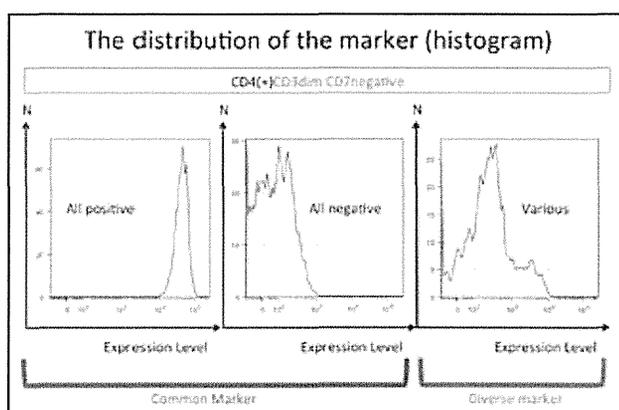域を選択し、CD7N 細胞と定義した。CD7N 細胞における評価対象抗原を図2のようにヒストグラム展開し、その陽性発現率を患者検体ならびに健常人検体で評価し、比較した。なお、健常人においては、CD7N 細胞は極めて少ない。

<図2>

C. 研究結果

今回、ATL 細胞における細胞表面抗原の発現レベルを評価した102の抗原は、以下の通り分類された。（CCR4 は ATL 細胞ではほぼ全てが陽性であったが、今回使用した蛍光抗体では発現レベルが低く、正確に陽性細胞率を計算することができなかったため、最終的な解析から除いた。）

① 健常人の CD7N 細胞ではほとんど発現していないが、ATL 細胞で高率に発現している抗原（6抗原:CCR4 を除いて）

② ATL 細胞における発現レベルに、陽性～陰性と幅広く分布があり、多様性をもって発現している抗原（２２抗原）

③ 健常人の CD7N 細胞ではほぼ高率に発現しているが、ATL 細胞で高率に発現が低下している抗原（9抗原）

④ ATL 細胞でも健常人の CD7N 細胞でも高率に発現しているもの（8抗原）

⑤ 健常人の CD7N 細胞では少数が発現しているが、ATL 細胞では全く発現していない抗原（8抗原）

⑥ ATL 細胞でも健常人の CD7N 細胞でも発現していないもの（４９抗原）

D. 考察

急性型 ATL の ATL 細胞においては、レトロウィルスである HTLV-1 の DNA への組込み部位(integration site)の差異を用いて Inverse PCR 法などでクローナリティーを評価しても Major clonal band のみが検出されるのが通常であり、急性型 ATL の ATL 細胞は一般的に均一な集団と考えられてきた。

過去に我々も CD4 陽性細胞に対して細胞表面抗原を調べ、heterogeneity を評価してきたが、急性型 ATL など病期の進行した患者においても CD4 陽性細胞の領域には健常な CD4 陽性リンパ球(Th1/Th2/Treg など)が一定数混じっていることが証明されている。そのため、より精度の高いゲーティングを用いて、さらに評価抗原の数を大きく増やして解析した。

ATL 細胞に限局して詳細に解析した結果から、急性型 ATL 細胞は細胞表面抗原の発現からも決して均一ではなく heterogeneous な集団であることが分かった。

特にグループ□や□に示した抗原においては、発現レベルが陽性～陰性と広く分布しており、ATL における CSC マーカーである可能性も示唆される。今後発現レベルの差異によってフローサイトメトリー等によるソーティングにて細胞を分離し、in vitro/in vivo における増殖能を評価することを検討している。

また、グループ□に代表されるような抗原は ATL 腫瘍細胞において比較的特異性高く発現しており、抗 CCR4 抗体 (Mogamulizumab)療法に代表されるような腫瘍細胞全体に対する抗体療法につながることが期待される。

一方、グループ□に代表される抗原は、健常人の CD7N 領域に含まれる細胞の多様性が、腫瘍細胞の均一性により失われたためと考えられる。

E. 結論

今年度の我々の研究では、ATL 細胞に高率に発現している細胞表面抗原や、逆に高率に発現

を落とす抗原、さらには、CSC マーカーである可能性のある細胞表面抗原をリストアップすることに成功した。今後は検体数ならびに評価抗原数を増やして検討するとともに、リストアップしたマーカーが CSC を規定するマーカーであるか in vitro/in vivo の系を用いて検討する必要がある。

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（国内学会）
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（その他）
特になし

G. 知的財産権の出願・登録状況
１．特許取得
　特になし

２．実用新案登録
　特になし

網羅的解析を基盤とした新規分子標的の探索

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研究要旨：
ATL は HTLV-1 感染不死化 T 細胞に遺伝子変異が蓄積した結果発症する多段階発癌が示唆されている。本分担研究においては、高速シーケンス技術を用いた全エクソン解析による網羅的な遺伝子変異解析により ATL 腫瘍化に関与する多岐にわたるゲノム異常を同定した。非常に興味深いことに他の末梢性 T リンパ系腫瘍で高頻度に変異している TET2 の体細胞性変異が同定された。TET2 は DNA の脱メチル化に関わっており、多数検体で変異解析したところ約 10%の頻度で変異が認められた。すなわちこれらの変異がエピゲノムな変化を引き起こすことにより腫瘍化に関わる可能性が示唆された。

A. 研究目的

成人 T 細胞白血病(ATL)は HTLV-1 のキャリアに発症する末梢性 T 細胞腫瘍である。有効な治療法や発症予防法は現時点で知られておらず、予後は極めて不良である。日本では推定 120 万人のキャリアから今後 5-10 万人の ATL 新規発症が予測されており、本疾患に対する画期的な治療法及び発症予防技術の開発を行うことは極めて重要かつ緊急の課題である。

本分担研究は、ATL の早期診断・画期的根治療法開発のための基盤構築に向けて、ATL 患者中の腫瘍細胞及びがん幹細胞特性を有する細胞群(ATL-CSCs)について、マイクロアレイならびに高速シーケンス技術を用いたゲノム解析を行い、特性を明らかにすることを目的とする。

B. 研究方法

本分担研究では、ATL 患者中の腫瘍細胞やがん幹細胞の特性をゲノムの視点から明らかにするために、マイクロアレイ技術・高速シークエンス技術を用いたゲノム異常の解析を行う。
(1)腫瘍特異的な変異を同定するためには患者固有の多型を除外する必要があり、ATL 患者 1 例について腫瘍検体と同一患者からの正常検体を Agilent 社の Sureselect を用いて全エクソン領域を濃縮し、次世代シークエンサー Hiseq (Illumina 社) を用いて網羅的な遺伝子変異解析を行った。
(2)全エクソン解析で体細胞性変異が同定された興味深い遺伝子は、高速シークエンス技術を併用したプールドターゲットシークエンスを用いることにより、大規模なコホートにおいて変異頻度が調査された。得られた変異データは予後や病型などの臨床情報との関連について統計学的な解析が行われた。また、認められた変異については深い読み取り深度で、正確なアレル比の測定を行った。

（倫理面への配慮）

本研究は東京大学の倫理委員会の承認を得ている。本分担研究で用いた検体はインフォームドコンセントを得た後に連結可能匿名化を施して検討に用いた。

C. 研究結果

(1)腫瘍側、正常側ともに平均して 110 回の深度で全エクソン領域が読まれ、解析の感度は十分と考えられた。エクソン領域の濃縮率は 72%であり、20 回以上読めている領域は全体の 85%であった。正常検体と比較し、77 個の腫瘍特異的な変異が同定された。
(2) 非常に興味深いことに他の末梢性 T リンパ

系腫瘍で高頻度に変異している TET2 の体細胞性変異が ATL においても同定された。多数検体で変異解析したところ 145 人中 14 人に変異が認められ、約 10％の頻度であった。それらは様々な病型に分布しており、急性型 47 例中 6 例、リンパ腫型 46 例中 5 例、慢性型 36 例中 3 例にそれぞれ変異が認められた。両アレルが変異している症例が 14 例中 4 例認められ、変異の有無に関して生存解析が行われたが、有意差は認められなかった。

D. 考察

ATL の腫瘍細胞で特異的かつ高頻度にゲノム異常を来す ATL-1 については、ゲノムコピー数解析に加え、全エクソン解析を行うことで点突然変異など従来の手法では同定できなかった多数の変異を同定した。新規に同定された変異遺伝子の 1 つである TET2 は DNA の脱メチル化に関わっており、エピゲノムな変化を引き起こすことにより ATL が腫瘍している可能性が示唆された。多くの TET2 変異はアレル頻度について解析したところ腫瘍のメジャークローンとなっていたが、一部の症例はマイナークローンとなっていた。すなわち、腫瘍が発達していく過程の中で TET2 変異がより後期のイベントとなっている可能性が示唆された。

E. 結論

マイクロアレイによるコピー数解析で既に確認されているゲノム異常以外に、次世代シークエンサーによる網羅的変異解析により、多数の新規の変異遺伝子が確認された。これらについて機能的実験を進めると同時に多数の変異遺伝子の中から腫瘍発症に直接関わる遺伝子の同定を行い、多検体における頻度の確認や予後、病型など臨床情報との相関について検討することも重要な課題と考えられた。さらに将来への展望としては、全ゲノム解析や RNA 解析により、転座や逆位などの構造異常や新規の融合遺伝子を見出すことでさらなる病態解明につながることが期待される。

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G. 知的財産権の出願・登録状況

1. 特許取得
   特になし

2. 実用新案登録
   特になし

網羅的解析を基盤とした新規分子標的の探索

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研究要旨：
ATL は悪性度が高く、薬剤抵抗性や治療後再発が多いため、現在標準的な治療法は存在しない。ATL 細胞の生存に関与する標的遺伝子に対する特異的 siRNA を ATL 細胞に運搬するためのドラッグデリバリーシステムとしての核酸キャリアー（各種ミセル、リポソーム、PEI、アテロコラーゲン）を候補として評価を進めた。現在、抗 CCR4 抗体(モガムリズマブ)による治療が奏功しているが、今回、ATL の治療法の選択肢として、核酸創薬は候補となり得ることが示されたことから、CCR4 以外の ATL 細胞の生存に関与する標的遺伝子を対象とした新規治療法を提供できる可能性が高まる。

A. 研究目的
siRNA を ATL 細胞に運搬するための DDS(ドラックデリバリー)に関する基盤研究

B. 研究方法
ATL 細胞の生存に関与する標的遺伝子に対する特異的 siRNA を合成する。遺伝子抑制効果を既存の手法により in vitro で評価した後、未知である核酸 DDS のキャリアーを候補から選択する。

C. 研究結果
各種ミセル、リポソーム、PEI、アテロコラーゲンをキャリアーとして上記 siRNA と混合し、in vitro, in vivo モデルを使用して ATL 細胞増殖能への影響、治療モデルによる評価を進めた。

D. 考察
ATL は悪性度が高く、薬剤抵抗性や治療後再発が多いため、現在標準的な治療法は存在しない。同種造血幹細胞移植は高齢者が多い本疾患の患者群にはリスクが高い。現在、抗 CCR4 抗体(モガムリズマブ)による治療が奏功しているが、さらに、核酸創薬の樹立により、複数の治療法を提供できる可能性が高まると考える。

E. 結論
ATL の治療法の選択肢として、核酸創薬は候補となり得ることが示された。

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２．学会発表
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G. 知的財産権の出願・登録状況

１．特許取得　なし

２．実用新案登録　なし

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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<u>Yamagishi M</u>, <u>Watanabe T</u>	Molecular Hallmarks of Adult T Cell Leukemia (Review Article)	*Front Microbiol*	3: 334	doi: 10.3389/fmicb.2012.00334	2012
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<u>山岸　誠</u>	Person人と研究「成人T細胞白血病の分子レベルの全体像と、Polycomb—miR-31—NF—κB経路の異常」	*Trands in Hematalogical Malignancies*	4(3)	16-19	2012
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43

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IV. 研究成果の刊行物・別刷

Clinical & Cellular
Immunology

New Paradigm of T cell Signaling: Learning from Malignancies

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Abstract

T cells are key mediators of cell-mediated immunity. Their functions and proliferation result from T cell-specific receptor signaling (TCR/CD28) that activates the NF-κB, NFAT, Ras-MAPK, and PI3K-Akt pathways. Their development and activation also involve a complex array of signaling pathways that regulate gene expression networks, including signaling of mTOR, Notch, Wnt, Hedgehog, TGF-β, and toll-like receptors. Furthermore, recent discoveries have provided two molecular hallmarks of potential generality: miRNA patterns and polycomb-mediated epigenetic reprogramming, which can strongly coordinate the balance between molecular networks in lymphocytes. Their deregulation apparently causes T cell disorders, such as T cell acute lymphoblastic leukemia (T-ALL), and human T cell leukemia virus (HTLV-1)-induced adult T cell leukemia (ATL). This review continues with a description of our understanding of crosstalk among the signaling pathways, which contribute to the highly orchestrated development of T cell fate specification under both normal physiological and pathological conditions.

Introduction

T cells use diverse genetic programs to direct the development of distinct lineages and the generation of several effector functions required for innate and adaptive immunity. The molecular networks in T cells are strictly controlled by the input from intercellular and extracellular signals. T cell activation is basically achieved by antigen-presenting cells (APCs). Antigen recognition by and signaling through the T cell receptor (TCR) are crucial processes for T cells and provide the molecular underpinning for the specificity and execution of immune system responses.

TCR-mediated signaling pathways are the bare bones of T cell activation. However, several other signaling pathways are intimately associated with T cell development, activation, and homeostasis. In addition, newly emerging molecular characteristics have been proposed on the basis of studies of normal T cells and T cell malignancies. This new paradigm includes miRNA-mediated gene regulation and epigenetic reprogramming.

In this review, by dissecting each signaling pathway, we summarize the molecular hallmarks of T cells. We first briefly introduce the fundamental molecular mechanisms involved in T cell activation and gene expression related to T cell functions [1,2]. These signaling pathways are also involved in T cell development and disorders. We next discuss the newly emerging signaling pathways involved in T cell biology. Genetic and physiological studies have indicated that the pathways primarily identified in the areas of development, regeneration, and innate immunity are also closely associated with T cell functions and their appropriate development. Finally, we discuss regarding a conceptual advance, i.e., crosstalk between several signaling pathways.

The complexity of signaling networks within T cells confers robustness to specific and diverse gene expressions and biological functions. The complex signaling regulation that is involved within different environments has been suggested from investigations in immature and mature T cell malignancies, including T cell acute lymphoblastic leukemia (T-ALL) and adult T cell leukemia/lymphoma (ATL).

Proximal Signaling of TCR-mediated T cell Activation

T cells are instructed to use their developmental and effector programs through stimulation of the TCR complex and costimulatory molecules, which are presented by professional APCs, e.g., macrophages, dendritic cells, and B cells. TCR signaling is initiated by CD3 phosphorylation. The CD3 proteins comprise a series of dimers, including γε, δε, and ζζ, which are associated with a single TCRαβ heterodimer (Figure 1). After the recognizing cognate complexes of foreign peptide and self major histocompatibility complex (MHC) class II (pMHC) molecules, TCR/CD3 affects signaling cascades, including phosphorylation of proximal TCR components, Ras–mitogen-activated protein kinase (MAPK) signaling, activation of nuclear factor κB (NF-κB) by protein kinase C θ (PKCθ), and Ca^{2+} flux-mediated signaling [1]. In response to stimulation, signaling through these mediators is integrated with input from other signaling pathways. This provides the biological output after TCR recognition of a ligand.

CD3 phosphorylation is regulated by the local balance of the tyrosine kinase Lck, which is associated with the monomorphic coreceptors CD4 and CD8 and phosphatases in the TCR signaling complex. Functional key motifs within CD3ε, γ, δ, and ζ chains, designated immunoreceptor tyrosine-based activation motifs (ITAMs), are phosphorylated by Lck after TCR ligation, which is an early, prerequisite step for TCR-directed T cell activation. Importantly, conformational changes in CD3 molecules are directly induced by CD3 phosphorylation after pMHC–TCR ligation. These conformational changes in CD3ε and CD3ζ make ITAMs more accessible for phosphorylation and are required for efficient T cell activation.
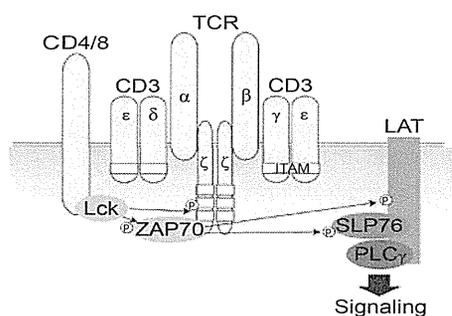
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Figure 1: TCR/CD3-mediated proximal signaling. After engagement of TCR/CD3 complex, conformation change and following phosphorylation cascade is induced. Activated PLCγ coordinates the signaling required for T cell activation.

Phosphorylated CD3ζ (and other ITAM-containing proteins) provides recruitment sites of the 70-kDa phosphoprotein ZAP-70, a Syk kinase family member. That is, TCR engagement by pMHC leads to the activation of Src family PTK, such as Lck, which results in ITAM phosphorylation and ZAP-70 recruitment (Figure 1). The tandem SH2 domains of ZAP-70 are engaged by the phosphorylated ITAMs of CD3ζ, where in turn ZAP-70 is activated by Lck-mediated phosphorylation.

Among the most important ZAP-70 targets are the transmembrane adapter protein linker for the activation of T cells (LAT) and the cytosolic adapter protein Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). Phosphorylated LAT in turn serves as a docking site to which a number of signaling proteins can bind. These two adapters form the backbone of a complex that includes several effector molecules to allow for the activation of multiple signaling pathways. Proteins that are incorporated in these assemblies include other scaffold molecules, such as Grb2, Gads, and enzymes like phospholipase C γ1 (PLCγ1), and phosphoinositide 3-kinase (PI3K). The coordination of these interactions and the ensuing signaling result in a stable, but dynamic, zone of contact between APCs and T cells; this zone has been designated the immunological synapse (IS). IS has become a paradigm for studying the signals exchanged between the two cell types.

A lipid raft is a microdomain within the plasma membrane that is rich in cholesterol, glycosphingolipids, and sphingomyelin. Lipid rafts accumulate at IS. Initial TCR activation and an early phosphorylation cascade precedes the formation of IS, which not only provides the sustained signaling required for gene regulation in T cells but may also control the eventual halt of the signaling pathways. It is important that the ability of a kinase to support TCR signaling critically depend on its lipid modification. Indeed, the accumulation of lipid rafts and Lck in these areas can accelerate increased phosphorylation. Within a lipid raft, the signaling backbone established by LAT and SLP-76 coordinates downstream signaling by controlling PLCγ1 activity. However, a large amount of data suggests that the formation of this complex is more complicated. For its optimal activity, PLCγ1 directly binds to SLP-76, LAT, and Vav1 as well as to its activating kinase Itk. Lck, which binds to CD4/8 and activates ZAP-70, also phosphorylates Itk. Itk in turn phosphorylates PLCγ1. Activated PLCγ1 then hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2), producing the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG), that are essential for T cell function (Figure 2).

TCR-mediated Gene Regulation via NFAT, NF-κB, and MAPK Cascades

TCR engagement results in the induction of expression of numerous genes required for T cell activation by triggering several signaling pathways. These pathways are described follows. Each of them acts specifically, but collaboratively, during T cell activation process.
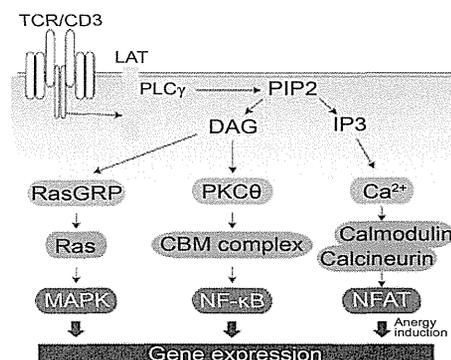
Ras–MAPK pathway

TCR-induced DAG production results in the activation of two major key molecules: Ras and PKCθ. DAG recruits both RasGRP and PKCθ to the plasma membrane. Activated Ras, a guanine nucleotide-binding protein, initiates the MAPK phosphorylation and activation cascade by activating of the serine/threonine kinase Raf1 [1]. Raf1 is a MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPK kinases (MAPKKs), including MEK. This in turn phosphorylates and activates MAPK extracellular signal-regulated kinase 1 (Erk1) and Erk2 (Figure 2) [3]. Erk kinase activity is regulated by its phosphorylation. The transcription factor Elk1 is one of downstream molecule of Erk1 and induces Fos expression. Consequently, the activation of the activator protein-1 (AP1) complex constituted by Jun and Fos is sustained by the DAG–Ras pathway. Jun is activated via the Vav1–Rac pathway. In addition, Erk activation results in phosphorylation of signal transducer and activator of transcription 3 (STAT3) and Lck.

PKCθ-dependent NF-κB and AP1 pathways

Increased cellular concentrations of DAG also activates NF-κB signaling through PKCθ, a PKC family member that contains a lipid-binding domain specific for DAG. This domain is required for recruiting PKCθ to the lipid raft after TCR engagement. Moreover, Lck-mediated PKCθ phosphorylation appears to contribute to conformational changes required for binding of PKCθ to the lipid membrane. This concentration of PKCθ within the lipid raft enhances binding of PKCθ to DAG and results in PKCθ activation.

NF-κB plays its most important and evolutionarily conserved role in the immune system by regulating genes involved in inflammatory and immune responses as well as in some aspects of cell growth, survival and differentiation [4]. Both canonical (classical) and noncanonical (alternative) activation of this pathway in T cells are intimately involved in TCR-mediated T cell activation. PKCθ-mediated NF-κB induction has been shown to be selectively mediated by IKKβ that is

Figure 2: TCR-mediated signaling cascades. Engagement of TCR consequently induces expression of numerous genes required for T cell activation by triggering several signaling pathways, including Ras–MAPK pathway, NF-κB pathway, and NFAT pathway. Each of them specifically but collaboratively acts during T cell activation or anergy induction processes.

associated only with the canonical pathway, whereas noncanonical activation in T cells remains elusive. One study showed that MAP3K14 (also called NF-κB-inducing kinase; NIK), which is an essential factor for the noncanonical NF-κB cascade, was required for complete T cell activation [5].

Following TCR stimulation, PKCθ regulates the assembly of a CBM complex (CARMA1/Bcl10/MALT1) through its phosphorylation of CARMA1, which is required for CARMA1 oligomerization and association with Bcl10 [6]. MALT1 physically binds to Bcl10 and induces polyubiquitination of IKKγ, the regulatory subunit of the IKK complex, via activation of the E3 ubiquitin ligase TRAF6. K63 polyubiquitination appears to have a role in IKKγ activation, which results in IκB phosphorylation by IKK catalytic subunits and subsequent IκB degradation. Nuclear localization of NF-κB heterodimers induces gene activation (Figure 2). NF-κB activation is also regulated by costimulatory signaling (described below).

The original analysis of PKCθ knockout mice revealed that two transcription factors, NF-κB and AP1, are targets of PKCθ in TCR/CD28-costimulated T cells [7-9]. The pathway leading from PKCθ to AP1 activation is less clearly understood. One study has reported that SPAK, a Ste20-related MAP3K, is a direct substrate of PKCθ in the pathway leading to AP1, but not NF-κB, activation [10]. SPAK is most likely a mediator of PKCθ signals leading to AP1 activation, but intermediates downstream of SPAK in AP1 activation remain to be identified and functionally characterized.

Ca²⁺-dependent NFAT pathway

PLCγ1-mediated IP3 generation stimulates Ca^{2+} permeable ion channel receptors (IP3R) located on the endoplasmic reticulum (ER). This leads to the release of ER Ca^{2+} stores into the cytoplasm. Because Ca^{2+} ions are universal second messengers in eukaryotic cells, TCR-induced increases in intracellular Ca^{2+} levels result in the activation of Ca^{2+}- and calmodulin-dependent transcription factors, including phosphatase calcineurin and the Ca^{2+}/calmodulin-dependent kinase (CaMK). Activated calcineurin then dephosphorylates members of the nuclear factor of activated T cells (NFAT) family, which results in their translocation to the nucleus. In the nucleus, NFATs form cooperative complexes with various other transcription factors, and this results in differential gene expression patterns and functional outcomes, depending on the context in which the TCR signal is delivered [11]. AP1 proteins are the main transcriptional partners of NFAT during T cell activation. Cooperation between NFAT and AP1 integrates two of the main signaling pathways i.e. the Ca^{2+} signaling and RAS-MAPK pathway. The NFAT-AP1 cooperation during T cell activation is responsible for a specific pattern of gene expression, which induces the functional changes that characterize an activated T cell. In the absence of AP1, NFAT proteins activate a distinct program of gene expression. The products of these genes inhibit T cell function at different levels and induce a status of T cell unresponsiveness, which is one of processes to induce the immune tolerance [12]. Thus, NFAT proteins control two opposing aspects of T cell function i.e. activation and anergy [13] (Figures 2 and 3).

Costimulation Signaling

A most important gene induced by TCR-mediated signaling is *IL-2*, which can activate STAT pathways. However, full induction of IL-2 expression is not achieved by TCR signaling alone, i.e., a nonresponsive state (anergy) exists in which T cells are refractory to restimulation. Additional engagement of other cell surface receptors provides and

integrates signals required for anergy avoidance and productive T cell activation. Although many cell surface receptors can participate in costimulation signaling, CD28 provides more robust signals than other costimulatory molecules.

Weak TCR ligation does not lead to cell proliferation and differentiation; rather, it results in anergy or cell death. However, if engaged, CD28 strongly amplifies a weak TCR signal. In contrast, triggering of CD28 alone results in the transient expression of only a few genes and has no obvious biological consequences. A question has arisen whether TCR and the coreceptors induce separate signaling pathways (qualitative model) or whether the signaling pathways triggered by both receptor systems are entirely overlapping (quantitative model) [14].

Many studies have indicated that the signals generated by CD28 ligation have no unique effects. A microarray study showed that TCR-induced expression of thousands of genes in primary T cells was amplified (or suppressed) to varying degrees by CD28 costimulation, but that no new gene was induced by CD28 costimulation [15]. Indeed, all of the factors identified as components of the CD28 signaling pathway are those that are implicated in TCR-mediated signaling. These include PI3K, the Tec family of PTKs, such as Itk, Vav1, and the serine/threonine kinase Akt (also known as PKB), and NF-κB signaling.

CD28-mediated PI3K–Akt pathway

PI3K recruitment is a key event for coupling CD28 to several signaling pathways. Following engagement of CD28 with its ligands CD80 or CD86 expressed on APCs, the p85 regulatory subunit of PI3K associates with the phosphorylated cytoplasmic tail of CD28. This regulatory subunit recruits the p110 catalytic subunit of PI3K, which can convert PIP2 to PIP3 in the cell membrane. Then, the locally generated PIP3 serves as a docking site for the PH domains of PDK1 and its target Akt (Figure 3).

Akt phosphorylates multiple proteins involved in numerous cellular responses. Activated Akt enhances the nuclear translocation of NF-κB by associating with CARMA1 and facilitating the assembly of the CBM complex, a step critical for NF-κB activation [16]. In addition, one well-known Akt target is GSK-3, a serine/threonine kinase that influences the nuclear export of NFAT as well as the Wnt pathway. Thus, Akt-mediated GSK-3 inactivation might be a pathway responsible for prolonged NFAT nuclear localization and thus *IL-2* transcription following CD28 costimulation (Figure 3).
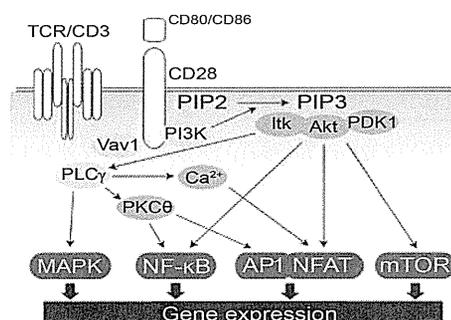
Figure 3: Costimulation signaling. Additional engagement of cell surface receptors such as CD28 provides and integrates signals required for anergy avoidance and productive T cell activation. The signaling generated by CD28 ligation has no unique effects but enhances the T cell signaling, including pathways of PI3K–Akt–mTOR, NF-κB, NFAT-AP1, and MAPK.

CD28/PI3K-generated PIP3 also provides a docking site for the PH domain of Itk. As described above, Itk associates with the TCR downstream complex consisting of LAT/SLP-76/Gads/PLCγ1 (Figure 3). Importantly, the localization and activation of this signaling complex depends on PI3K-generated PIP3.

A novel signaling mechanism has been suggested where by CD28 regulates cAMP degradation in T cell rafts through the recruitment of an Akt/β-arrestin/PDE4 complex [17]. CD28/PI3K-mediated PIP3 production recruits this supramolecular complex to lipid rafts, with recruitment occurring through the Akt PH domain.

Thus, although many of these pathways are activated by TCR ligation alone, the magnitude and maintenance of T cell activation signaling required for an appropriate response appear to be considerably regulated by costimulatory signals. This suggests that CD28 engagement primarily functions in a quantitative rather than a qualitative change in T cell activation.

CD28-mediated NF-κB pathway

Another important CD28 mediator is NF-κB signaling. CD28/PI3K generates the second messenger PIP3 that binds to various proteins harboring PH domains, including PDK1 and Akt. At present, we know that Akt is not an essential component of NF-κB signaling, at least in T cells. Akt appears to function as a rheostat; Akt can gently modulate the strength of the NF-κB cascade through its interaction with CARMA1 (Figure 3) [16]. Of note, PDK1, which is recruited to newly generated PIP3, can efficiently bind to both PKCθ and CARMA1 [18]. CD28 facilitates NF-κB activation by regulating the recruitment and phosphorylation of PDK1, which are necessary for the efficient binding of PDK1 to PKCθ and CARMA1 and thus for NF-κB induction. Furthermore, TCR/CD28 confers the noncanonical NF-κB activation in naïve T cells [19]. Taken together, NF-κB signaling is one of the major signaling pathways regulated by costimulation. Full activation of the NF-κB pathway requires CD28-mediated costimulatory signals in T cells.

CD28–Vav1 pathway

Vav1 is a guanosine exchange factor (GEF) for several small GTPases, including RAC1, RAC2, and RHOG. Vav1 controls several biochemical processes, such as those involved in cytoskeletal rearrangements. In addition, Vav1 strongly amplifies CD28-mediated costimulation-dependent activation of NFAT, NF-κB, CD28 response element, and JNK [20,21].

T cell development is markedly reduced in Vav1-deficient mice [22]. Of note, a CD28 mutant, which is unable to activate Vav1, does not alter TCR-directed ZAP-70 and LAT phosphorylation, but it does affect SLP-76, PLCγ1, and Itk phosphorylation and Akt activation [23]. This phenotype, which is remarkably similar to that of Vav1-deficient T cells [24], supports a role for Vav1 as a crucial signaling effector of CD28 costimulation.

TCR/CD28 and MAPK pathways

TCR engagement activates the ERK, JNK, and p38 cascades in T cells. With regard to costimulation effects, one study demonstrated that T cell activation involves these three MAPK cascades [25]. pMHC ligation directly activates the Ras–MEK–ERK pathway, as described above. JNK activation stimulated by TCR engagement requires CD28 coligation in T cell clones. However, JNK activation is not observed in mouse primary T cells. TCR and CD28 synergize after coligation to elicit enhanced p38 MAPK activation. p38 MAPK, but not JNK, is

involved in signal integration during costimulation of naive mouse primary T cells. Indeed, the p38 MAPK inhibitor SB203580 blocks CD28-dependent proliferation and IL-2 production in human T cells [26]. We currently do not know how p38 modulates TCR signaling and IL-2 production.

Studies using both pharmacological and genetic manipulations have provided evidence that p38 is a crucial mediator of T cell development, activation, and inflammation [27]. In addition, p38α function in T and B cells has been addressed using the RAG-deficient blastocyst complementation method. Surprisingly, p38α-deficient T and B cells developed in normal numbers and proliferated normally in response to various stimuli, such as antigen–receptor ligation [28]. Moreover, mice that lack p38β appear to be completely normal with no obvious defects in T cell development or LPS-induced cytokine production [29]. To reconcile the genetic and pharmacological data with the paucity of observable abnormalities in p38 isoform-specific knockouts, further characterizations and detailed analyses of the p38 pathway in both normal T cells and T cell malignancies will be necessary.

Signaling Pathways Linking to T cell Activation, Development, and Disorder

In addition to TCR/CD28 engagements, several other signaling inputs participate in the regulation of T cell fates. These comprise pathways responsible for the development and differentiation of several cell types. Of note, deregulation of these signaling pathways lead to T cell disorders.

NF-κB signaling

In addition to TCR engagement-mediated NF-κB activation, pathological studies have strongly demonstrated that NF-κB activation is often sustained in T cell disorders. ATL cells show strong, constitutive NF-κB activation that contributes to their prolonged survival, proliferation, and invasiveness [30]. In particular, human T cell leukemia virus type 1 (HTLV-1) Tax is an intracellular stimulator of IKK. This stimulation is based on the physical interaction between Tax and IKK, which leads to the persistent activation of NF-κB-mediated transcription. Formation of the Tax/IKK complex relies on the physical interaction between Tax and the IKK regulatory subunit IKKγ. The Tax–IKKγ interaction is required for recruiting Tax to IKK catalytic subunits and for Tax-mediated IKK activation (Figure 4) [31].

In leukemic cells in which Tax is not expressed, a noncanonical NF-κB cascade appears to be important for the cellular characteristics of ATL. Both the canonical and noncanonical NF-κB pathways are persistently activated because NIK is aberrantly expressed in ATL cells [32]. NIK plays a central role in noncanonical NF-κB signaling through IKKα phosphorylation [33], and its constitutive expression leads to aberrant NF-κB activation in various malignancies, including B cell lymphoma, multiple myeloma, breast cancer, pancreatic cancer, and ATL [30].

Recently, we identified a novel molecular link between NF-κB activation and miRNA deregulation. Comprehensive gene expression analysis and in vitro experiments showed that miRNA-31 (miR-31) could regulate NIK expression through the 3′ untranslated region (UTR) in several cell types. In ATL cells, miR-31 expression was genetically and epigenetically silenced, which in turn induced constitutive NF-κB activation through NIK expression (Figure 4) [34]. Because current evidence clearly indicates that miR-31 dominates NF-κB activity in T cells, manipulating cellular miR-31 levels may be a novel molecular approach to reduce NF-κB activity and induce cellular apoptosis.
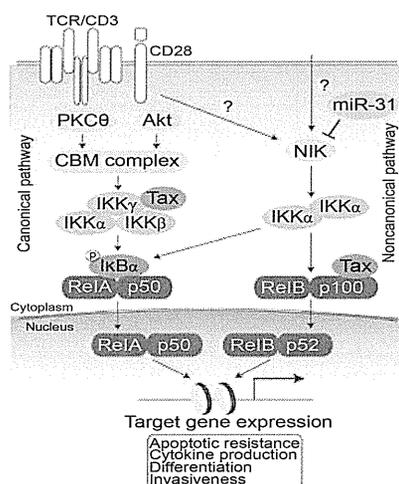
Figure 4: NF-κB signaling in T cell. Canonical and noncanonical pathways contribute to gene expression required for apoptotic resistance, inflammatory cytokine production, appropriate differentiation, and invasiveness. In HTLV-1 infected cells, viral protein Tax binds and drastically activates both pathways. In transformed leukemic cells, loss of miR-31 leads to NIK accumulation and persistent NF-κB activation.

Why is NF-κB signaling important for ATL cell survival? One of the target genes is Bcl-xl, which is expressed when NF-κB is activated. In HTLV-1-infected cell lines, Bcl-xlis expressed through the Tax–NF-κB pathway. Interestingly, fresh ATL samples exhibit Bcl-xl overexpression. Given that Bcl-xl is a principal anti-apoptotic protein as a Bcl-2 family member, persistent Bcl-xl expression is one of the molecular means to resist apoptosis, which may contribute to clinical chemoresistance. Indeed, inhibition of the NF-κB pathway by NIK depletion leads to impaired Bcl-xl expression and apoptotic death of ATL cells [34].

Because prevention of NF-κB activation showed good results in a xenograft model of cell lines with and without Tax [30], molecular targeting therapy based on the NF-κB pathway is a promising new treatment for ATL. Of note, we found that specific inhibition of NF-κB by DHMEQ could also remove virus-carrying cells from carrier peripheral blood mononuclear cell (PBMC) samples [35].

mTOR pathway

The signaling pathway target of rapamycin (TOR) is at the intersection between cell growth and starvation. The evolutionarily conserved kinase mammalian TOR (mTOR; officially known as the mechanistic target of rapamycin) controls cell growth and metabolism-related response to environmental inputs by regulating gene expression, which has been implicated in disease states, such as cancer, metabolic diseases and ageing. In T cell regulation, mTOR signaling is also involved in immune signals and metabolic cues for the proper maintenance and activation of T cells. Under resting conditions, mTOR signaling is strictly controlled by multiple inhibitory mechanisms, which enforces normal T cell homeostasis. T cell activation through antigen recognition triggers mTOR activation, which in turn influences the differentiation patterns of these cells.

mTOR, which belongs to the family of PI3K-related protein kinases (PIKK), is a conserved serine/threonine kinase and plays a central role in the regulation of cell growth and metabolism [36]. Diverse environmental signals, such as nutrients and growth factors, deliver their signaling inputs to the PI3K–Akt axis. Many upstream signals

activate the mTOR complex 1 (mTORC1) via the GTP-loaded small GTPase RHEB. The activated PI3K–Akt pathway phosphorylates the Thr1462 residue of tuberous sclerosis 2 (TSC2), which in turn inhibits the TSC1/2 complex. Because RHEB is tightly regulated by the TSC1/2 complex, TCR/CD28 engagement activates RHEB/mTORC1 via the PI3K–Akt pathway. Recent studies have shown that RHEB deficiency in T cells reduced mTORC1 activation in response to TCR stimulation [37]. In addition, loss of TSC1 disrupts the TSC1/2 complex and enhances basal and TCR-induced mTORC1 activity [38,39]. Therefore, these results suggest a crucial role for the TSC–RHEB axis in T cell responses.

TCR engagement can activate both mTORC1 and mTORC2 within minutes. The strength of mTOR activation appears to be directly correlated with the duration of T cell–APC interaction and the cognate antigen dose. It should also be noted that mTOR activity is further conferred by costimulatory signals. CD28-mediated costimulation is a major driving force for the PI3K–AKT axis (described above), which in turn upregulates TCR-induced mTOR activity to facilitate productive T cell activation (Figure 5) [40,41]. In addition to CD28, another costimulatory receptor, OX40, which is a member of the tumor necrosis factor receptor (TNFR) family, assembles a signaling complex by recruiting PI3K–AKT to augment TCR-dependent Akt signaling; this may also affect mTOR signaling in T cells [42].

Several genetic studies have strongly suggested that the mTOR pathway is closely associated with T cell differentiation and homeostasis [43]. In particular, CD8⁺ T cell differentiation is strictly regulated by mTOR signaling [44]. In addition, mTOR signaling appears to be involved in T helper (Th) cell differentiation and proliferation [43]. In sum, mTOR dictates the cell fate decisions of effector and regulatory T cells.

A common hematological malignancy, T-ALL, frequently harbors activating mutations in *NOTCH1* and/or loss-of-function mutations in a gene encoding phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN plays critical roles in cell growth, migration, and death because it can inhibit the PI3K–Akt pathway by catalyzing PIP3 dephosphorylation. It is noteworthy that both of these mutations can activate mTOR signaling, which suggests a pivotal role for mTOR in T-ALL development. Taken together, mTOR-mediated T cell fate decisions are of particular importance because of the unique functions of the mTOR pathway. Recent experimental advances have established that mTOR is a fundamental determinant of T cell homeostatic and functional fates. In addition, therapeutic targeting of mTOR may have beneficial effects for treating T cell disorders.
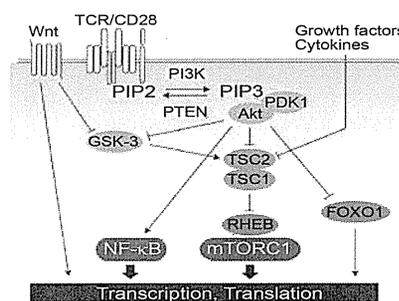
Figure 5: PI3K–Akt–mTOR axis. After TCR/CD28 stimulation, activated PI3K enhances phosphorylation of PDK1 and Akt. The Phosphorylated Akt in turn inhibits tumor suppressor TSC2, resulting in activation of mTORC1. In addition, Akt supports NF-κB signaling through CBM complex.

Notch pathway

Notch proteins are cell-surface receptors and their expression is widely conserved in numerous species. It has been shown that Notch signaling is essential for T cell lineage development, thymocyte survival, and proliferation of T cell progenitors [45]. In addition, Notch signaling has been extensively studied in T-ALL because several lines of genetic evidence highlight the Notch signaling cascade as a central player in T-ALL pathogenesis [46].

Notch proteins are synthesized as a single polypeptide (approximately 300 kDa). After intracellular modifications that include fucosylation, glysocylation, and cleavage, a Notch heterodimer associates with the plasma membrane. Signaling is triggered by the interaction of Notch with Delta-like ligands (DLLs) or Jagged ligands on the surface of instructing cells. This ligation induces proteolytic cleavage of the Notch receptor by disintegrin and metalloproteinase (ADAM) and mono-ubiquitination of the intracellular portion of the transmembrane Notch fragment. This final cleavage leads to the release of the Notch intracellular domain from the plasma membrane and its translocation to the nucleus.

CBF1/suppressor of hairless/Lag1 (CSL) represses transcription of Notch target genes. Following activation by Notch, CSL is converted into a transcriptional activator and activates the transcription of the same genes. Intracellular Notch displaces the corepressors from CSL and recruits coactivators, such as Mastermind-like 1 (MAML1), to activate the expression of target genes (Figure 6). Intracellular Notch is then degraded by a polyubiquitination–proteasome pathway.

A landmark study published in 2004 changed our perspectives dramatically by showing that more than 50% of T-ALL cases harbored Notch1 mutations, which resulted in the hyperactivation of the Notch pathway. This finding implicated Notch1 has a very important role in the pathogenesis of T-ALL [47]. This suggests that Notch likely interacts with several important cellular pathways and can cooperate with other oncogenes during leukemogenesis (discussed above).

Although the importance of Notch signaling activation in T-ALL is well established, the detailed molecular mechanisms by which NOTCH1 induces T cell transformation remain unclear. The best-characterized direct target genes are the bHLH transcriptional repressor Hes1 and the transcription factor c-Myc. Recently, Hes1 was shown to be a key regulator in the induction and maintenance of T-ALL. Notch-mediated c-Myc expression is also crucial for maintaining T-ALL.

Recently, activating mutations in Notch were identified in more than 30% of ATL patients [48]. Of note, compared with the activating frameshift mutations observed in T-ALL, ATL showed single substitution mutations activating the Notch pathway. These gene mutations could reduce CDC4/Fbw7-mediated degradation, which resulted in stabilization of the intracellular NOTCH1. They also resulted in Hes1 expression in ATL patient samples. In addition, γ-secretase inhibitors reduced tumor cell proliferation and tumor formation in ATL-engrafted mice. Collectively, these findings suggest that activated Notch may be important for ATL leukemogenesis.

There is mounting evidence that Notch signaling is also an important pathway for mediating cell fate decisions in developing thymocytes and peripheral T cells. Strong Notch signaling restricts the multilymphoid progenitors of the T cell lineage and promotes thymocyte development [49]. Results from several studies support a role for Notch in the generation of single-positive cells from double-positive precursor thymocytes [50].

Notch proteins are now emerging as potentiators of TCR/CD28 signaling in mature T cells. Some studies have confirmed the roles for Notch receptors in T cell activation, proliferation, and cytokine production. Pharmacological inhibition of the Notch pathway in CD4$^+$ and CD8$^+$ T cells significantly inhibited their proliferation and IFNγ production after TCR stimulation [51]. In addition, Notch signaling was shown to be involved in a positive-feedback loop of IL2–IL2R that regulated T cell proliferation [52]. Important pathways activated through Notch1 signaling include the PI3K–Akt and mTOR signaling cascades. In addition, NF-κB signaling, which can be activated through TCR/CD28 signaling, appears to be sustained by the Notch pathway. These details are discussed in a later chapter.

Wnt pathway

Wnt signaling is subject to strict molecular control. Thus, dysregulated Wnt signaling has been implicated in the development of several malignancies, including the leukemias and lymphomas [53]. To date, at least three different Wnt pathways have been identified: the canonical Wnt pathway, which involves β-catenin and the T cell factor (TCF)/lymphocyte-enhancer-binding factor (LEF) family; the planar cell polarity pathway; and the Wnt/Ca^{2+} pathway. Among these, the canonical (Wnt/β-catenin) pathway is primarily involved in T cell development and proliferation [54].

One important study demonstrated that a T cell-specific defect in Wnt signaling by deletion of β-catenin impaired T cell development at the β-selection checkpoint, which resulted in a decrease in splenic T cells. In addition, β-catenin appeared to be a target of TCR signals in thymocytes and mature T cells. These results indicate that β-catenin-mediated signals are required for normal T cell development [55].

TCF1 is highly expressed in T cells. A recent study showed that β-catenin expression was stabilized after TCR-mediated T cell stimulation, resulting in the upregulation of TCF1-dependent gene expression in T cells [56]. It also suggested that TCF-1 initiated Th2 differentiation of activated CD4$^+$ T cells by promoting GATA-3 expression and suppressing IFN-γ expression. In addition, during T cell development, TCR signaling was shown to stabilize β-catenin and regulate the response to αβTCR engagement [57]. In sum, several genetic studies have suggested that the canonical Wnt pathway participates in T cell development. In CD8$^+$ T cells, activation of Wnt/
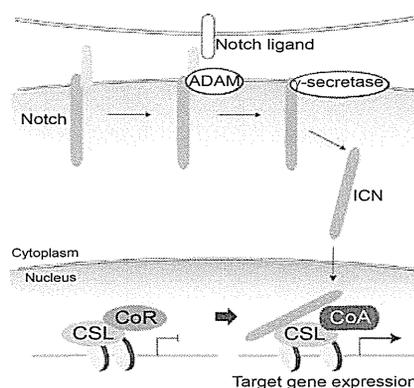
Figure 6: Notch signaling. After intracellular modifications, the Notch heterodimer associates with the plasma membrane. Signaling is triggered by the interaction with Notch ligands, which induces proteolytic cleavage of the Notch and leads to the nuclear translocation of the Notch intracellular domain (ICN). The intracellular Notch displaces the co-repressors (CoR) from CSL and recruits co-activators (CoA) to activate the expression of target genes.

β-catenin signaling inhibited effector differentiation and promoted the generation of self-renewing CD8+ memory stem cells and central memory T cells. In CD4+ T cells, Wnt/β-catenin signaling facilitated Th2 polarization and enhanced the survival of naturally occurring regulatory T cells (nTreg). Canonical Wnt signaling has also been suggested as important for the self-renewal of hematopoietic stem cells (HSCs) and progenitor compartments. β-catenin deletion perturbs HSC survival, self-renewal, and their subsequent development into T cells [58,59].

Because the Wnt pathway is important for normal T cell development and signaling pathways, constitutively active Wnt signals can lead to T cell acute malignancies. An experimental model of Wnt activation demonstrated that the constitutively active form of β-catenin led to development of an aggressive T cell lymphoma that could invade the bone marrow and was transplantable into irradiated recipient mice [60]. These data suggest that Wnt-dependent c-Myc expression is required for lymphomagenesis. Interestingly, this appears to be independent of Notch signaling that has been thought to be an analog of the Wnt pathway. Crosstalk between these pathways will be discussed in a later chapter.

Interestingly, modest transgenic expression of β-catenin in thymocytes enhances the generation of CD8+ thymocytes and accelerates thymic involution [61]. Some evidence indicated that oncogenic β-catenin induced p53-independent oncogene-induced senescence and p53-dependent apoptosis, which protected developing thymocytes from transformation [62]. The molecular mechanisms induced by the Wnt pathway in T cells remain to be elucidated.

Hedgehog (Hh) pathway

The Hh family of secreted intercellular molecules, including Sonic, Indian, and Desert Hh, regulates target gene transcription through GLI transcription factors. This family is of pivotal importance in embryogenesis and the homeostasis of adult tissues, as well as cell differentiation, cell cycle progression, and cell survival [63].

Hh signaling is important in T cell development in both humans and mice [64].The generation of mature functional T cells in the thymus requires sonic Hh (Shh) signals from the thymic epithelium. Shh-induced signaling is important for the differentiation and proliferation of early thymocyte progenitors. Shh also modulates TCR signaling during repertoire selection.

Hh signaling is involved in the conversion of immature DP cells to mature SP thymocytes and can affect TCR repertoire selection and CD4/8 lineage choices. Depletion of Hh signaling promotes CD4 lineage commitment and results in an increased CD4:CD8 ratio. In contrast, increased Hh signaling results in a reduced CD4:CD8 ratio, suggesting that Hh signaling is an inducer of CD8+ T cell development [64,65].

Hh is also involved in peripheral T cell activation. Additional Shh supports TCR/CD28 signaling and enhances T cell activation and subsequent proliferation [66-68]. In contrast, Hh activation by the expression of the constitutive active form of Gli2 results in inhibition of TCR/CD28-mediated T cell activation. In this case, Hh appears to inhibit TCR-dependent Erk phosphorylation [65]. Modulation of TCR signal strength by Hh has profound implications for immunity. Thus, the precise molecular involvement of Hh in T cell signaling needs to be examined.

Hh signaling is implicated in several hematological malignancies,

including chronic lymphocytic leukemia (CLL), plasma cell myeloma, mantle cell lymphoma, diffuse large B-cell lymphoma (DLBCL), ALK-positive anaplastic large cell lymphoma (ALCL), chronic myelogenous leukemia (CML), and acute leukemias (AML). ALK+ ALCL is an aggressive type of non-Hodgkin's lymphoma of the T cell/null lineage. One study demonstrated that Hh ligands and GLI1 were highly expressed in ALK+ ALCL [69]. NPM-ALK activates the PI3K–Akt pathway, which contributes to GSK-3β inactivation. Because GLI1 and GLI2 are the targets of GSK-3-mediated proteasomal degradation, NPM-ALK activates Hh signaling through the PI3K–Akt–GSK-3 cascade. Of note, we have demonstrated that the NPM-ALK abrogates CD30 signaling and constitutive NF-κB activation in ALCL cells [70]. The reciprocal relationship between Hh and NF-κB signaling in T cells remains unclear.

TGF-β-mediated signaling

The TGF-β superfamily regulates many cellular functions, including cell growth, differentiation, migration, and apoptosis. TGF-β-mediated signaling is essential for embryonic development, embryogenesis, and cell fate decisions [71].

TGF-β is synthesized in an inactive form consisting of a TGF-β dimer in association with the latency-associated protein (LAP). Signaling is initiated by a conformational change in a tetrameric complex consisting of TGF-β receptor II (TGF-βRII), TGF-β receptor I (TGF-βRI), and activated TGF-β dimer. Activated TGF-βRI then phosphorylates the transcription factors Smad2 and Smad3 and triggers their translocation into the nucleus. Smads then bind to the specific regulatory sequences of target genes and regulate gene expression by recruiting transcription cofactors.

TGF-β regulates T cell development, homeostasis, tolerance, and immunity [72]. TGF-β signaling promotes the differentiation of thymic T cells into natural killer T cells, nTreg cells, and CD8+ T cells. TGF-β signaling is likely to be pivotal for the survival of peripheral CD4+ and CD8+ T cells with low affinity. In contrast, it inhibits the proliferation and differentiation of CD4+ and CD8+ T cells with high affinity.

In particular, TGF-β plays central roles in Treg cell regulation. It inhibits proliferation of Treg cells, although it supports their maintenance in peripheral lymphoid organs, which can control Treg cell survival. TGF-β promotes the differentiation of induced Treg (iTreg) cells, which is potentiated by IL-2 and retinoic acid (RA). In the presence of IL-6, TGF-β drives the differentiation of Th17 cells and supports their maintenance.

The interplay between TCR and TGF-β signaling has been implicated in the regulation of several genes, including FoxP3, which is important for Treg functions [73]. However, because most studies have mainly performed phenotypic analyses, the exact molecular signaling pathway induced by TGF-β in T cells is not completely understood.

Signalings from receptors of innate immune cells

Innate and adaptive immune responses have many interactions that are regulated by the balance of signals initiated by several activatory and inhibitory receptors. Toll like receptor (TLR) expression has been generally assigned to professional APCs, such as dendritic cells. However, several recent studies have demonstrated that the low expression levels of TLRs could also be detected on naïve and memory T cells [74,75]. In addition, TLR agonists can directly target TLRs expressed on T cells and function as signal inducers.

Engagement of the TLR1/2 complex was associated with T cell

proliferation, survival, and functions [76]. Ligation of TLR3 on effector CD8+ T cells increased IFN-γ secretion by these cells. Interestingly, the effects of CpG (TLR9 ligand) on CD4+ T cells were mediated by MyD88 and PI3K-dependent mechanisms [77]. Another study showed that ligands for TLR3 and TLR9 directly enhanced T cell survival, which was sustained by NF-κB signaling [78]. Collectively, these findings suggest that TLR agonists can directly target TLR signaling in effector CD4+ and CD8+ T cells. We do not understand the molecular mechanisms by which TLR signaling can affect the other sustained T cell signaling.

NKG2 receptors are expressed by natural killer (NK) cells and a subset of CD8+ T cells. They regulate the development and function of NK cells by inhibiting and activating cytotoxicity and promoting cell survival. The CD94/NKG2 heterodimers consist of one CD94 molecule and one NKG2 family member, including NKG2A and its splice variant NKG2B, which form inhibitory receptors, as well as NKG2C, NKG2E, and NKGH, which form activating receptors [79]. NKG2D is unique in that it does not dimerize with CD94; instead, it associates with the adaptor molecules DAP10 and DAP12 for its activating function [80]. Among these, the acquisition of CD94/NKG2A expression by CD8+ T cells could play a role during infection by pathogens. This has been observed in mice infected with a variety of pathogens, including lymphocytic choriomeningitis virus, herpes simplex virus (HSV)-1, influenza virus, polyomavirus, and *Listeria monocytogenes* [81]. Furthermore, HIV-1 infection has also been implicated in the upregulation of CD94/NKG2A [82,83]. The expression of CD94/ NKG2A, which is acquired early during the infection and persists after clearance of the pathogen, does not interfere with the activity of CD8+ T cells. However, the CD94/NKG2A expression inhibits antiviral CD8+ T cell responses [84]. In addition, high level of CD94/NKG2A expression on CD8+ T cells protects them from apoptosis, thereby perhaps inducing in the generation and maintenance of memory cells.

Emerging Regulatory Factors: Learning from Malignancies

Recent experimental evidence indicates the presence of additional molecular hallmarks of T cells. miRNAs regulate gene expression patterns in all cell types. Epigenetic gene regulation is based on chromatin regulation. Both of these are pivotal for development, differentiation, proliferation, and other cellular processes in hematopoietic lineage. Their deregulation is frequently observed in T cell disorders, which strengthens the fact that miRNA-mediated gene regulation and epigenetic mechanisms may be important for maintaining T cell homeostasis.

miRNA in T cells

One of the most significant recent advances in biomedical research has been the discovery of the 22-nt-long class of non-coding RNA designated miRNA that post-transcriptionally regulates gene expression by binding to the target mRNAs. miRNA is expressed by all metazoans and plants, as well as by several DNA viruses; it regulates cellular processes such as development, differentiation, growth, homeostasis, stress responses, apoptosis, and immune activation [85].

Some recent extreme studies have collectively suggested several concepts. First, miRNAs have unique roles as time-sensitive gene regulators. Second, miRNAs have distinct functions in distinct cell types. Third, miRNAs regulate gene expression by incompletely repressing their targets. These specific functions of miRNAs as "fine-tuners" are apparently involved in immune systems as well as

hematological cancers. It is not surprising that miRNA deregulation appears to play important roles in human cancers.

miRNAs provide modest changes in gene expression [86]. However, it is also true that gene expression patterns can occasionally be changed drastically by a change in a single miRNA that regulates a key transcription factor. For example, a decrease in a miRNA, miR-150, leads to drastic deregulation of the B cell transcriptome by regulating c-MYB [87]. Furthermore, given that miRNAs are upstream of gene regulation systems, miRNAs confer robustness to several biological processes by controlling several feed-forward and feed-back loops [86]. Several recent reports including our own have demonstrated crosstalk between miRNA-mediated gene regulation and several signal transduction pathways in T cells.

Specific knockout of *Dicer*, which is essential for processing miRNAs and siRNAs in the T cell lineage, leads to impaired T cell development and aberrant Th cell differentiation and cytokine production [88,89]. In addition, peripheral CD8+ T cell development was severely blocked after *Dicer1* deletion in the thymus. These cells were defective in miRNA processing, and after stimulation, they proliferated poorly, which resulted in their apoptosis. Dicer-deficient Th cells preferentially expressed INFγ. The levels of secreted IL-2 in *Dicer*-deficient and control cultures were comparable after primary stimulation, and addition of exogenous IL-2 to the growth media did not rescue the proliferative defect of *Dicer*-deficient T cells, which suggested that Dicer might be involved in IL-2 receptor (CD25) signaling in T cells [89].

An early study revealed that miR-181a acted as an intrinsic antigen sensitivity "rheostat" during T cell development [90]. miR-181a expression enhanced the basal activation of TCR signaling and IL-2 production because miR-181a repressed multiple negative regulators of TCR signaling (Figure 7). Thus, several miRNAs probably participate in each step of T cell regulation. This in turn suggests that miRNA deregulation may cause catastrophic gene regulation in T cells.

Individual miRNAs have been implicated in T-ALL. miR-19b is
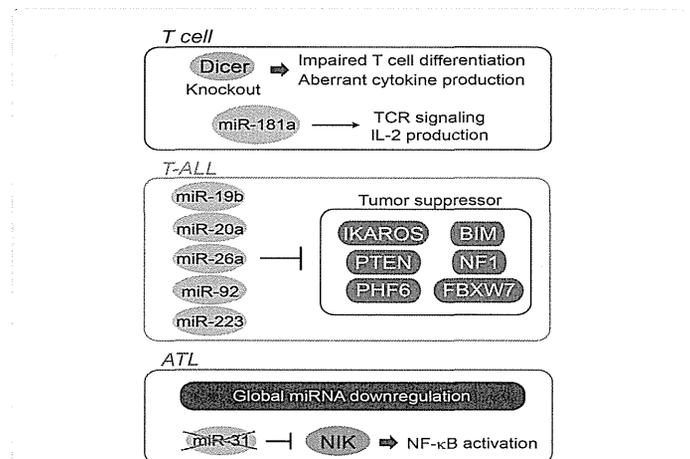
Figure 7: miRNA in T cell biology. Specific knockout of *Dicer* in T cell lineage leads to impaired T cell development and aberrant T helper cell differentiation and cytokine production. miR-181a expression enhances the basal activation of TCR signaling and IL-2 production (top box). In T-ALL cells, upregulation of five miRNA leads to repression of tumor suppressor genes implicated in T-ALL (middle box). Global downregulation of miRNA is the one of molecular hallmarks of ATL. In particular, miR-31 expression is genetically and epigenetically silenced, which is prerequisite condition of ATL cells. The loss of miR-31 consequently contributes to NIK over expression.

a member of the oncogenic miR-17-92 cluster, which is targeted by the t(13;14)(q32;q11) translocation in T-ALL [91]. Comprehensive methods have been used to address the miRNA candidates associated with abnormal gene expression and leukemogenesis. Recently, one study reported on miRNA signature in T-ALL [92]. A total of 430 types of miRNAs were analyzed in 50 clinical T-ALL and 18 T-ALL cell lines by quantitative RT-PCR, revealing that a small set of miRNAs was responsible for the cooperative suppression of several tumor suppressor genes. Cross comparisons of miRNA expression profiles in human T-ALL with the results of an unbiased miRNA library screen identified five miRNAs (miR-19b, miR-20a, miR-26a, miR-92, and miR-223), which were capable of promoting T-ALL development in a mouse model. Furthermore, these miRNAs produced overlapping and cooperative effects on tumor suppressor genes implicated in T-ALL, including *IKAROS, PTEN, BIM, PHF6, NF1*, and *FBXW7*. Thus, a comprehensive and unbiased analysis of miRNA actions in T-ALL reveals a striking pattern of miRNA–tumor suppressor gene interactions (Figure 7).

For ATL, some studies have reported several miRNA aberrations identified in HTLV-1-infected cells and ATL samples [93-95]. Very recently, we established global gene expression analysis in a large cohort ATL study that included analyses of mRNA expression, miRNA levels, and genomic copy numbers [34]. A strict threshold ($p < 1 \times 10^{-5}$) and two-dimensional hierarchical clustering analysis revealed 61 miRNAs with significantly altered expression levels in ATL cells (n = 40) compared with control CD4$^+$ T cells (n = 22). It is most important that primary ATL samples show global miRNA downregulation, similar to observations in other cancer researches [96,97]. Of the 61 miRNAs, 59 (96.7%) exhibited decreased expression in ATL (Figure 7).

Among these, miR-31 was the most profoundly repressed miRNA in all ATL individuals (fold change of 0.00403). This is a known tumor suppressor that may also be associated with metastatic breast cancer [98]. Other downregulated miRNAs found in ATL patients may also be involved in the hallmark capabilities of ATL because they were uniformly decreased in tested ATL samples and each miRNA may regulate a large number of genes.

Several predictions and experimental approaches have defined a novel miR-31 target gene, NIK, which acts as a persistent NF-κB activator in various malignancies (described above). Manipulation of the miR-31 level clearly indicated that the miR-31 level was negatively correlated with cellular NF-κB activity. Importantly, enforced miR-31 expression in B cells attenuated both BAFF- and CD40L-mediated NIK accumulation and subsequent canonical and noncanonical NF-κB signaling. Induced miR-31 expression or NIK knockdown reduces apoptotic resistant proteins such as BCL-XL and XIAP, which results in significant apoptosis among ATL cell lines as well as among primary leukemic cells from ATL patients [34]. Several lines of evidence definitively support two notions: (1) miR-31 acts as a tumor suppressor in T cells and (2) NIK-regulated NF-κB is of pivotal importance to cancer cell survival (Figure 7).Taken together, based on the results of genetic studies and those on T-ALL and ATL, abnormal miRNA expression has a critical impact on the development of hematological malignancies.

Epigenetic regulation

Technological advances in genomics and epigenomics have provided new methods to distinguish one cell type from another. The epigenetic code consists of the combined on–off states of hundreds of genes, which coordinately dictate cellular identity and function.

Increasing attention is being paid to global regulatory factors and molecular mechanisms by which gene transcription control is regulated. Within these environments, genome programming operates fundamentally through DNA methylation, histone chemical modification, and protein complex binding.

The appropriate control of gene expression must be maintained in normal T cells by utilizing transcriptional networks as well as epigenetic controls. Indeed, several specific, comprehensive studies have demonstrated the epigenetic regulation of genes encoding for cytokines that are required for T cell development, differentiation, and activation [99]. Collectively, several global analyses have proposed that naïve T cell activation, differentiation, and lineage commitment result in epigenetic changes and that a fine balance between different histone modifications is required. In contrast, memory T cells are already poised and do not require epigenetic changes for short-term activation. It has been suggested that transcriptional memory, particularly epigenetic marks on chromatin, forms the underpinning of immunological memory [100-102].

Integrated histone modifications consequently decide the degrees of chromatin condensation and subsequent transcriptional sensitivity. Trimethylation of the histone H3 Lys27 (H3K27) mark plays a central role in repressing transcription, primarily in the euchromatin region. The polycomb family is a master regulator of the H3K27me3 level by inducing and maintaining the histone mark. Polycomb repressive complex 1 (PRC1) and PRC2 are recognized as essential molecular machines involved in polycomb-mediated gene silencing [103].

The polycomb family participates in the control of T cell activation. Enhancer of zeste homolog 2 (EZH2) serves as the catalytic subunit in PRC2 and mediates gene silencing by catalyzing H3K27 trimethylation in the promoters of target genes. One study showed that triggering of TCR and costimulation signaling in *Ezh2*-deficicint T cells resulted in an impaired proliferative response [104]. The mechanism by which Ezh2 can affect T cell signaling is not completely understood.

Deregulation by the polycomb family confers a specific gene expression pattern that is responsible for chronic proliferation, survival, peculiar development, and cancer-associated stemness in various cancer types, including ATL [105]. The involvement of the polycomb family in ATL was first revealed by global gene expression analysis. Significantly higher levels of EZH2 as well as RING1- and YY1-binding protein (RYBP) transcripts with enhanced H3K27me3 levels were found in ATL cells compared with normal CD4$^+$ T cells [106]. EZH2 is highly expressed in many cancer types, including breast and prostate cancers and lymphomas, and its expression is often correlated with advanced stages of tumor progression and a poor prognosis. We recently identified a notable gene silenced by polycomb. A human gene that encodes for miR-31, *hsa-miR-31*, is located at 9p21.3, which is adjacent to clusters of the *CDKN2* and *IFNA* families. In addition to loss of this gene (12.5% of ATL cases), transcription of the miR-31 precursor was completely lost in ATL cells. Computational predictions and experimental evidence clearly demonstrated that an assembly of YY1-binding motifs upstream of the miR-31 region was responsible for the occupancy of the polycomb family at the target region, which resulted in H3K27me3-dependent transcriptional repression. Of note, given that miR-31 is a master regulator of the ATL-specific gene expression pattern described above, polycomb-mediated loss can influence gene expression downstream of miR-31in T cells and other cell types (Figure 8) [34].

Intriguingly, several recent lines of evidence have shown two faces