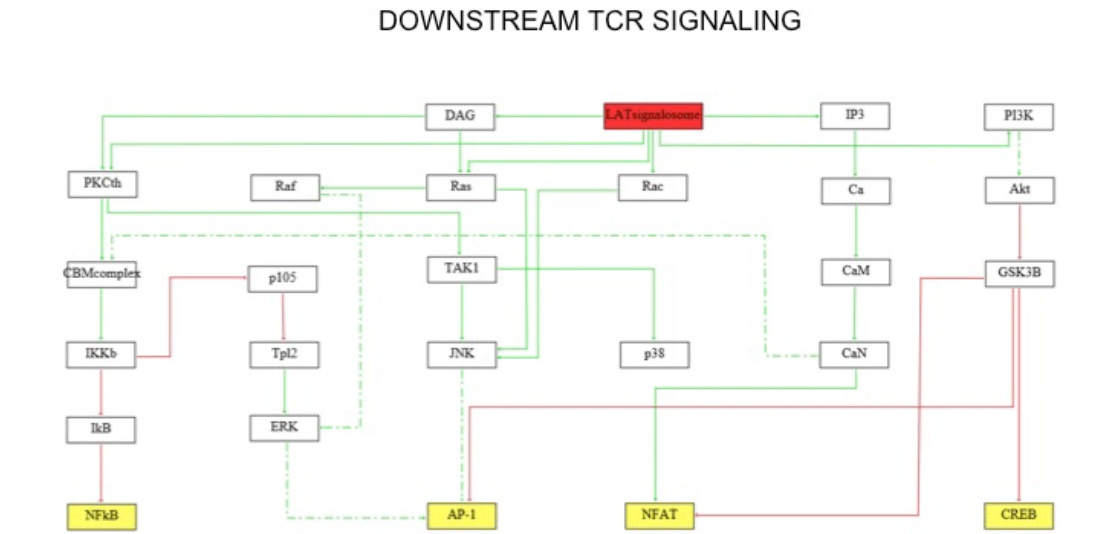

Figure 2: Downstream TCR signaling. Multi-protein complexes interactions allow the integration and the diversification of signals. LAT signalosome is an example of a multiprotein complex from where a series of signaling cascades branch in order to control several aspects of the cellular response. LAT signalosome favors the activation of signaling cascades leading to NFκB, AP-1, NFAT and CREB transcription factor activation. Dashed lines represent indirect and/or proposed pathways whereas continuous lines represent direct influence on the target protein.

LAT Signalosome

LAT is a transmembrane protein with a long intracellular tail, which can be phosphorylated in at least 5 different sites: Tyrosines 132, 171, 191, 226 and 127, in human cells. LAT phosphorylation allows the recruitment of SH2 containing proteins, among them several adapter proteins such as Gads and Grb2, to form multi-protein complexes that support the diversification of signaling [16].

The physiological role of LAT phosphorylation has been a focus of study for years and some mechanisms have already been elucidated. For instance, LAT phosphorylation in residues 171 and 191 allows the recruitment of multi-SH3 domain containing adaptor protein Gads, which in turn recruits adaptor SLP76, a protein with multiple partners. The same LAT residues can also recruit Grb2 adaptor that interacts with Sos proteins to mediate Ras activation. In the other hand, phosphorylation of Tyrosine 132 of LAT promotes PLCγ1 recruitment, whose presence is further stabilized by its interaction with SLP76. SLP76 is phosphorylated in 3 sites and this is important for Itk binding, which leads to Itk activation by Lck and then to Itk mediated PLCγ1 activation. Active PLCγ1 then cleaves PiP_2 in plasma membrane to generate IP3 and DAG second messengers, therefore promoting calcium dependent and PKCθ –dependent pathways [9,16].

SLP76 and LAT are also important for PI3 kinase (PI3K) activation. This kinase produces PiP_3 from PiP_2, and therefore mediates recruitment and activation of several proteins with Pleckstrin homology (PH) domains, Akt (PKB) among them. PI3K pathway is further enhanced by CD28 co-stimulus [17].

LAT signalosome is also important for regulating actin cytoskeleton and cellular adhesion. For instance, in the LAT signalosome SLP76 interacts with Vav1 and Nck, these proteins recruit Cdc42 to activate WASp, which interacts with Arp2/3 complex to mediate actin polymerization and cytoskeleton changes. Whereas ADAP is also recruited through its interaction with SLP76, coupling TCR signaling to cell adhesion [9].

The disassembly of LAT signalosome produces an uncontrolled T cell response rather than impeding the response, pointing to the existence of LAT independent TCR signaling pathways and to a regulatory role of LAT signalosome. In fact, increasing evidence points to the existence of LAT-independent signalosomes and its participation in TCR signaling [18].

Proteomic analysis has identified novel interactions in LAT-SLP76-Zap70 signalosomes as well as a LAT independent signaling pathway. One of them is mediated by CD6 whose function as a scaffold protein allows the recruitment of SLP76 and Vav, to mediate TCR signaling [19].

For a recent review on the cooperativity of protein interactions in the LAT signalosome and the role of LAT as an integrator and regulator of signaling see Bartelt 2013 [16].

PKCθ Central Role

A key component activated in response to early TCR and CD28 signaling is protein kinase C θ (PKCθ). A serine/threonine kinase highly regulated by phosphorylation in multiple sites. This protein is recruited to the center of the immunological synapse by its indirect interaction with the co-stimulatory receptor CD28. CD28 contributes to the strength of TCR signaling by recruiting several adaptor and signaling proteins such as Grb2, PI3K and Lck. It is through its association with Lck that PKCθ is recruited to the CD28 intracellular domain. There, GLK mediated phosphorylation of PKCθ in T538 and further auto-phosphorylation activates the protein kinase. Activity of PKCθ

is also regulated by its phosphorylation in Y90 by Lck. PKCθ activity has been reported to auto-phosphorylate in T219 and this modification is believed to mediate its own localization to the membrane. This step ensures downstream signaling, leading to the activation of the transcription factors NF-κB and AP-1. Recently, GLK has been reported to mediate PKCθ translocation to the plasma membrane but it is believed to happen through an indirect mechanism, possibly by inducing PKCθ auto-phosphorylation [20,21].

Another protein complex, mTORC2, mediates full activation of PKCθ through its phosphorylation at S696, adding to the complexity of PKCθ regulation [20]. For a full review of the regulation of PKCθ activation by phosphorylation see Wang 2012 [21].

MAP Kinases Activation

TCR signaling results in the activation of three different mitogen activated protein (MAP) kinases JNK, ERK and p38, which are responsible for the activation of several effector kinases and the formation of the AP-1 transcription factor dimmers [22]. The mechanisms underlying the activation of MAP kinases have been widely studied, because they control several cellular responses. The specific components responsible for the activation of each pathway are however not completely understood. An important activator of MAP kinases pathways is TAK1, a MAPKKK protein that is activated in response to TCR and other receptors signaling [23]. TAK1 is implicated and extensively studied in the context of IL-1R/TLR signaling pathways. Its activation is regulated by its interaction with TAB1 and TAB2 or TAB3 and by poly-ubiquitination by TRAF6. TAK1 can then phosphorylate several MAPKKs, such as MKKs, to activate JNK and p38 pathways [24,25]. Another MAPKKK that has been reported to participate in TCR signaling is MEKK.

Activation of JNK requires its phosphorylation by MKK4/7 (also known as JNKK) while P38 activation is dependent on the MKK3/6 mediated phosphorylation. MKK3/6 and MKK4/7 can be phosphorylated and activated by MEKK, which has to be previously activated by the small GTPases Ras and Rac, in contrast to ERK pathway, which cannot be activated by Rac. JNK phosphorylate Jun while cFos synthesis and phosphorylation depend on ERK signals. These two proteins form the heterodimmer AP-1 (among other possible partners).

Erk activation in response to TCR signaling occurs through the activation of Ras, which is recruited to the plasma membrane by Sos and activated by RasGRP and Sos itself. Ras activation mediates the activation of the Raf-Mek-ERK pathway. An additional mechanism for ERK activation was proposed, mediated by IKK through the phosphorylation of Tpl2 and the inhibition of Tpl2 inhibitor p105, which promotes MKK mediated ERK activation. TCR and CD28 stimulation have been shown to mediate AP-1 activation by means of IKKβ activation [26].

ERK sustained signaling is necessary for proper T cell activation, because it establishes a positive feedback loop through the phosphorylation of Lck [27,28]. ERK can induce cFos expression through the activation of Elk and thus favor AP-1 activation.

Calcium Mediated Signaling

IP3 generation due to the cleavage of PIP_2 by PLCγ is recognized by a calcium channel receptor in the endoplasmic reticulum and produces Ca^{2+} release from this intracellular compartment. This process triggers Ca^{2+} entrance from extracellular sources and an increase in its cellular concentration. Ca^{2+} binds Calmodulin and this complex activates the phosphatase Calcineurin and kinase CaMKII. Calcineurin then dephosphorylate NFAT proteins. Dephosphorylation of NFAT exposes its nuclear localization signal and therefore mediates its nuclear import and transcriptional activity. Inactivation of NFAT occurs through phosphorylation by several kinases, which induce its cytoplasmic re-localization, with GSK3 playing a major role. Other proteins with minor roles have been proposed as kinases for NFAT, such as CK1 and CK2 as well as the MAP kinases P38, ERK and JNK [29,30].

IKK-NF-κB Pathway

Activation of NF-κB transcription factor is indirectly mediated by PKCθ. It is believed to occur through the activation of CARMA1-Bcl10-MALT1 (CBM) and IKKα-IKKβ-IKKγ (IKK) complexes [26,31]. Phosphorylation of CARMA1 by PKCθ promotes its association with the preformed complex Bcl10-MALT1 and the recruitment of the resulting trimolecular complex to the TCR, where it will interact with IKK and mediate the complex activation. A direct interaction between PKCθ and IKKβ has been also reported, suggesting the existence of a direct path for NF-κB activation [31,32].

In the TCR micro-clusters or immunological synapse, CBM complex becomes K63 poly-ubiquitinated, a process known to mediate multi-protein interactions, possibly by TRAF6. This event can promote IKK complex recruitment to the TCR clusters due to IKKγ ability to interact with poly-ubiquitin chains. This will further promote IKK activation. Another suggested event is the participation of the adaptor protein ADAP that mediates CARMA1 and TAK1 interaction [33]. TAK1 can then phosphorylate and activate IKKβ. Several other kinases have been proposed to regulate IKK activation, one strong candidate is CaMKII, which implies that Calcium signals can influence NF-κB activation [34].

IKK- is responsible for inhibitor of κB (IκB) phosphorylation, a process that mediate IκB poly-ubiquitination and degradation by the proteasome, liberating NF-κB from its inhibitor and exposing its nuclear localization sequence, thus promoting its nuclear translocation [35]. For a recent revision on the signaling from TCR to NF-κB see Paul 2013 [31].

1. NF-κB dimmers are important for TCR and CD28 mediated T cell activation. TCR signaling induces the activation of early p65 containing and the late but sustained activation of c-Rel containing dimmers. NF-κB-mediated signals participates in all the canonical differentiation programs Th1, Th2, Th17 and Treg [36].

T CELL DIFFERENTIATION

Once T cells have been activated, they may differentiate into effector, memory or regulatory cells. The biological functions of these cells are the immediate immune function, the fast and potent immune response upon a next encounter and the control of the magnitude of immune response and self-tolerance, respectively.

Regulatory cells are a very heterogeneous group of cells involved in the control of immune response. Mechanisms include the bystander or directed secretion of anti-inflammatory cytokines, direct killing of activated cells or tolerization of dendritic cells. The best-characterized subsets are $CD4^+CD25^+FoxP3^+$ cells, which could differentiate in the thymus (tTregs) or in the periphery (pTregs). Other sorts of regulatory T cells have been described, such as Th3 (TGF-β producers), Tr1 (IL-10 producers) or Th35 (IL-35 producers). CD8 T cells can also have regulatory functions and are named suppressor T cells. Among the most studied CD8 suppressor cells are $CD28^-CD8^+$ T cells, and Qa-1 restricted cells [37,38].

Memory cells are able to survive for long periods of time, are able to self-renew to maintain their presence in different parts of the body and have the ability to mount a faster and stronger immune response upon a further encounter with the antigen. They were originally defined as $CD45RO^+$ and to belong to two big categories: T central memory T_{CM} or T effector memory T_{EM}, differentiated by the expression of the homing receptors CD62L and CCR7 that direct this cells to the lymph nodes in the T_{CM} cells. T_{EM} express low amounts of these receptors and thus remain in the affected tissues, while T_{CM}, with high expression of these receptors, localize in the lymph nodes. A third compartment of memory T cells, named stemcell memory like was later described, characterized by basically naïve T cell markers, but with a high expression of CD95, IL-2Rβ, CXCR3, and LFA-1. Later studies showed that the different memory cell lineages were in fact distinct maturational stages, being T_{SCM} the precursor cell and the T_{EM} the most differentiated lineage. Two additional maturational stages were later described, transitional memory cells T_{TM}, which refers to an intermediate stage between T_{CM} and T_{EM} and a final differentiated cells named Terminal effector T_{TE}, differentiated from the $T_{EM.}$ These memory differentiation stages and naïve T cells could be distinguished by the combined analysis of a set of just 4 markers (CD45RO, CCR7, CD28 and CD95), their phenotypes being as follows: Tn (naïve): $CD45RO^-CCR7^+CD28^+CD95^-$, T_{SCM}: $CD45RO^-CCR7^+CD28^+CD95^+$, T_{CM}: $CD45RO^+CCR7^+CD28^+CD95^+$, T_{TM}: $CD45RO^-CCR7\text{-}CD28^+CD95^-$, T_{CM}: $CD45RO^-CCR7^+CD28^+CD95^-$, T_{EM}: $CD45RO^+CCR7^-CD28^-CD95^+$, T_{TE}: $CD45RO^-CCR7^-CD28^+CD95^+$. Their homing receptors, indentifying their location could define additional stages and functions [39].

Effector T cells may differentiate into specific lineages, that are classified upon their patterns of cytokine production and expression of master transcription factors. CD4 expressing helper T cells (Th) have been better characterized than CD8 expressing cytotoxic cells (Tc) for their specific effector lineages, although both types of cells may differentiate into all the distinctive

effector cells. The following lineages of Th cells are broadly accepted and characterized: Th1, Th2 and Th17. Additional subsets include cells specialized in interaction with follicular B cells (Tfh), IL-22 producers (Th22) and IL-9 producers (Th9) [40]. Th1 cells are characterized by the production of Interferon gamma (IFNγ) and are specialized in the induction of cytotoxicity by CD8 T cells, NK cells and macrophages. This immunity is required for the elimination of intracellular pathogens- infected cells. The master transcription factor for these cells is T-bet, induced by STAT1 and STAT4 in response to IFN-γ and interleukin (IL)-12, respectively. Th2 cells produce IL-4, IL-5 and IL-13, and are required for the elimination of helminthes. Their master transcription factor is GATA-3, produced in response to STAT6 upon IL-4 sensing. Th17 cells secrete IL-17A, IL-17F, IL-21 and IL-22. They are important for the control of extracellular bacteria and fungi and their master transcription factor is RORγT in response to STAT3 among other transcription factors and the cytokines IL-6, IL-1 and TGFβ. Thf are characterized by high expression of CD40L and the chemokine CXCR5, which allows them to migrate to the follicles and interact with B cells. Their master transcription factor is Bcl-6. This subset is particularly heterogeneous, responding to STAT-1, STAT3 and STAT4 signals. Consequently, these cells may produce different cytokines, influencing the switching into different immunoglobulin types [41]. Th22 cells are involved in host defense in mucosal tissues and epithelia. They differentiate in the presence of IL-6 in the absence of TGF and their master transcription factors are T-bet and AhR. Th9 are closely associated with Th2 cells, but their signature cytokine is IL-9. They are involved in defense against parasites and like Th2 cells and could be involved in pathogenic asthma and allergies [42]. Two signature transcription factors have been proposed for these cells PU.1 and BATF [43].

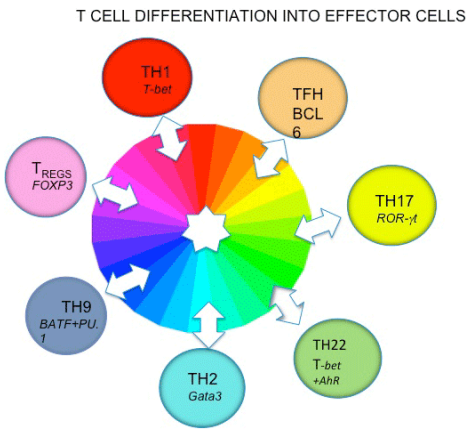

Figure 3: Linages of effector T cells. Effector T cells and their master transcription factors are plastic and cells with mixed expression of the master transcription factors and effector cytokines are observed. Plasticity is the response of T cells to a plethora of signaling events, enabling the cells to adapt to environmental factors, including cytokines, nutrients, pollutants, microbiota and the nature of the antigen. The immunological history of the individual and his genetic predisposition (as cytokine promoters polymorphisms) also play a role.

Having described the different sorts of effector T cells, it is clear now that their phenotypes are not pure, and that there are cells in which more than one of the master transcription factor are expressed, as well as a mixed profile of effector cytokines [40]. Protein expression in eukaryotes is due to a complex interplay between promoter and enhancer activities, chromatin condensation and the expression of multiple non-coding RNAs. Genome wide studies and epigenome of effector T cells have shown that effector T cells maintain dual epigenetic marks in the control elements of master transcription factors expression, which confer the cells a great deal of plasticity to change their phenotype according to environmental clues and the dominating sort of immune activity. In this way, T_{REGS} may express T-bet in chronic inflammatory conditions [44-46], and instead of controlling immune response, become inflammatory themselves. Th17 cells could give rise to Th1 cells [47]. Even mixed Th1-Th2 cells have been described, capable of secreting simultaneously IFNγ and IL-4 [48-50].

PERSPECTIVES

The identification of the molecular entities that participate in TCR mediated signaling is with no doubt important to the understanding of immune response. The mechanism by which these different entities exert their functions has been the main focus in the T cell signaling field. In recent years, the number of players in TCR signaling network has increased, moreover, with the application of new technologies such as proteomic analysis, particularly mass spectrometry. These techniques have helped to understand the complexity of the regulation of signaling cascades by protein interactions and the identification of new players. Application of these techniques in several time points after TCR stimulation would be invaluable to better understand the dynamics of the process, but great efforts would be also needed to analyze such massive data. Now, the picture of TCR signaling is becoming bigger and more complex, because there seems to be redundant pathways and partially redundant players. In recent years, we have gained power of observation by means of high-resolution microscopy and imaging. This has allowed the resolution of specific interactions between players, such as the existence of TCR oligomers or nano-clusters that form micro-clusters in response to TCR stimulation and their migration along the plasma membrane in microtubules-dependent mechanisms. Temporal imaging has been used to study specific processes in TCR signaling such as protein clustering. Applying these kinds of studies to the more important signaling hubs would be of great use to understand how multi-protein complexes integrate up-coming signals to promote downstream signals.

Investigations have shown TCR signaling cascades are coupled to metabolic, cell adhesion and cytoskeleton re-modeling networks. More studies are, however, needed in order to fully understand the interactions between all of these networks. Also, the contribution of early and late TCR mediated signaling to specific events of the cellular response has not been fully understood.

T cell differentiation leading to the formation of effector, regulatory or memory cells is also under intense investigation. Big laboratories consortia, like Roadmap and Blueprint have the

ambitious goal of deciphering the process of immune cell differentiation through genome wide epigenetic analysis of pure human cell populations, which will allow us to identify unique and shared regulatory elements and the mechanisms for cell differentiation.

T cells are important players in the maintenance of human health and the knowledge of the basic mechanisms of T cell activation and differentiation will have a huge impact in the medical treatments in the future.

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