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GENETIC AND PHENOTYPIC CHARACTERIZATION OF THE LYMPHOCYTIC VARIANT OF THE HYPEREOSINOPHILIC SYNDROME : A MODEL OF T LYMPHOMAGENESIS

CARACTERISATION GENETIQUE ET PHENOTYPIQUE DE LA VARIANTE LYMPHOCYTAIRE DU SYNDROME D'HYPEREOSINOPHILIE : UN MODELE DE LYMPHOGENESE T

Thèse soumise en vue de l'obtention du titre académique de Docteur en Sciences Biomédicales

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List of abbreviations

ACAD	activated T cell autonomous death
Acgh	array-comparative genomic hybridization
AICD	activation-induced cell death
ALL	acute lymphoblastic leukemia
AP-1	activating protein-1
APC	antigen presenting cell
BM	bone marrow
CCR	cc chemokine receptor
CEL	chronic eosinophilic leukemia
CMML	chronic myelomonocytic leukemia
DAG	diacylglycerol
DC	dendritic cell
DLBCL	diffuse large B cell lymphoma
FAS	TNF-family member receptor Fas (CD95)
FAS-L	TNF-family ligand Fas ligand (CD178)
FGFR	fibroblast growth factor receptor
FISH	fluorescence *in situ* hybridization
HES	hypereosinophilic syndrome
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
L-HES	lymphocytic variant of hypereosinophilic syndrome

LOH	loss of heterozygosity
M-HES	myeloproliferative variant of hypereosinophilic syndrome
NFAT	nuclear factor of activated T cell
NFkB	nuclear factor kappa-B
NHL	non-hodgkin lymphoma
PCNSL	primary central nerve system lymphoma
PDGFR	platelet-derived growth factor receptor
PKC	protein kinase-C
RMD	region of minimal deletion
STAT	signal transducer and activator of transcription
TARC	thymus and activation related chemokine
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TSG	tumor suppressor gene

RESUME

Le syndrome d'hyperéosinophilie (SHE) est une maladie orpheline regroupant un grand nombre d'affections hétérogènes dont le dénominateur commun est la présence d'une hyperéosinophilie morbide et persistante. Après avoir exclu toutes les causes d'hyperéosinophilie secondaire, on distingue 3 catégories majeures dans ce syndrome telles que la variante myéloïde (M-SHE), la variante lymphocytaire (L-SHE) et la variante idiopathique (I-SHE). Le M-HES se caractérise par la prolifération d'éosinophiles malins générés sous l'effet du gène de fusion FIP1L1-PDGFR présent dans une cellule souche lympho-myéloïde tumorale et constitue la cible thérapeutique de l'inhibiteur imatinib mésylate. Par contre, l'hyperéosinophilie persistante observée dans le L-SHE est le résultat de l'expansion monoclonale d'une population aberrante lymphocytaire T CD4+, le plus souvent CD3-CD4+, secrétant en abondance des cytokines éosinophilopoïétiques telles que l'IL-5. Les patients L-SHE bénéficient de traitements symptomatiques souvent mixtes avec des résultats variables et inconnus à long terme visant à réduire le nombre d'éosinophiles responsables des lésions tissulaires. Toutefois, alors que le décours clinique du L-SHE semble bénin, son pronostic est terni par la progression possible vers un lymphome T chez un certain nombre de patients. Dès lors, cette propension à la lymphogenèse T propre au L-SHE en fait un rare modèle d'étude procurant l'opportunité d'identifier les changements génétiques et phénotypiques associés aux différentes étapes du processus transformant.

Par la collaboration établie avec le Prof. Goldman et les Drs F. Roufosse et K. Willard-Gallo, nous avons participé au diagnostic génétique des patients L-SHE constituant la cohorte qui a servi de base indispensable aux différents travaux rapportés dans cette thèse. Nous avons ensuite complété l'identification des caractéristiques phénotypiques des clones lymphocytaires Th2 aberrants CD3-CD4+ de ces patients confirmant leur homologie immunophénotypique en l'absence d'infection virale sous-jacente. Nous avons parallèlement étudié les caractéristiques cytogénétiques des cellules T CD3-CD4+ de 2 patients L-SHE (P1 et P2) au moment de leur diagnostic et décelé une délétion commune de la région q13-q22.1 d'un chromosome 6. En étudiant rétrospectivement les clones T CD3-CD4+ de P1 et P2 lors des stades cliniques successifs de leur maladie, nous avons constaté que cette anomalie chromosomique était non seulement présente au diagnostic mais persistante durant la phase chronique pour enfin prédominer lors du développement d'un lymphome T chez la patiente

P1. Ces résultats ont donc suggéré un rôle précoce et pathogène de la délétion 6q dans le L-HES dotant les clones T CD3-CD4+ d'un potentiel lymphoprolifératif.

Dans le but d'identifier les changements s'opérant dans les clones L-SHE, nous avons comparé le profil d'expression moléculaire des cellules T CD3-CD4+ en phase chronique de 3 patients L-SHE (P1, P2 et P3) avec celui de cellules T CD3+ CD4+ provenant de 5 individus contrôles. Cette analyse a permis de mettre en évidence une grande similitude dans les dérèglements transcriptionnels caractérisant les clones L-SHE de ces 3 patients. De plus, le phénotype Th2 effecteur-mémoire à tropisme inflammatoire des cellules CD3-CD4+ a pu être établi et confirmé sur les échantillons provenant d'une cohorte de 6 patients L-SHE. Enfin, ces résultats ont révélé des altérations de la voie d'action du TGFβ et de plusieurs voies pro-apoptotiques au bénéfice de voies alternes anti-apoptotiques et pro-inflammatoires compatibles avec la survivance des clones T CD3-CD4+.

La délétion 6q a été rapportée dans une grande variété de tumeurs solides et d'affections lymphoprolifératives suggérant la présence de plusieurs gènes suppresseurs de tumeur situés sur le bras long du chromosome 6. Afin d'identifier un gène oncosupresseur potentiellement localisé dans la région 6q13-q22.1, nous avons établi une corrélation entre les gènes situés dans la délétion et le profil transcriptionnel génique des clones T CD3-CD4+ des 3 patients L-SHE en phase chronique et lors de la phase de transformation en lymphome de P1. Nos résultats indiquant la répression commune et continue du gène *BACH2* lors de la progression de la maladie chez P1 et coïncidant avec les données de la littérature ont convergé vers l'identification de ce gène en tant que principal candidat anti-oncogène. Nos analyses fonctionnelles ont démontré le rôle oncosuppresseur du gène *BACH2* en dévoilant sa capacité à moduler l'apoptose des cellules Jurkat CD4+ par sa répression en présence d'agents génotoxiques. De plus, il s'est avéré que cet effet modulateur de *BACH2* est médié via la régulation du gène *FAS-L* important acteur de la voie apoptotique extrinsèque des lymphocytes T suggérant ainsi un rôle crucial de BACH2 non seulement dans la lymphogenèse T du L-HES mais aussi dans l'homéostasie des cellules T CD4+.

En conclusion, nos travaux de caractérisation phénotypique et génétique des cellules T CD3-CD4+ ont permis d'identifier de nouvelles cibles thérapeutiques et diagnostiques pouvant s'appliquer potentiellement au L-SHE ainsi qu'à d'autres pathologies autoimmunes, lymphoprolifératives et infectieuses ou l'homéostasie des cellules T CD4+ serait perturbée.

INTRODUCTION

1. T Cell Immune Response

1.1 Generalities

Immunity is defined as the resistance against disease, orchestrated by cells of the immune system that are designated to combat pathogens. The function of the immune system is to prevent and eradicate all declared invaders. Defence mechanisms used by the host are divided essentially into innate immunity (epithelial barriers, monocytes, NK cells and proteins of the complement) and adaptive immunity. The characteristic of adaptive immunity is an extremely high specificity for a large variety of antigens and a long lasting memory of anterior priming. Moreover, sophisticated mechanisms control the rapid initiation and termination of the immune response to prevent lymphoproliferative disorders and host damage. Homeostatic balance is thus required to prevent either lymphoproliferative responses leading to autoimmunity or insufficient responses responsible for immunodeficiency. The main adaptive immune system cells are B and T lymphocytes with clonal receptors, respectively specialized for humoral and cell-mediated immunity. B lymphocytes recognize preferential epitopes from complete antigenic molecules, whereas peptides, which come from intracellular antigenic degradation, are presented by major histocompatibility complex (MHC) class I or MHC II molecules for recognition by either cytotoxic CD8+ or CD4+ helper T-cells, respectively.

1.2 CD4+ T-Cell Development

The lymphoid organs where the lymphocytes differentiate and mature from stem cells are the primary lymphoid organs including the thymus dedicated to T-cell development and the fetal liver or postnatal bone marrow for B cell development. After maturation, T and B cell lineages migrate to the secondary lymphoid organs: lymph nodes, the spleen and mucosa-associated lymphoid tissues. Thymocytes development includes central tolerance mechanisms that eliminate “useless” and self-reactive T lymphocytes by multistep positive and negative selections. Ultimately, T-cell lineages progress from double-negative to single-positive CD4 or CD8 expressing cells, whereas CD4+ versus CD8+ subset commitment is believed to be modulated by the strength and duration of receptor signalling [1]. Later, the

remaining autoreactive cells are eliminated or modified by peripheral tolerance mechanisms involving deletional or non-deletional pathways.

1.3 T-cell Activation and the Immunological Synapse

1.3.1 T-cell receptor and CD3 co-receptor signalling

Antigen priming of CD4+ naïve T-cells occurs in secondary lymphoid tissues where peptide-MHC class II complexes presented mainly by dendritic cells are specifically recognized by the T-cell receptor/CD3 complexes together with the CD4 co-receptor. This activation step associated with co-stimulatory signals generates a cascade of events leading ultimately to the differentiation and clonal proliferation of effector CD4+ T-cells.

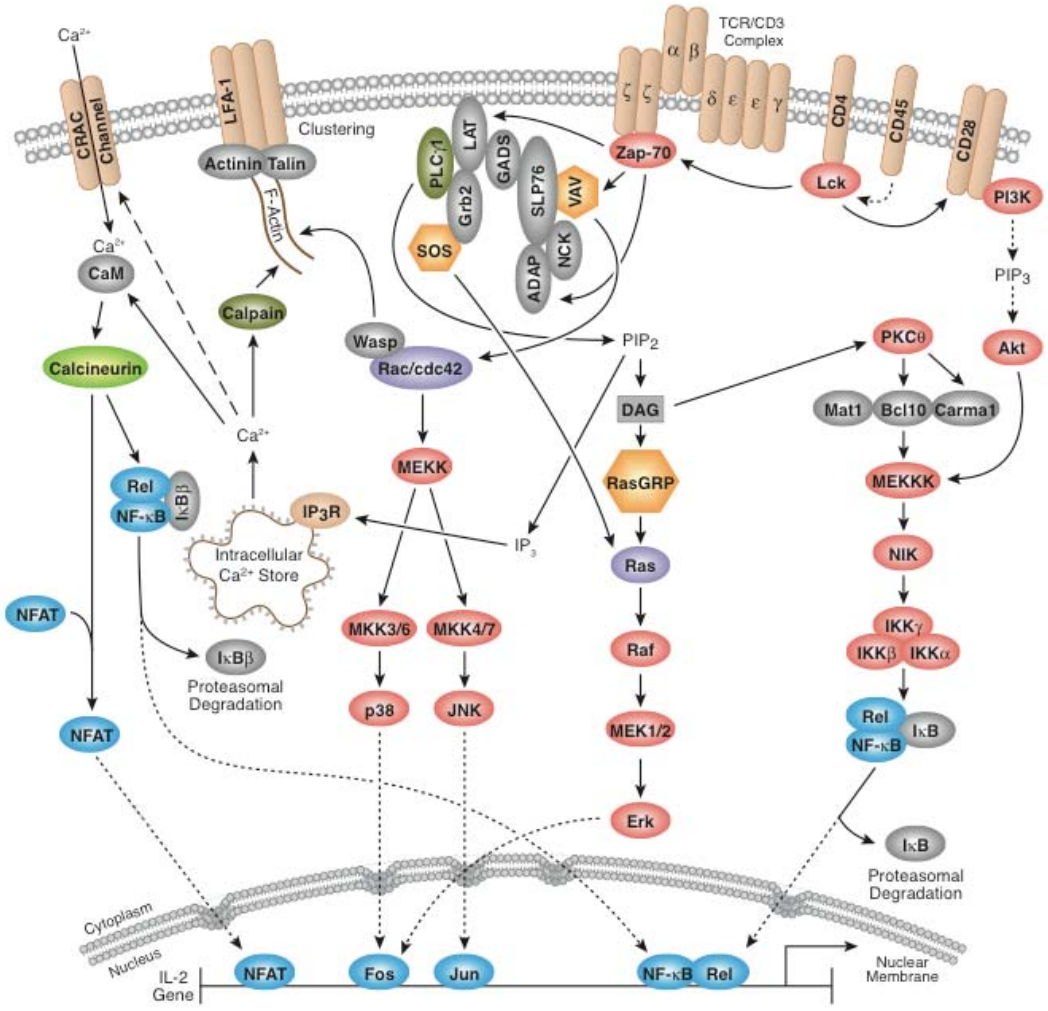


Figure 1. Structure and signalling of the TCR/CD3 complex (Source: *www.cellsignal.com*)

Structure of the TCR/CD3 complex

The TCR/CD3 complex is composed of heterodimers of highly polymorphic TCR alpha and beta chains (or gamma and delta) for the antigen-binding function associated with dimers of the invariant CD3γ, CD3δ, CD3ε and CD3ζ molecules [2] (see Figure 1). For appropriate surface expression, a series of dimers γε, δε and ζζ have to bind non-covalently to a single TCR αβ heterodimer before glycosylation and transport [3].

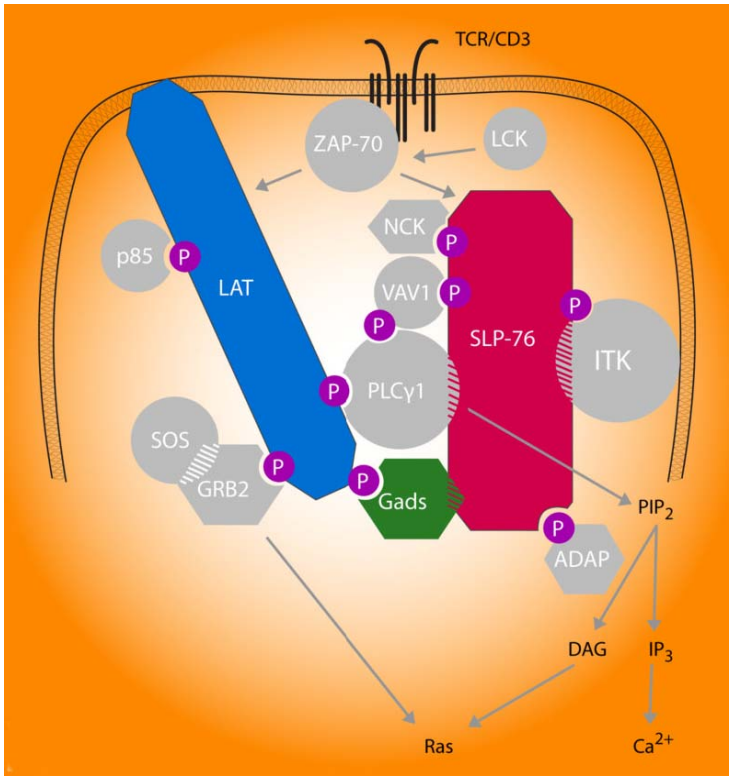


Figure 2: Adaptors of the TCR/CD3 signalling (Source: Smith-Garvin J.E.,[4]).

Signalling of the TCR/CD3 complex

Recognition by the TCR of an antigen-MHC II molecule complex initiates the signalling events. The earliest events involve lymphocyte tyrosine kinases of the Src family (Lck), associated with CD4, and Fyn which phosphorylate 10 immunoreceptor tyrosine-based activation motifs (ITAMS) located on the four CD3 chains [5] Unlike growth factor receptor whose intrinsic enzymatic activity is increased by phosphorylation, the CD3 molecules have no such function; therefore, they serve as docking sites for protein kinases and adaptators for signal transmission. Thus, the most important step after CD3 ITAMs phosphorylation is the recruitment of the 70kDa z-chain associated protein tyrosine kinase (ZAP-70 PTK) followed by the assembly and activation of different proteins in a multi-molecular signalling complex.

Among the 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 76Kda (SLP-76) [6-7]. These two adapters, which interact with the indicated proteins in Figure 2 (p85 subunit of PI3K, GRB2, GADS, NCK, VAV, ITK, ADAP), form the nucleus of the proximal signalling complex that stabilizes phosphorylated phospholipase Cγ1 (PLCγ1) in an active conformation for signal transduction [8]. Activated PLCγ1 hydrolizes the membrane lipid phosphatidylinositol 4,5 biphosphate (PI(4,5)P2) producing the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG) that are essential for T-cell function. On one hand, DAG production results in the activation of two major pathways depending on the guanine nucleotide-binding protein Ras and protein kinase Cθ (PKCθ), respectively. Ras stimulates the serine-threonine kinase Raf-1 which leads to the mitogen-associated protein kinases (MAPKs), extracellular signal-regulated kinase 1 (Erk1), and Erk2 activation cascade with final triggering of the activator protein-1 (AP-1) transcription complex [9]. PKCθ regulates transcription factor NFκB activation and its translocation in the nucleus by degradation of inhibitor molecules (IκB) [10]. On the other hand, IP3 generated by TCR-stimulated PLCγ1 increases intracellular Ca^{2+} levels and leads to a nuclear factor of activated T-cells (NFAT) dephosphorylation by calcineurin and nuclear translocation [11]. These transcriptional factors cooperate together to mediate the T-cell activation process. The most well-known interaction is NFAT/AP-1 cooperation resulting in IL-2 production, whereas the genes activated by NFκb are involved in the function, survival and homeostasis of T-cells.

1.3.2 Co-stimulation

For productive T-cell activation, interaction at the single TCR level is not sufficient. On the contrary, it can induce a negative or tolerogenic signal [12]. Co-ligation of other cell surface receptors is thus required not only for binding, but more importantly, for activation signalling to allow the threshold lowering of stimulation complexes. CD28 is a major co-stimulatory molecule expressed on naïve, resting and activated CD4+ T-cells (figure 3). Following binding to its ligands CD80 or CD86, CD28 recruits the activating phosphoinositide 3 phosphate kinase (PI3K), converting PIP2 to PIP3 at the membrane that ultimately activates PDK1/Akt [13]. Activated Akt enhances NFkb nuclear translocation with a positive effect on the expression of pro-survival genes like Bcl-xl [14]. Akt also

increases also the transcription of NFAT-regulated genes such as IL-2 by inhibiting glycogen-synthase kinase 3 (GSK-3), a serine–threonine kinase that promotes the nuclear export of NFAT [15]. Many of the activation pathways described solely for TCR ligation are mildly but reproducibly obtained with CD28 ligation in the absence of TCR/CD3 triggering. This observation suggests that CD28 engagement results primarily in a quantitative rather than qualitative change in T-cell-activated pathways. However, it has been recently shown that CD28 ligation alone enhances NFAT transcriptional activity through an independent process [16].

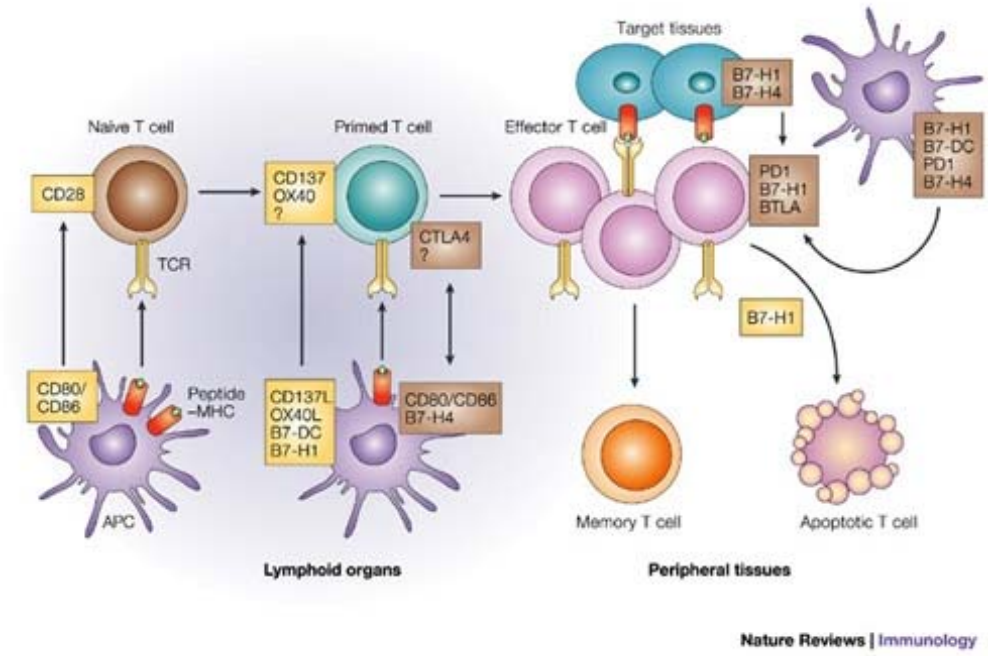


Figure 3.Co-stimulators (yellow) and co-inhibitors (brown) have overlapping but preferential functions in controlling one or more stages of T-cell activation, including priming, differentiation, maturation and potentially memory responses. Naive T cells in lymphoid organs constitutively express CD28. After ligation by its ligands, CD80 or CD86, initial priming occurs. (Source : Chen L., [17]).

While CD28 knock-out models demonstrated reduced immune response to various infectious agents, not all responses were severely impacted by its loss, therefore suggesting that other molecules can provide the co-stimulation for T-cells. Among these co-stimulators, inducible co-stimulator (ICOS) is expressed on activated T-cells [18]. Two members of the tumor necrosis factor receptor (TNFR) family, OX40 (CD134) and 4-1BB (CD137),

respectively, induce the activation of Akt, NFκB, JNK and MAPK. However, this involves downstream signalling mediated by TRAF (TNFR associated factor) adaptater proteins rather than direct association with protein kinases [19]. These co-stimulators are also important for memory T-cell formation and are involved in the negative regulation of TCR signalling (Figure 3).

1.3.3 Adhesion molecules

Integrins are αβ heterodimeric receptors that mediate stable cell/cell or cell/matrix adhesion. They also participate in cell surface signalling and rebinding events, thus allowing the cell movement and migration reviewed in [20]. Key integrins in the immune response are the leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) binding their ligands, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), respectively. Defects in the integrin β 2 (*ITGB2*) gene encoding the LFA-1 beta chain are the cause of leukocyte adhesion-deficiency syndrome (LAD1) leading to severe infectious diseases [21]. The avidity of integrins for their ligands is critically dependent on events initiated by the TCR and defined as “inside-out signalling” [21]. The main regulator of TCR-induced adhesion to ICAM1 is the small GTPase Ras-proximity-1 (Rap1) [22]. However, downstream TCR-signalosome effectors have been shown to be critical as ADAP-knockout mice are deficient for TCR-induced LFA-1 clustering and adhesion to ICAM-1 [23]. In addition, signals from the TCR also regulate cytoskeletal attachments to integrins [24].

The area of contact between a T-cell and an antigen presenting cell is the immunological synapse where adhesion and co-stimulatory molecules are believed to cooperate to stabilize peptide-MHC-TCR interactions. The formation of the immunological synapse is an active dynamic process forming a central cluster of TCRs interacting with MHC molecules ringed mainly by ICAM-1-LFA-1 interacting complexes. A cluster of differentiation (CD2) is a cell adhesion molecule expressed on all human CD4+ T-cells binding to CD58/LFA3 (CD48 in mice) on resting or activated APCs. CD2 has been shown to hamper the quantitative thresholds of antigen required for TCR activation [25]. Furthermore, CD2 exhibits an additive co-stimulatory function. Indeed, CD2/CD58 interactions dramatically enhance TCR/CD3-initiated intracellular signalling [26] in response to antigenic stimulation. These events are mediated by PLCγ1 activation.

1.4 Naïve CD4+ T Helper 2 Differentiation

1.4.1 T helper polarization

The differentiation of naïve CD4+ helper T-cells into fully functional effectors requires activation of the T-cell receptor (TCR) and cytokine production by the activated cells from both the adaptive and innate immune system. The Th1/Th2 paradigm was first proposed by Mosmann in 1986 [27] on the basis of the following observation: specific diseases generating exclusive immune responses could be mediated by a different subset of effector cells classified by their lymphokines profiles. In particular, they include Th1 cells, producing mainly the IL-2 and IFN-γ involved in delayed type hypersensitivity reactions distinct from Th2 cells, making IL-4 implicated in IgE secretion and allergic reaction. Since then, multiple different Th cell lineages have been identified and are summarized in a simplified concept of T helper differentiation. CD4+ T helper cells normally differentiate into Th1, Th2, Th17 or Treg, depending on the strength of the antigen-TCR ligation, the nature of the cytokine liberated in the environment, and the expression of specific transcription factors summarized in figure 4 [28-31]. In addition, T helper cells from different lineages may secrete common cytokines such as IL-2, Il-9 and IL-10 [32].

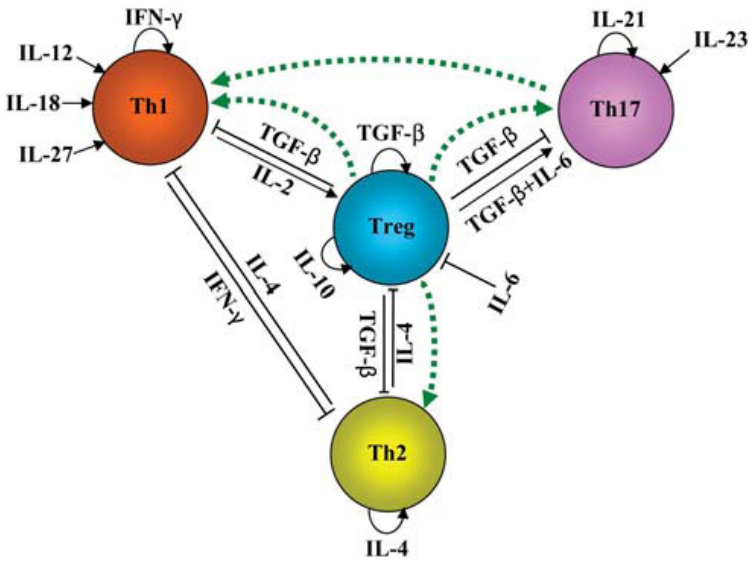


Figure 4.T helper polarization. (Source: Wan Y.Y., [28]) .

Th1-cell lineage

Th1-cell lineage induces immune response against intracellular pathogens like bacteria and viruses and promotes isotype IgG2a secretion by B cells upon stimulation. The Th1 cytokines secretion profile includes IFN-γ, IL2, TNF and lymphotoxin-b (LT-b)[33-36]. For their differentiation, Th1 cells require cytokines IFN-γ, IL-12, IL-18 and IL-27 in the medium and increased expression of transcription factors such as T-bet, Hlx, STAT4 and IRF1. T-bet is the master regulator of Th1 differentiation by increasing IFN- γ production. In addition, the transcription factor T-bet suppresses the secretion of Th2 cytokines IL-4 and IL-5. Th1 cells have also been implicated in anti-tumour immunity and in autoimmune diseases such as graft-versus-host (GVH) disease, inflammatory bowel disease (IBD), type I diabetes and rheumatoid arthritis (RA) [37-39].

Th-17 cell lineage

Th17 produces IL-17A, IL-17F, IL-6, G-CSF, GMCSF and TNFa. Il-17 mediates the activation and recruitment of neutrophils. [40]. For their differentiation, both TGFβ and IL-6 are required. The master transcription factor of Th17 subset differentiation is RORC2 [41]. Th17 contribute to the resistance to specific extracellular bacterial and fungal infections and are associated with autoimmune disease such as multiple sclerosis (MS), psoriasis and allergic responses [42-44]. A new role in tumourigenesis has also been recently reported for IL-17A-secreting CD4+ T lymphocytes [45].

Th2-cell lineage

Th2-cell lineage induces responses against extracellular parasites, and promotes IgG1 and IgE class-switching and eosinophils recruitment [46-47]. Th2 cells usually secrete IL-4, IL-5, IL-9, Il-10 and IL-13 cytokines [44]. IL-4 and IL-13 are involved in B cell class switching to the IgE required for activation of basophils and mast cells through their FcεRI. IL-5 and IL-13 are directly responsible for tissue eosinophilia and inflammation with IL-5 specifically involved in eosinophil maturation from progenitors [48]. Activated eosinophils release cytotoxic proteins from their granules and secrete mediators such as cytokines, leukotriens and prostaglandins, thus amplifying the Th2 response. Furthermore, IL-9 increases Th2 differentiation by inhibiting the Th1 pathway and enhancing IgE production or mast cell proliferation and by preventing the apoptosis of Th2 cells from Il-2 deprivation

[49]. Th2 cells require IL-2 and IL-4 in the environment and enhanced expression of GATA3, c-maf, STAT6 in concert with NFAT, NFkB and AP-1 transcription factors for their differentiation [4, 44]. IL-2 induces the STAT5a necessary for the upregulation of IL-4 production and maintains Th2 proliferation. In addition, IL-4 is the major cytokine of Th2 differentiation by inducing early the activation of STAT6 by the janus kinases (Jak1/3) pathway [50]. Activated STAT6 then transactivates several IL-4 responsive genes that encode IgE, IL-4, IL4-R, FcR, and MHC II molecules, and most importantly, induces the master regulator of Th2 cell differentiation, the transcription factor GATA3 [51]. GATA3 induces chromatin remodelling of the Th2 cytokines locus and promotes production of Il-4, IL-5 and IL-13. In parallel, GATA3 represses the Th1 transcription factor STAT4 and IL-12R gene expression inhibiting the Th1 cell differentiation [52].

Weak TCR/peptide ligation specifically favours the Th2 differentiation of naïve CD4+ T-cells. It has been shown that low peptide concentrations induce GATA3 expression, STAT5 phosphorylation and IL-4 production [53]. Moreover, it is clear that Th2 polarization is reinforced by CD28 signalling. Indeed, CD28 ligation activates the STAT6-inducing IL-4 gene promoter, and also stimulates GATA3 expression and Th2 cytokines production [54-55].

The transcription factor c-maf specifically stimulates also IL-4 production by binding itself to the MARE sequences of the IL-4 promoter [56]. NFAT1 and NFAT2 transcription factors further contribute to the sustained IL-4 production in association with IRF-4, which is activated by c-rel, AP-1 and c-maf [56-58]. The importance of NFκb in the induction of GATA3 [59] and Th2 differentiation [60] has been further demonstrated. Thus, both GATA3 and these transactivators contribute to the amplification and maintenance of Th2-cells differentiation. Th2 cells have been implicated in the immune responses to parasites such as helminthes and nematodes [61] and in models of autoimmune diseases such as atopic asthma [62-63].

1.4.2.1 Migration of Th2 effector cells

In the absence of inflammation, naïve CD4+ T-cells do not adhere to the blood vessel wall and circulate constantly between the blood and the secondary lymphoid organs through endothelial venules [64-65]. In particular, the rolling of naïve CD4+ T-cells is

mediated by interaction between the L-selectin (L-62) and the peripheral-node addressin (PNAd) expressed on endothelial cells [65]. By this weak interaction, the chemokine receptor CCR7 is constitutively expressed on naïve CD4+ T cells that can bind to two types of ligands, CCL19 and CCL21, both located on the endothelial wall [66]. Following this step, LFA-1 integrin is activated and firmly binds its ligand ICAM-1 inducing CD4+ T-cells diapedesis and migration to the T-cell area within the lymph node. During infection, activated DCs (dendritic cells) upregulate their CCR7 expression and enter to the T-cell area where they co-localize with naïve T-cells via afferent lymphatic vessels. After the priming process, T-cells decrease CD62L and CCR7 expression and finally highly differentiated CD4+ T cells exit the lymph node. Upon differentiation, Th effector cells acquire adhesion molecules and specific chemokine receptors to determine tissue homing [67]. Th1 cells preferentially express CCR5, CXCR3 and CXCR6, whereas Th2 express CCR3, CCR4, CCR8 and sometimes the prostaglandin D2 receptor (CRTH2) [68]. Given that Th2 immune response is linked to allergic inflammation, it is not surprising to find corresponding ligands like CCL11 (eotaxin), CCL22 (MDC), CCL17 (TARC) and prostaglandin D2 (PDGD2) produced at the site of inflammation by cells such as eosinophils, epithelial cells, basophils, monocytes and mast cells that attract Th2 lymphocytes to the site of inflammation for perpetuating the allergic response [69].

1.4.3 Memory CD4+ T cells

Memory T-cells have a much higher survival potential and possess the ability to proliferate and produce cytokines after re-stimulation with a much lower dose of antigens than effector cells [70]. The memory CD4+ T-cell population is characterized by the CD45RO+ isoform expression. It has been shown that two types of memory T-cells exist : central memory T-cells (T_{CM} cells), CCR7+ and CD62L high, homing lymphoid tissues without having an immediate effector function on antigen stimulation, and effector memory cells (T_{EM} cells), CCR7- and CD62L low, that migrate to non-lymphoid tissues with the immediate recovery of effector functions upon antigenic contact [71].

The new concept of T helper differentiation has revealed more plasticity among T helper subsets than it was generally assumed. If one considers the evolution from naïve T-cells to terminal effectors like a progressive epigenetic mechanism reversibly modifying the master regulators of T helper differentiation, it explains why memory T-cells, shortly arisen

after initial antigen stimulation, retain a more flexible repertoire compared to terminal effector cells [72]. Indeed, with continued polarizing activations, specific master regulators become exclusively silenced or activated maintaining cells with a fixed cytokine production phenotype. Thus, in contrast to terminal effector cells, memory T-cells exist as non-committed cells (CXCR3-CCR4-), Th1 lineage cells (CXCR3+CCR4-), or Th2 lineage cells (CXCR3-CCR4+). Moreover, depending on the restimulation environment, Th1 and Th2 memory cells can invert their cytokine production [73]. However, it is important to note that a subset of Th2-commited T effector memory (T_{EM}) cells characterized by CRTH2 expression are "Th2-fixed" thereby creating an exception to this rule [74].

In addition, Th2 memory cells express the IL-25R (IL-17RB) on their surface. It was recently shown that IL-25 (IL-17E) secreted by activated eosinophils, basophils and mast cells is the ligand for this receptor [75]. Upon IL-25R engagement, Th2 cells proliferate and secrete cytokines increasing eosinophils activation [76]. Consequently, an activation loop between Th2 effectors and cells from the innate immune system can be induced, which further amplifies the inflammation process.

1.5 Negative regulation of activated CD4+ T lymphocytes

1.5.1 Negative costimulatory signalling

Once the antigenic stimulation resumes, a homeostatic balance is required to prevent the overactivation and uncontrolled expansion of CD4+ effector cells. An important homeostatic pathway is active anergy mediated by several co-receptors represented by the CD-28 homologue, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) and programmed death (PD-1), as illustrated in Figure 3. The major inhibitor, CTLA-4, binds to CD80 and CD86 with twenty-fold more affinity than CD28, therefore, antagonizing CD28/TCR signalling [77-78]. It has been shown that signalling through CTLA-4 blocks IL-2 production, down regulates CD25 surface expression and induces cell cycle arrest [79]. Upon restimulation, anergic T-cells are unable to proliferate, even with appropriate co-stimulatory signals. Mutations in the CTLA-4 gene are associated with multiple autoimmune diseases in humans, and knockout mice develop fatal T cell proliferative disorders [80].

Potential immunotherapeutic applications are considered based on the manipulation of these potent inhibitory networks [81].

1.5.2 Apoptosis of activated T-cells

Two major pathways are described to induce the apoptosis of activated cells: the extrinsic pathway illustrated by the activation-induced cell death (AICD) and the intrinsic pathway including cytokine deprivation, as shown in Figure 5.

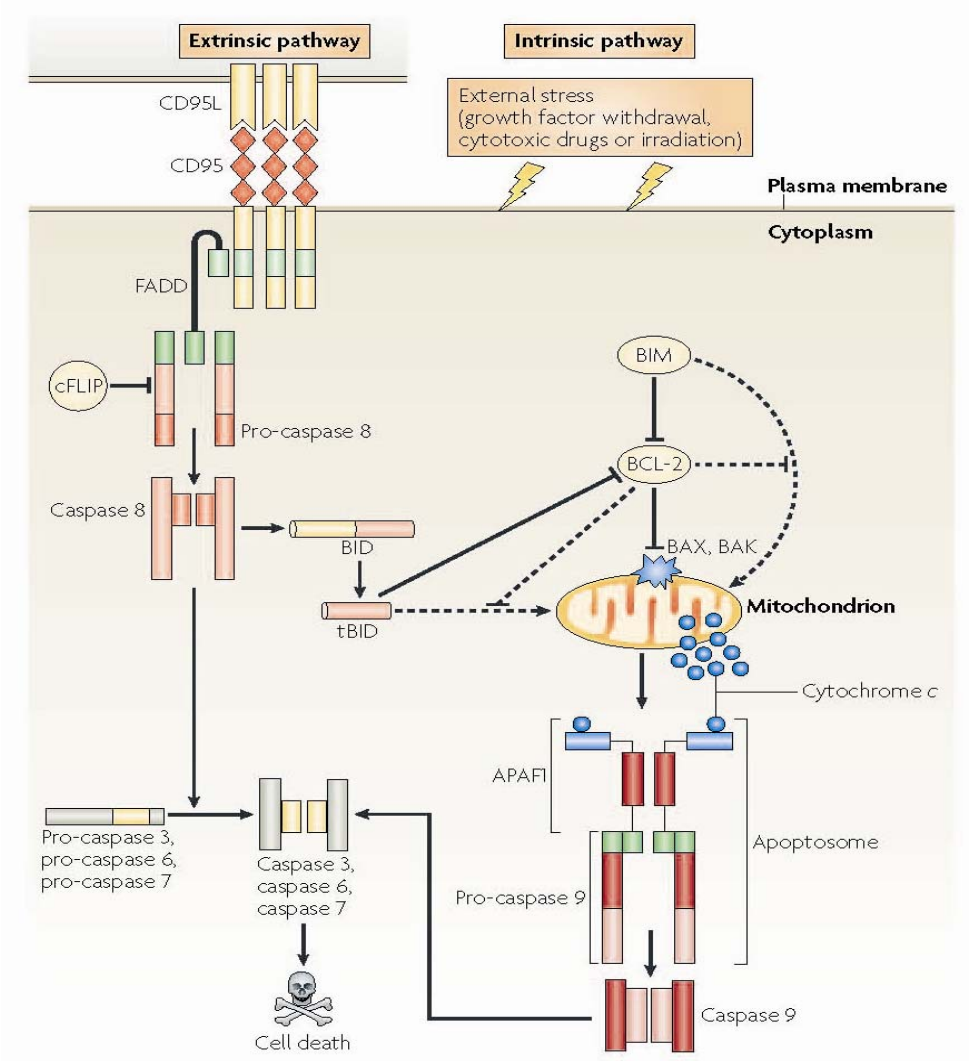


Figure 5.Extrinsic and intrinsic apoptosis pathways (Source: Bouillet P., [82])

AICD

AICD is a death receptor-mediated or extrinsic apoptosis of activated T lymphocytes to ensure the rapid elimination of effector cells after antigen clearance. This apoptotic process is classically triggered by TCR stimulation in activated effector FAS+CD4+ T-cells by upregulating the FAS-ligand (FAS-L/CD95-L), which interacts with the death receptor (DC) FAS-receptor (FAS-R/CD95) expressed on their surface [83] [84]. This autocrine FAS-L/FAS-R activation loop recruits the adaptator protein Fas-associated death domain (FADD) and procaspases-8 and -10 molecules, thus inducing formation of a death-inducing signalling complex (DISC) reviewed in [85]. On one hand, low levels of DISC generate the truncated Bcl-2 homology 3-only protein Bid (t-Bid) release of cytochrome c from mitochondria, which mediates the mitochondrial or extrinsic apoptosis [86]. This leads to apoptosome formation and procaspase-9 activation, which in turn cleaves the downstream effector responsible for cell death : caspases-3, -7 and -6.

This process might be blocked by B-cell CLL/lymphoma 2 (BCL2) family members BCL2 like-1 (BCL2L1) and BCL2 [87]. On the other hand, high levels of DISC formation lead to an increased amount of active caspase-8 and 10, which directly activate downstream caspases-3 and -7, inducing DNA fragmentation and cell death. In this process, the FLICE-inhibitory protein (c-FLIP) inhibits extrinsic activation of procaspase-8 by blocking its processing [88]. Therefore, the intrinsic or extrinsic pathways of death induction depend on the balance between anti-apoptotic and pro-apoptotic mediators, illustrated here by c-FLIP/Bcl2 or caspase-8/-9, respectively. AICD is thus a complex mechanism concentrating opposite positive and negative influxes and ultimately determining T-cell faith.

Reflecting also the complexity of AICD, there is actually no clear explanation why antigen-primed CD4+ T cells are resistant to AICD whereas the same TCR stimulation promotes death of the subsequent effector CD4+ T-cells. Differential susceptibility to AICD described for Th1 and Th2 cells also exists [89]. It is believed that AICD sensitivity is increased by progressive NFκB inactivation through the hematopoietic kinase 1C (HPK1-C) phosphorylation pathway [90].

It is also important to note the dual role for cytokine IL-2 in AICD revealed by IL-2 and IL-2R knockout mice models. Although IL-2 induces T-cell proliferation and is a critical survival factor, it sensitizes activated T-cells to FAS-mediated apoptosis by upregulating

FAS-L expression and suppressing two FAS signalling inhibitors, c-FLIP and FAS apoptotic inhibitory molecule 3 (FAIM3/TOSO), respectively [91-92]. Interestingly, CD4+ memory T-cells producing Il-15 resist IL-2 mediated AICD; this suggests the opposite effects of lymphokines in T-cell survival [93]. Other members of the TNF and TNF-R superfamily are also involved in AICD, such as TNFSF10/TNFRSF10B (APO2/TRAIL). However, the major role of FAS and FAS-L has been underlined *in vivo* by the lethal autoimmune lymphoproliferative diseases developed in mice with mutated *FAS* (*lpr*) or *FAS-L* (*gld*) genes [94-95].

Cytokine deprivation

Cytokine deprivation is another important apoptosis pathway also known as “death by neglect”, that results from the absence of a signal linked to the progressive withdrawal of survival promoting cytokines. This results in increased mitochondrial membrane permeability liberating cytochrome c, with which, the apoptotic protease-activating factor 1 (Apaf-1), binds procaspase-9. The resulting apoptosome with active caspase-9 cleaves downstream caspases-3, -6 and -7, thus promoting cell death. This intrinsic pathway is associated with decreased concentrations of Bcl2 related anti-apoptotic proteins, relative to pro-apoptotic proteins such as bax, bcl-x, Bcl2L11 (BIM) and Bcl2-L10. This ongoing process of apoptosis can be reversed by the addition of IL-2, IL-4, IL-7 and IL-15, which all up-regulate bcl2 expression [96].

2. Hypereosinophilic Syndrome (HES)

2.1 Definition of the Hypereosinophilic Syndrome (HES)

Eosinophilia is the hallmark of the coloration acquired by eosinophils nucleated cells when observed with light microscopy, and first described by Paul Ehrlich in 1879 (La leukocytose, XIII Congrès International, Paris, 1900). Chronic eosinophilia is a symptom present in a vast variety of human diseases extending from allergic, infectious to malignant conditions, and therefore requiring a clear definition of the entity studied. The first reference to diseases collectively labelled as "hypereosinophilic syndromes" comes from a review article of Hardy and Anderson in 1968 [97]. Furthermore, the "hypereosinophilic syndrome" (HES) was clearly defined by Chusid and colleages in 1975 with strict diagnosis criteria [98]:

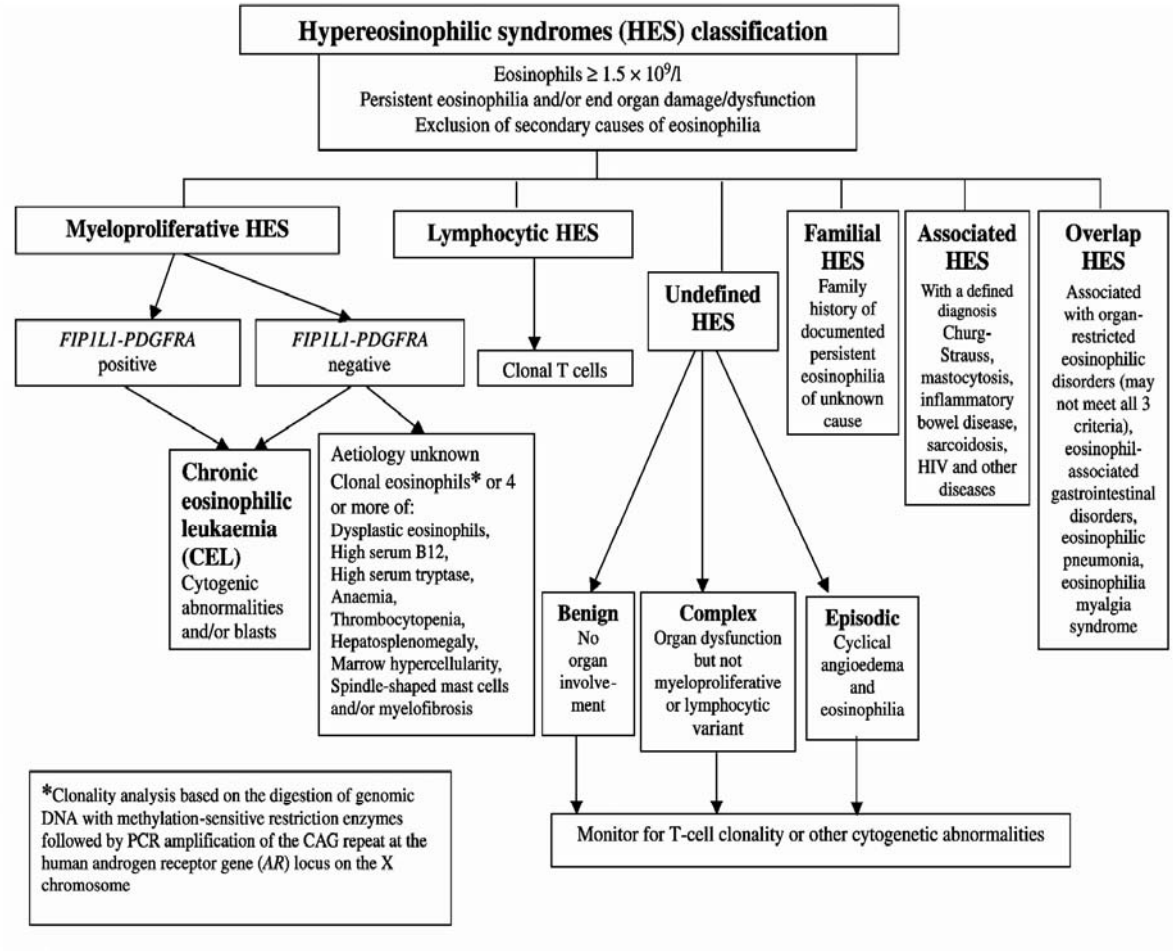
1) Peripheral blood eosinophilia greater than 1500 cells/mm^3, sustained for more than six months
2) Signs or symptoms of end-organ (heart, lungs, gastrointestinal tract, skin, bone marrow and brain) involvement with eosinophil tissue infiltration/injury
3) Exclusion of known secondary causes of eosinophilia (including allergic diseases and parasitic infections).

These three criteria remain the basis of the actual definition of HES.

2.2 HES classification

HES encompasses a heterogeneous spectrum of disorders, and since recent advances in clinical or genetic diagnosis, has been divided into six clinical entities of which three are major: myeloproliferative variant (M-HES), lymphocytic variant (L-HES) or undefined/idiopathic (I-HES) [99]. Three minor separate conditions with prolonged peripheral blood hypereosinophilia were also included, although they did not completely meet the criteria or were associated with familial or defined diseases (Figure 6).

Different classifications coexist based on new molecular findings, but they are usually missing clinical cases belonging to the three minor variants [100-101]. Work is still in progress and awaiting refinement from what is learned from new diagnostic techniques and therapeutics.



Classification of the hypereosinophilic syndromes. A workshop summary report of the 2005 Hypereosinophilic Diseases Working Group (meeting in conjunction with the International Eosinophil Society). Modified from *J Allergy Clin Immunol*, 117, Klion, A.D., Bochner, B.S., Gleich, G.J., Nutman, T.B., Rothenberg, M.E., Simon, H.U., Wechsler, M.E., Weller, P.F. & The Hypereosinophilic Syndromes Working, G. Approaches to the treatment of hypereosinophilic syndromes: a workshop summary report., 1292–1302. Copyright 2006, with permission from Elsevier.

Figure 6.Classification of HES (Source: *Hypereosinophilic Diseases Working Group*[99]).

2.3 Pathogenesis, Diagnosis and Treatment of the Major Clinical Variants of HES

2.3.1 M-HES variant

Following the World Health Organization (WHO) classification revised in 2008 and shown in Table 1, the myeloproliferative hypereosinophilic syndrome or M-HES variant can be divided into two types. The first type is myeloid and lymphoid neoplasms with eosinophilia molecularly defined (including platelet-derived growth factor receptor *PDGFRA*/*B* and *FGFR1* fibroblast growth factor receptor 1 fusion genes), and the second is molecularly undefined (chronic eosinophilic leukemia, not otherwise specified, CEL-NOS) (Table 1).

TABLE. **World Health Organization Classification of Myeloid Malignancies**

Acute myeloid leukemia and related disorders
Myeloproliferative neoplasms
Chronic myelogenous leukemia, *BCR-ABL1* positive
Chronic neutrophilic leukemia
Polycythemia vera
Primary myelofibrosis
Essential thrombocythemia
Chronic eosinophilic leukemia, not otherwise specified
Mastocytosis
Myeloproliferative neoplasms, unclassifiable
Myelodysplastic syndromes
Refractory cytopenia with unilineage dysplasia
Refractory anemia
Refractory neutropenia
Refractory thrombocytopenia
Refractory anemia with ringed sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess blasts
Refractory anemia with excess blasts 1
Refractory anemia with excess blasts 2
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Myelodysplastic syndromes/myeloproliferative neoplasms
Chronic myelomonocytic leukemia
Atypical chronic myeloid leukemia, *BCR-ABL1* negative
Juvenile myelomonocytic leukemia
Myelodysplastic syndromes/myeloproliferative neoplasms, unclassifiable
Refractory anemia with ringed sideroblasts and thrombocytosis
Myeloid and lymphoid neoplasms associated with eosinophilia and genetic abnormalities
Myeloid and lymphoid neoplasms associated with *PDGFRA* rearrangement
Myeloid neoplasms associated with *PDGFRB* rearrangement
Myeloid and lymphoid neoplasms associated with *FGFR1* abnormalities

Table 1. Source: *WHO classification of haematopoietic and lymphoid tissue tumours*, 2008.

Characterizing the first type of M-HES, a neoplastic clone (neoplastic myeloid-lymphoid precursor stem cell) of myeloid cells expressing the fusion gene *FIP1L1-PDGFRA* produced by cryptic deletion at 4q12 was identified in 2003 [102]. This fusion gene encodes an activated tyrosine kinase responsible for the clonal proliferation of malignant eosinophils (to a lesser extent mast, neutrophil and lymphoid lineages) that can be inhibited by imatinib with durable remission [102]. Subsequently, it was found that PDGFRA and PDGFRB also participate in variant translocations similarly sensitive to imatinib [103-104]. Furthermore, a separate group of eosinophilic leukemias negative for *PDGFR* fusion genes was also included in the molecularly defined group of M-HES. This is characterized by a specific translocation t(8;13)(p11;q12), involving the FKHR1 chimeric gene resistant to imatinib therapy [105]. The second type of M-HES, which is the group of molecularly undefined chronic eosinophilic leukemias (CEL-NOS) involves either clonal eosinophils (detected by human androgen receptor gene analysis (HUMARA)) or displays the four or more criteria detailed in the HES classification (Figure 6) [99]. These conditions are mostly resistant to imatinib treatment.

Specific clinical features of the M-HES variant are predominant in affected males between 25 and 55 years old with hepatosplenomegaly, pruritis, cardiac tissue damage, elevated serum B12 or tryptase levels, anemia, thrombocytopenia, dysplastic mast cells and myelofibrosis. The most serious threat arises from irreversible cardiac (myocardium, endocardium) fibrosis which requires early diagnosis and treatment [106].

2.3.2 L-HES variant

Pathogenesis.

In contrast to M-HES, where the eosinophilic population is issued from a neoplastic myeloid clone, the lymphocytic variant (L-HES) is characterized by an increased but normal eosinophilic population that proliferates polyclonally and is secondary to a cytokine-driven process. Two discoveries have converged to uncover the underlying mechanism driving eosinophilic reactive proliferation in L-HES. Firstly, the recognition of distinct T helper subsets, Th1 and Th2 with clonal growth corresponding to their cytokine profiles and the identification of IL-5 as a major growth and activation factor for eosinophils [27, 107]. The production of IL-5 by Th2 lymphocytes was quickly recognized as responsible for eosinophilia in multiple allergic conditions and parasitic infections [108]. Furthermore, several case reports described eosinophilia associated with T lymphomas in HES patients as a

consequence of the eosinophilopoietic cytokines production by a malignant T-cell clone [109-110]. Subsequently, evidence that T cells from HES patients could stimulate the growth of eosinophils in vitro by producing IL-5 established the importance of Th2 cells in HES pathogenesis [111]. Shortly thereafter, in 1994, an abnormal CD4+ T cell population was identified with a TCRβ clonal rearrangement in a HES patient with increased serum IgE. Flow cytometry revealed an aberrant CD3-CD4+ immunophenotype associated with a marked production of IL-4 and IL-5, thus suggesting a direct role in HES pathogenesis [112]. Following this observation, numerous HES cases associating hypereosinophilia with a circulating abnormal T-cell clone were reported and finally categorized with the unifying qualification of "lymphocytic variant" [113]. Currently, the reported prevalence of L-HES ranges from 14 to 31 percent of all HES patients depending on the techniques used for diagnosis [114-116]. However, the exact cause for the development of these abnormal T cell populations in L-HES patients remains unknown.

Diagnosis.

An important diagnostic progress in L-HES is the identification of T-cell clonality by molecular analysis of the TCR rearrangements associated with an extended immunophenotyping of the T-cell clones. By combining these techniques, three types of L-HES clones have been defined including CD3-CD4+CD8-, CD3+CD4-CD8- and CD3+CD4+CD7- [114, 116-117]. The most prevalent is the CD3-CD4+CD8- subset representing 60% of the L-HES T cell clones [113]. However, it should be noted that in some cases, depending on the percentage of abnormal circulating T-cells, clonality can only be certified on the purified aberrant T-cells because of the sensitivity of the techniques applied (qPCR, southern blotting and FACS) [117]. Therefore, standardized techniques are needed to accurately establish the prevalence of L-HES in future prospective studies. Noteworthy, is the fact that intracytoplasmic TCRVβ repertoire analysis of CD3-CD4+ cells from L-HES patients has not provided evidence of a specific Vβ subset by using a panel of antibodies covering 60 percent of the families [113]. Therefore, it cannot be used to complement a L-HES diagnosis. A common feature of L-HES T-cell clones is the production of Th2-cytokines and, in particular, of the IL-5 driving proliferation of eosinophils [114, 118]. A variable amount of IL-4 is also observed, explaining the high level of IgE detected in the serum of L-HES patients. However, neither IL-5 secretion nor IgE detection are absolutely specific to the L-HES variant, since both have been occasionally reported in M-HES [114,

119]. In contrast, extremely elevated levels of serum thymus and activation-regulated chemokine (TARC) have been shown to be a hallmark of L-HES and can be added to the potential specific biomarkers of the disease [120-121]. Chromosome studies have been infrequently realized on L-HES T-cells. Among the chromosomal aberrations reported, besides 6q chromosomal anomalies described in three L-HES patients, only isolated aberrations such as one trisomy 7 and one chromosome 16 derivative, der(16), have been found [121-124]. Given the reduced number of cases, no correlation with prognosis significance could be obtained for these chromosomal aberrations. Therefore it is necessary to await results from larger prospective studies.

Clinical features.

Contrasting with the M-HES characterized by a marked male predominance, the ratio of male to female patients is approximatively the same in L-HES [113]. The L-HES patients are predominantly affected by cutaneous manifestations, including eczematous dermatitis, pruritus, erythroderma, urticaria and angioedema sometimes indistinguishable from episodic angioedema or Gleich's Syndrome [116-117, 125-127]. The presence of CD3-CD4+ or CD4+CD7- T-cells together with eosinophils in skin lesions from L-HES patients, suggests that their crosstalk plays an important pathogenic role in the cutaneous manifestations of the disease. In addition, the absence of CD7 expression, which is a hallmark of normal skin CD4+ T-cells and is shared by epidermotropic cutaneous T-lymphoma cells, may explain the favoured dermo-epidermal homing of CD3-CD4+CD7- T-cells [128-129]. Rheumatologic and connective tissue involvement such as arthralgia, arthritis, vasculitis and tenosynovitis is also described [106, 118, 130]. Asthma, pulmonary infiltrates, eosinophilic pleuritis or gastritis and colitis have also been reported but surprisingly, endomyocardial fibrosis or thrombi in the cardiac cavity are an extremely rare complication [106]. In summary, immediate life-threatening complications are much less frequent in L-HES compared to M-HES. [124] However, L-HES long term prognosis depends on the patient's response to the treatment and an eventual transformation in T lymphoma, which has been reported in several cases (20 to 40 percent of L-HES patients) with variable prodrome's duration [109, 113, 124, 130]. Therefore, despite an indolent course of the disease, L-HES patients should be considered at risk of developing T-cell malignancies and L-HES T-cell clones as having a transformation potential. In particular, lymph node enlargement should alert clinicians to a possible disease progression to T-cell lymphoma.

Treatment.

An accurate diagnosis of the HES subtype is critical for effective management of the disease, and a recently published algorithm is provided in Figure 7 as an example [131]. Other diagnostic algorithms have been proposed with a similar view dictated by technical progress in diagnosis [132]. With a few exceptions, once diagnosed L-HES patients usually benefit from corticosteroids (CS) as a first-line therapy, depending on the morbidity caused by the eosinophilic expansion [133]. The CS effect results in the apoptosis-induction of the eosinophils and inhibition of T-cell functions including Th2 cytokine production and IL-2 dependant proliferation [134-136]. *In vivo*, CS efficiently decrease eosinophil levels in the majority of L-HES patients and thereby control clinical manifestations. However, excepted in two reported cases, the proportion of circulating CD3-CD4+T cells was not modified by CS therapy [133, 137].

The calcineurin inhibitor, cyclosporin, has been used efficiently to treat several autoimmune diseases but only for one reported case of HES [138]. Hence, this approach is not favoured as a first line therapy for L-HES particularly in light of the severe side-effects observed including secondary lymphomas and virus reactivation associated with its inefficiency in retrospective multicentric studies on HES [139] and L-HES patients (A.Klion, personnal observation). Given the long-term side effects and CS-resistance, second-line therapies has been used for L-HES including IFN-α, cytotoxic agents (hydroxyurea) and monoclonal antibodies (anti-IL-5, anti-CD52). IFN-α has been shown to antagonize Th2 responses, mainly IL-5 production [140]. Moreover, IFN-α therapy has been associated with the partial regression of pathogenic CD3-CD4+ T-cells in two patients [141]. However, a subsequent report indicated that IFN-α prolongs the survival of CD3-CD4+ T-cells *in vitro* by inhibiting their spontaneous apoptosis [142]. Therefore, considering the >30% probability of progression to T lymphoma in L-HES patients, it is recommended that IFN-α be avoided as a monotherapy and that other treatments are favoured for CS-sparing purposes.

Other agents include antibodies that recognize surface receptors. For example, anti-CD52 (Alemtuzumab), which binds to a receptor expressed on the surface of both eosinophils and T cells, has been used to successfully treat one L-HES patients with a CD3-CD4+ T-cell clone [143-144]. Moreover, endogenous expression levels for the CD52 gene are high (based on estimate from mRNA expression) in the CD3-CD4+ T cells from P1, P2

and P3 L-HES patients [145]. A recent analysis revealed that CD52 is well expressed on the surface of clonal CD3-CD4+ T cells from a newly diagnosed patient. Thus, Alemtuzumab's potential as a therapeutic agent for L-HES needs to be further evaluated in clinical trials.

An anti-IL-5 antibody (Mepolizumab) was recently tested in a large randomized cohort of HES patients who were negative for the *FIP1L1-PDGFRA* fusion gene [146]. This treatment showed rapid control of eosinophilia and prevented a recurrence or progression of tissue lesions. Moreover, it permitted a reduction of glucocorticoid use without any adverse events. Prospective and retrospective multicenter studies of a response to therapy in HES confirmed these promising results in a majority of L-HES and I-HES patients (Roufosse F. et al., JACI, in press and [139]). However, the long term effects of anti-IL-5 therapy on the aberrant T-cells population remain unknown.

Finally, since L-HES represents a distinct clinical entity whereby the clonal expansion of aberrant T cell population leads to T lymphomas in about one-third of patients, it is important to identify effective therapies. L-HES patients with lymphoma have been treated with classical cytotoxic regimens such as fludarabine, which failed indicating that more aggressive or targeted therapy is necessary, including conventional or reduced-intensity conditioning allogeneic hematopoietic cell transplantation [113].

2.3.3 Idiopathic/undefined HES

It is critical to differentiate this HES subtype from other pathologic situations like parasitic infection, allergies or tumour diseases also generating secondary hypereosinophilia through Th2 lymphokines overexpression. In Figure 6, patients in I-HES are grouped into three categories: benign, episodic and complex [99]. Patients who are fulfilling the 3 criteria and do not have the *PDGFR* rearrangements or myeloproliferative disorders's symptoms nor signs of L-HES, are included in the complex undefined HES subset. Patients with hypereosinophilia without organic lesion and patients with angioedema fall into the benign or episodic I-HES categories, respectively. Diagnostic algorithm is given in Figure 7 [131].

2.3.4 Diagnostic algorithm for HES

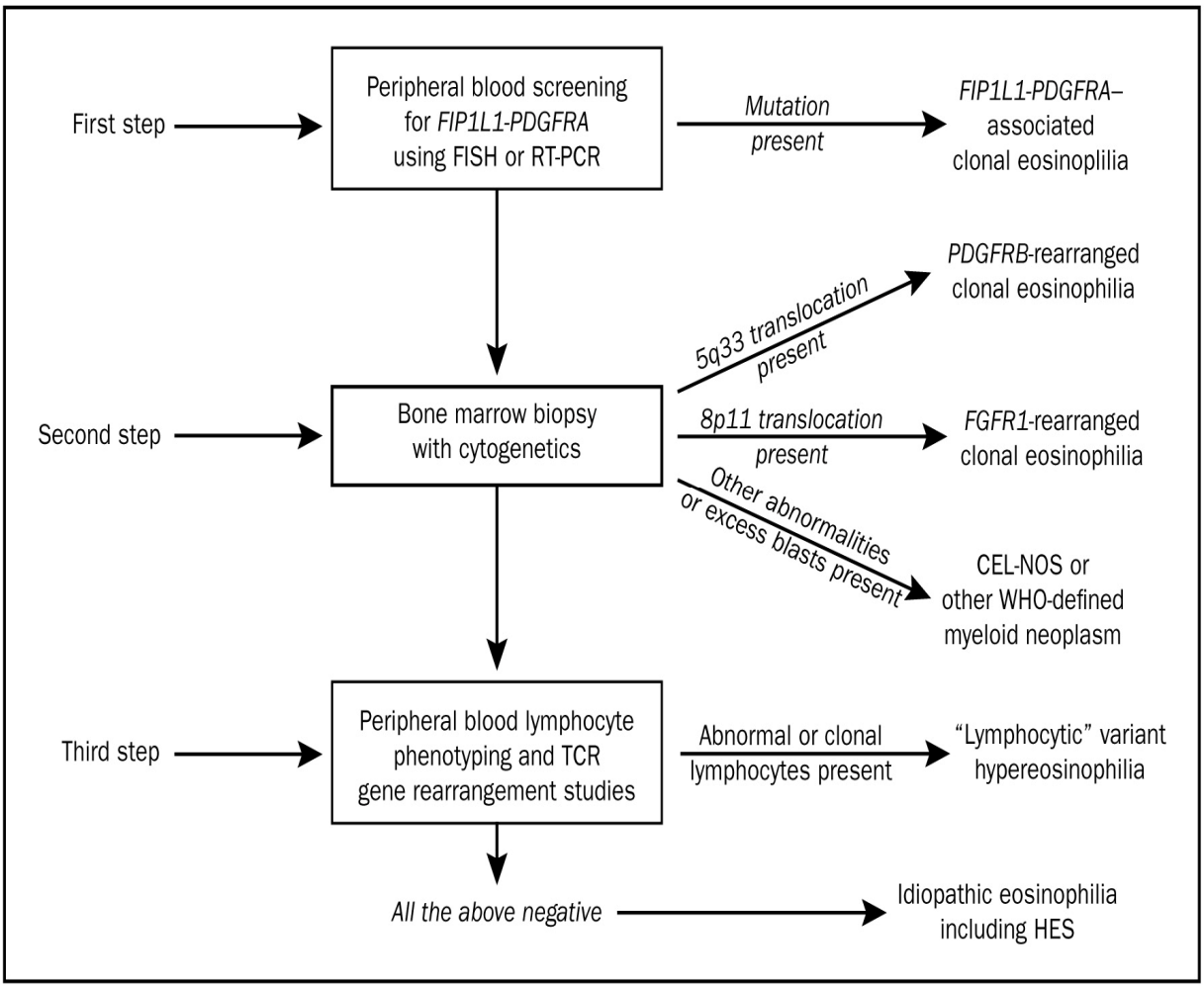


Figure 7.Diagnostic algorithm for HES (Source: Tefferi A., [131]).

When excluding secondary eosinophilia, the stepwise approach (described in figure 7) defines five specific diagnostic entities for HES that each dictates a distinct therapeutic approach. They require assessment of the peripheral blood smear, bone marrow morphologic aspects, cytogenetic and molecular analysis, peripheral blood phenotyping and T-cell receptor gene rearrangement studies.

First, after finding a peripheral blood smear indicating an underlying myeloid malignancy, it is reasonable to start with the detection of FIPL1-PDGFRA using FISH or RT-PCR on the peripheral blood, followed by bone marrow screening if the mutation is present. If peripheral blood screening for FIP1L1-PDGFRA is negative, the next diagnostic step is to

perform cytogenetics and morphologic examination to identify the presence or absence of 5q33 or 8p11.2 rearrangements, which, must be then confirmed by FISH and/or RT-PCR to definitively establish the involvement of the PDGFRB or FGFR1 genes.

Morphological analysis helps to differentiate systemic mastocytosis from chronic myelomonocytic leukemia and chronic eosinophilic leukemia (CEL-NOS). Definitive diagnosis of systemic mastocytosis requires the detection of morphologically abnormal mast cells, aberrant CD25 surface expression and /or detection of the *KIT*D816V mutation. The diagnosis of CMML requires the presence of more than 1 x 10^3/microL of peripheral blood monocytes associated with cytogenetic or morphologic features of a myeloid malignancy that is non-classifiable. CEL-NOS is characterized by the presence of cytogenetic abnormality or more than 2% or 5% blasts in peripheral blood or bone marrow, respectively.

Finally, the diagnosis of lymphocytic, idiopathic and minor subcategories of hypereosinophilia requires peripheral blood lymphocyte phenotyping and T-cell receptor gene rearrangement studies following exclusion of clonal eosinophilia.

3. T lymphomagenesis

3.1 Introduction

Lymphomas and leukemias are cancers of the blood. These diseases are in very rare cases associated with inherited conditions such as Bloom syndrome (OMIM 210900) or Ataxia telangectasia (OMIM 208900) resulting from germinal gene mutation. However, the vast majority of the hematologic malignancies occur as sporadic cancers arising from new mutations in a previously normal somatic cell. Lymphoid neoplasms are characterized by an abnormal proliferation of cells generally issued from the lymph nodes but also from the spleen, tonsils or the skin that often expands into the bone marrow. Normal maturation of lymphoid lineage, as seen before, involves many complex and highly controlled processes that regulate differentiation and proliferation of B and T-cells. Thus, as for any cancer cell, disruption of these regulatory pathways may confer a proliferative advantage to a lymphoid cell at any stage and leading ultimately to leukemia- or lymphomagenesis.

3.2 Principles of Cancer Development

3.2.1 Generalities

One of the earliest hypotheses that tumours originate from genetic alterations is rooted in the early 1900 with Theodor Boveri's postulate [147]. The idea that tumour growth could be linked to chromosomal rearrangements was strongly reinforced by the discovery of the Philadelphia chromosome and the identification of the translocation t(9;22)(q34;q11.2) in chronic myeloid leukemia [148]. Since that time and with the development of cytogenetics, recurrent chromosomal aberrations have been discovered and incorporated in cancer diagnosis and treatment (Mitelman F., 1994, *Catalog of chromosome aberrations in cancer*, 5th edition, Wiley-Liss, NY). For decades, cancer research has then focused on identification of the major actors of oncogenesis. So far, only percent of human genes have been identified as pivotal players in cancer pathogenesis, such as the control of growth, apoptosis, differentiation, senescence, angiogenesis, invasion and metastasis [149]. The functional categorization of "cancer genes" into dominant oncogenes and recessive tumour suppressors comes from the time of discovery of the first viral oncogenes [150-151]. The utility of this

classification is that it provides a functional link between genetic defects and carcinogenesis. In cancer cells, cellular proto-oncogenes are activated in oncogenes by a gain-of-function event promoting cell proliferation whereas anti-oncogenes or tumour suppressor genes limiting cell survival are inactivated by a loss-of-function event.

3.2.2. Oncogenes

Retroviral genes are easily identified as oncogenes by their ability to transform normal cells following infection [151]. Similarly, their cellular relatives or proto-oncogenes have been found to drive tumour formation when mutated [152]. Other typical gain-of-function events commonly found in haematologic malignancies are reciprocal translocations generating chimeric fusion genes with new oncogenic properties. Classical breakpoints observed in T lymphoid diseases involve the T-cell receptor alpha, beta or delta chain loci and the anaplastic lymphoma kinase gene (ALK) [153]. Identification of such translocations contributes to establishing the diagnosis of malignant T lymphoid disorders by correlating cytogenetics and immunophenotypic characteristics such as acute lymphoblastic leukemia, non Hodgkin lymphoma, chronic lymphocytic leukemia and adult T-cell leukemia (summarized by J.Rowley in Medical Genetics. *Emmery and Rimon's*. Churchil-Livingstone. Fifth edition, 2007, p.1755).

3.2.3 Tumour suppressor genes

Early experiments in 1969 with somatic cell fusion established the existence of antioncogenes that were recessive and could suppress tumourigenicity [154]. Later, A. Knudson predicted the existence of tumour suppressor genes (TSG) and proposed the "two-hit" model of tumorigenesis, which was soon confirmed by Comings et al. [155-156]. The inactivation of tumour suppressor genes varies and can result from partial or complete gene deletions, point mutations or epigenetic events such as microRNA targeting, promoter methylation or chromatine modification reviewed in [157]. These inactivation events produce either somatic or germinal mutations, with the latter dominantly implicated in a hereditary predisposition to cancer [158].

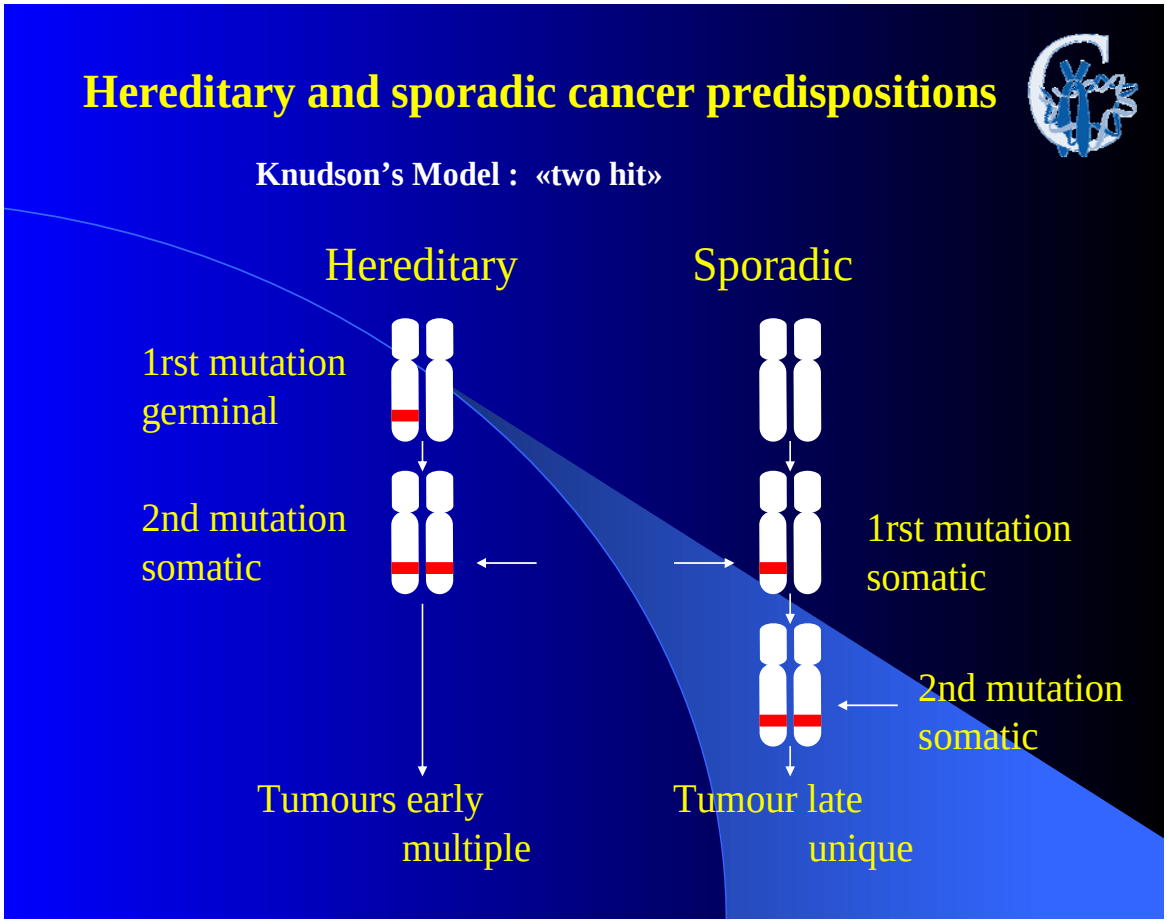


Figure 7.Two-hit model of A.Knudson (Source: Sibille C., *Club de Génétique, XXIX* , 2005)

3.2.3.1 Tumour suppressor gene function

Tumour suppressor gene (TSG) products possess numerous properties and are involved with the key pathways of cell physiology sometimes executed by the same protein, such as apoptosis, DNA reparation, cell cycle or differentiation. Depending on their normal function, TSG are usually classified as gatekeeper, caretaker or landscaper [159].

Gatekeepers. Gatekeeper genes regulate cell proliferation by negatively controlling the cell cycle. They induce cell death or the cell cycle arrest of potentially tumorigenic cells. Alteration of a gatekeeper gene results in an immediate and persistant disequilibrium in favour of cellular division, which leads to uncontrolled cell proliferation and malignant transformation. Therefore, the mutation of gatekeepers is associated with tumour initiation. However, tumour initiation is not necessarily the primary mutation event. Indeed, several

somatic mutations can accumulate in a normal tissue before a mutation in a gatekeeper initiates neoplastic transformation. Every cell type has one or more gatekeeper genes. For example, adenomatous polyposis coli gene (*APC*) (OMIM 175100) is the gatekeeper of the colon epithelium, retinoblastoma 1 gene (*RB-1*)(OMIM 180200) of the retinoblasts and von Hippel-Lindau gene (*VHL*)(OMIM608537) of the renal cells.

***Caretakers*.** Caretakers suppress cancer by maintenance of genome integrity. Upon DNA damaging events, cell cycle arrests to checkpoints G1/S or G2/M to allow the DNA-repair enzymes to correct genomic defects. If DNA breakages are too important, the gatekeepers promote cell apoptosis. Caretakers include either genes directly involved in double strand-DNA-break reparation such as mutL homolog 1, colon cancer, nonpolyposis type 2 (*MLH1*) (OMIM120436) or mutS homolog 2, colon cancer, nonpolyposis type 1 (*MSH2*) (OMIM609309) genes or genes responsible for the coordination of cell cycle arrest with DNA reparation such as breast cancer 1 (*BRCA1*) (OMIM113705) or ataxia telangiectasia mutated (*ATM*) (OMIM607785) genes. The mutation of caretakers does not lead directly to tumour initiation. However, their alteration induces multiple somatic mutations, which ultimately occur in the two alleles of a gatekeeper suppressor gene, resulting in neoplastic transformation. Notably, Knudson's "two hit" model does not apply here, considering that four hits are necessary for inducing tumour initiation.

***Landscapers*.** Landscaper genes modulate the microenvironment of the tumour cell and directly or indirectly promote the neoplastic conversion of the adjacent cells whose growth is polyclonal. For example, stromal cells with a mutation or T-cell mutation in the SMAD family member 4 (*SMAD4*) gene contribute to polyp initiation in the epithelial cells of gastrointestinal cancers of patients with juvenile polyposis syndrome [160]. Similarly, partial loss in smooth muscle cells of the *STK11* gene causing Peutz Jeghers syndrome results in polyp formation by generating a permissive environment [161]. Inversely, tumour cells harbouring mutated landscapers are able to modify the micro-environmental compartment. Several TSG, such as *BRCA1* gene, harbour both caretaker and landscaper properties [162].

3.2.3.2 Knudson theory

The Knudson model is based on epidemiological studies of sporadic and familial retinoblastomas proposing a mechanistically link for these two forms. Knudson postulated that in familial form, one mutation (the first hit) in a TSG is inherited, while a second mutation (the second hit) occurs in somatic cells, thus significantly delaying the onset of retinoblastoma [155]. In the nonhereditary form, two mutations (two-hit) are necessary prior to retinoblastoma initiation with an occurrence delay. Soon after, biallelic mutations were found in both sporadic and hereditary retinoblastomas supporting the two-hit's theory [163]. These observations have led to the longstanding paradigm of the obligate recessivity of tumour suppression imposing biallelic neutralization for oncogenesis. Although, this model has served the cloning of major TSG with potential cancer predisposition diagnosis, it no longer unifies the complex view of tumour suppression, including haplo-insufficiency and dominant negative mutations concepts.

3.2.3.3 Haplo-insufficiency

Haplo-insufficiency was defined by C. Stern in 1943 as a condition where the inactivation of one single allele is sufficient to change the phenotype of a diploid cell (Stern C. 1943, *Genetics*, 28, 441—475, "genic action as studied by means of the effects of different doses and combinations of alleles"). Translation of this concept in tumour suppression genetics does not reconcile with Knudson's "two-hit" model. Indeed, haplo-insufficiency at the locus of a TSG produced by a first hit, may overcome the need for a second hit at the remaining allele in order to generate a cancer phenotype [157, 164]. Evidence of haplo-insufficiency in cancer genetics has been obtained for several genes such as cyclin-dependent kinase inhibitor *CDKN1B* (p27kip1), *TP53*, phosphatase and tensin homolog (*PTEN*), *APC*, NK3 homeobox 1 (*NKX3.1*), and *SMAD4* genes [165-170]. Notably, the haplo-insufficiency concept was solidified through the analysis of tumours from knockout mice models or from hereditary cancer patients carrying heterozygous null mutations [171-172]. For example, when compared to normal or homozygous CDKN2B-/- knockout mice, CDKN2B+/- heterozygous mice were shown to display an intermediate number of tumours upon genotoxic exposure while retaining the wild type CDKN2B allele. Similarly, Nkx3.1+/- mice developed prostatic epithelial hyperplasia and intraepithelial neoplasia (PIN) without any loss of the remaining normal allele [173]. Moreover, analysis of the hyperplastic regions demonstrated

an extreme sensitivity of gene circuits controlling cell proliferation to Nkx3.1 dosage. Finally, as seen in the "just right" model of APC, the requirement of a second hit to promote tumourigenesis is clearly depending on the molecular nature and pathogenesis of the first mutation [174]. Haplo-insufficiency is thus dependant on a gene-dosage effect that, when exceeding a certain limit, interferes with a fundamental regulatory pathway and directly or indirectly induces oncogenesis through environmental modifications that promote cell proliferation.

3.2.3.4 Dominant negative mutations

Several mechanisms can explain haplo-insufficiency in cancer cells. Among them, the dominant negative effect of mutations in tumor suppressor genes such as *TP53*, Wilms tumour 1 (*WT1*), has been demonstrated [175-176]. Their derived proteins, which act as oligomeric complexes, are dominantly inactivated by one mutant protein that blocks the normal function of the all complex. Single truncating *APC* mutations have been shown to act dominantly by initiating spindle checkpoint defects and providing a cell growth advantage [177]. Thus, tumour suppressor genetics is more complex than expected, since most gene loci (like *TP53* or *APC*) discovered through biallelic recessive mutations display *bona fide* haplo-insufficient characteristics [178].

3.2.3.5 Identification of tumour suppressor genes

The identification of TSG has progressed with the evolution of genomic analytic tools. The first discovered TSGs such as CDKN2A, BRCA1, RB-1 or PTEN were isolated either by linkage analysis on a large cohort of patients or by identification of biallelic losses using locus-specific techniques such as chromosome analysis with fluorescence in situ hybridization (FISH) or by microsatellite analysis on tumour cells carrying chromosomal deletions [163, 179-181]. Recently, high-throughput methods have been introduced, allowing the large-scale analysis and identification of DNA copy number variations with a significant increase in resolution [182]. Both oligonucleotide arrays, originally restricted to detect single-nucleotide polymorphisms, or BAC–based arrays have been proven effective in assessing genomic imbalances in cancer cells with high resolution [183]. In addition to high resolution array-based comparative genomic hybridization (aCGH), other microarray-based procedures have

led to major breakthroughs in the detection of gene expression alterations, epigenetic changes and loss of heterozygosity (LOH) in tumours. Notably, combined information generated by genomic arrays with that provided by expression arrays has permitted the localization of novel candidate suppressor genes in several conditions such as NK /T lymphomas, and B lymphomas [184-185]. However, these genes, classically flagged by their transcriptional downregulation within monoallelic or biallelic deleted chromosomal regions, need to be further tested for their tumour suppressive properties before being labelled as *bona fide* tumour suppressors.

Usually, the criteria used for determining tumour suppressor properties are based on the phenotypic reversion of tumour cells. The expected phenotypic changes are: decreased growth or growth arrest, morphological changes, loss of independent growth *in vitro* and decreased clonogenicity. The major change is represented by the loss of tumourigenicity in animal models (immunodeficient nude mice). The phenotype obtained by tumour suppressor gene activity is mediated through multiple cellular pathways, including cell cycle inhibition, cellular growth, apoptosis or DNA reparation. Practically, not all criteria are necessarily present for one TSG. A demonstration of suppressive activity can be obtained either through inactivation or reintroduction of the tumour suppressor gene. *In vivo*, inactivation is obtained with knockout mice models, whereas *in vitro* loss-of-function is mediated by si or shRNAs at the cellular level. In the past, the restoration of tumour suppressive activity was performed by somatic cell hybrid fusion techniques that have since been replaced by microcell fusion with a chromosomal fragment or BAC containing specific genomic regions.

3.3 Frequency of the Chromosome 6q Deletions

3.3.1 Chromosome 6q deletions in solid tumours

Data from the literature indicate that the long arm of chromosome 6 is recurrently lost in a vast panel of tumours. 6q imbalances are frequently detected in solid and soft tissue tumours including breast carcinoma, epithelial ovarian carcinoma, prostate cancer, colon carcinoma, gastric carcinoma, pancreatic cancer, hepatocellular adenoma, squamous cell carcinoma, melanoma, osteosarcoma, chondrosarcoma, liposarcoma, mesothelioma,

subependymoma, medulloblastoma and salivary gland carcinoma [186-200]. Such abundant reports of 6q loss strongly suggest that this chromosomal region is harbouring at least one or more tumour suppressor genes awaiting further identification. The first indication that chromosome 6 carries gene(s) with suppressor properties arises from observation of the transformation induced by the spontaneous loss of chromosome 6 and the senescence produced by reintroduction of normal 6q arm in SV-40 immortalized fibroblastic cells [201]. Further demonstration that 6q loci are involved in tumour suppression comes from somatic cell hybrid experiments with microcell-mediated chromosome 6 transfer within breast and ovarian cancer cell lines [202-204]. For example, these experiments have refined minimal suppressive regions at 6q15-24.1, 6q23.3-24.3 and 6q25.3-qter for breast cancer, or recently at 6q27 for primary ovarian tumours, respectively [187]. However, the extraordinary multiplicity of 6q deletions revealed by the genome-wide aCGH studies of solid tumours suggests that the potent suppressor genes located there are either tissue-specific or display different roles at different tumour stages, in concert or separately.

3.3.2 Chromosome 6q deletions in lymphoproliferative diseases

The first observations of chromosome 6 long arm deletions in lymphoid malignancies were made in 1985 and 1989 with Burkitt lymphomas and mature T-cell leukemias, respectively [205-206]. Importantly, although frequently observed in lymphoid conditions, 6q alteration is generally not detected in myeloid leukemias. Numerous studies on various lymphoid diseases have reported 6q deletions in large patient cohorts in order to define minimal commonly deleted regions susceptible to home antioncogene(s). Despite a great technical heterogeneity, deletions of four major regions have been reported, including 6q14-15 [185, 207-208], 6q16-21[208-215], 6q23 [209, 216-218] and 6q25-27 [209, 216, 219]. However, for most of these regions, no direct correlation has been demonstrated between a particular gene locus and a specific malignant process, except for the tumour suppressor genes discussed below.

3.4 6q Candidate Tumour Suppressor Genes

BTB and CNC homology 1, basic leucine zipper transcription factor 2 (BACH2) is located at chromosomal band 6q15 [220]. It encodes a transcription factor member of the broad complex tramtrack bric-a-brac (BTB)-leucine zipper (bZip) factor family, which binds to the Maf recognition element (MARE), including the AP-1 sequence [221]. BACH2 forms heterodimers with a small Maf transcription factor to inhibit usually target genes, as illustrated by the repression of the 3'-enhancer of the immunoglobulin heavy chain [222]. BACH2 protein normally localizes in the cytoplasm, but nuclear accumulation is induced under oxidative stress, resulting in cell apoptosis [223]. BACH2 is expressed in B-cell compartment before the plasma cell stage, neuronal cells, umbilical cord blood and adult CD4+ T-cells [220, 224]. Mice deficient for BACH2 are severely impaired in class switch recombination (CSR) and the somatic hypermutation of immunoglobulin genes demonstrating the crucial role of BACH2 in B-cell mediated immunity [225]. Several reports have pinpointed *BACH2* as a candidate tumour suppressor gene in B cell compartment by showing a frequent loss of heterozygosity in non Hodgkin lymphomas and a reduction of clonogenicity with increased sensitivity to apoptosis in *BACH2* over-expressing Raji cells [220, 226]. Moreover, the transcription factor *BACH2* has also been described as a useful prognosis marker to predict disease-free and overall survival in patients with diffuse large B-cell lymphomas [227]. Furthermore, observation of non-random BACH2 gene inactivation either by Epstein-Barr virus (EBV) integration into the 6q15 chromosomal band of Burkitt lymphoma cell line (Raji) or recurrent insertions of the human immunodeficiency virus (HIV) at the BACH2 locus in resting CD4+ T-cells from patients receiving antiretroviral therapy have suggested that target-site preference contributes to lymphomagenesis or viral persistency [220, 228].

Glutamate receptor, ionotropic, kainate 2 (GRIK2) gene is positioned at 6q16.3 band. Glutamate receptors are the predominant excitatory neurotransmitter receptors mainly expressed in the brain. Mutations in this gene have been associated with autosomal recessive mental retardation [229]. Furthermore, *GRIK2* has been identified as a potent tumour suppressor gene by finescale mapping of a minimally deleted region defined at 6q16 among a large cohort of ALL cases [215, 230]. Repression of *GRIK2* was also reported recently in a gene expression profiling study of adult T-ALL. Although an inactivating germline mutation

was identified on the second allele of *GRIK2* in one case, its relationship with the malignant process has not yet been clearly established for this gene.

HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1 (HACE1) is located at the 6q16.3 chromosomal band. This gene has been proposed as a tumour suppressor gene in multiple human cancers. For example, a high rate of aberrant methylation of HACE1 gene has been reported in colon carcinomas [231], gastric carcinomas [232] and Wilms tumours [233]. Additionally, re-expression of the HACE1 gene in several human tumour cell lines abrogates tumorigenicity both in vivo and in vitro [234]. Finally, HACE-/- deficient mice spontaneously develop late onset cancers with higher multiplicity in a P53-/+ context [234]. Interestingly, a significant repression of the HACE1 gene associated with frequent deletion of band 6q21 was recently reported in gene profiling survey of extranodal NK/T-cell lymphomas, nasal type [235]. The authors suggest that deregulation of HACE1 gene expression might be involved in NK/T-cell lymphomas pathogenesis.

PR domain containing 1, with ZNF domain (PRDM1) gene located at 6q21 was first identified as a repressor of the human interferon (IFN)-β promoter [236]. Two alternatively spliced transcript variants that encode different isoforms have been reported, namely *PRDM1* α and β [237]. The PRDM1α encodes the full-length *PRDM1* protein whereas the *PRDM* β isoform lacks the PR domain preventing repressor activity [238]. PRDM1 protein is required for the formation of germ cells and the early embryonic development as *PRDM1*-null mice die at day 10.5 [239]. In the adult life, *PRDM1* has shown to be a master regulator of a wide variety of cell lineages. In B cells, *PRDM1* is necessary for terminal differentiation into antibody-secreting plasma cells, while in T-cells, it has been shown to regulate the homeostasis of effector and memory T-cells [240-242]. Published data have indicated that *PRDM1* is induced by IL-2 production in primed T cells and that *PRDM1* feeds back to inhibit IL-2 production by repressing the IL-2 promoter. This suppression mechanism is proposed to attenuate the proliferation of activated T-cells and to promote apoptosis [243]. Together, these data indicate that *PRDM1*'s deregulation provides to T- or B-cells with a proliferative advantage by blocking their full differentiation and expansion. Evidence that PRDM1 functions also as a tumour suppressor gene comes from several studies conducted on myeloma, Waldenström disease, diffuse large B-cell lymphomas (DLBCL), Hodgkin's lymphoma, T-cell lymphoma and leukaemia, respectively [237, 244-246]. Consistent with Knudson's theory the gene alterations observed are homozygous deletions or biallelic

inactivations such as heterozygous deletions, mutations and transcriptional silencing. It is notheworthy that high expression of the inactive *PRDM1* β isoform is preferentially detected in myelomas and T-cell lymphomas [247].

Sestrin 1 (*SESN1*) located at 6q21 is a DNA damage-inducible gene (GADD). Expression of two of its three protein isoforms is modulated by p53 after genotoxic stress [248]. These proteins are cysteine sulfinyl reductases required for peroxiredoxin regeneration and as such, they represent a crucial antioxidant defense that protects the cell from DNA damage. Indeed, the suppression of *SESN1* generates an accumulation of the intracellular reactive oxygen species, inducing cell death [249]. Moreover, the introduction of a mutant RAS gene in normal fibroblasts downregulates the two active SESN1 isoforms resulting in an increase of ROS levels, DNA oxidation and accelerated mutagenesis in the transformed cells [250]. This effect is transient in mutant cells expressing functional p53 which accumulate in response to oncogenic RAS expression and reverse SESN1 inhibition.

REV3-like, catalytic subunit of DNA polymerase zeta (*REV3L*) gene situated within band 6q21 encodes a subunit of the polymerase zeta specialized in translesion DNA synthesis (TLS) allowing cells to tolerate damaged DNA by genotoxicity without repair [251]. This gene, which resides at band 6q21 has been previously described as possessing tumour suppressor gene properties [252]. Furthermore, mice deficient for the *REV3L* gene in a *P53* -/- or -/+ context have been recently shown to develop earlier thymomas or multiple breast cancers, thus demonstrating that the loss of *REV3L* can promote tumorigenesis [253].

Protein tyrosine phosphatase, receptor type, K (PTPRK) gene locus is positioned at chromosomal band 6q22.33. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family, which regulates a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. The PTPRK gene was identified as a candidate suppressor gene in primary central nervous system lymphomas by a loss of heterozygosity mapping and RT-PCR [218]. Furthermore, deletion of this gene is associated with poor disease prognosis [254]. Expression of this gene is controlled by TGFβ and by downregulating the PTPRK gene, Epstein-Barr virus (EBV) contributes to the growth of infected Hodgkin lymphoma cells [255]. Conversely, enforced expression of PTPRK gene in EBV infected cell lines restrains cell growth there by demonstrating suppressive properties of this gene.

Tumour necrosis factor, alpha-induced protein 3 gene (TNFAIP3/A20) is a negative regulator of the NF-kappaB pathway positioned within chromosomal band 6q23.3 [256], which has shown to be associated with autoimmune risks [257-258]. This gene has recently been identified as a tumour suppressor gene whose homozygous deletions and/or mutations preferentially promote B lineage lymphoma development [259-260]. In particular, two reports described frequent biallelic somatic mutations in mucosa-associated tissue (MALT) lymphomas, Hodgkin's lymphomas (nodular sclerosis type) and at a lower frequency in the DLBCL of activated-B-cell-like (ABC) type and in follicular B lymphomas. Furthermore, reintroduction of the A20 gene in the A20-inactivated DLBCL cell line resulted in cell growth arrest and apoptosis indicating a tumour suppressor role. This was confirmed by in vivo inoculation of A20 reconstituted cells in nude mice showing suppressed tumorigenicity.

Parkinson disease (autosomal recessive, juvenile) 2, parkin (PARK2) gene located at 6q25.2-27 encodes a protein which is a E3 ubiquitin ligase that mediates the targeting of substrate proteins for proteasomal degradation such as cyclin E. Mutations in this gene are known to cause Parkinson's disease and autosomal recessive juvenile Parkinson's disease. However, one recent report has shown that inactivating somatic mutations and frequent intragenic deletions of PARK2 occur in multisite human malignancies, thereby abrogating the growth-suppressive effects of the PARK2 protein [261].

3.5 T Lymphomas

3.4.1 WHO classification

In the WHO 2008 classification of tumours of haematopoietic and lymphoid tissues, T-cell neoplasms reproducing the stages of normal T-cell differentiation are, to some extent, stratified according to the corresponding normal stage. However, as seen in the preceding section, T-cell lymphomas are heterogenous and functionally complex, showing lineage plasticity. The classification presented below combines all information currently available from the clinic, morphology, immunophenotype and genetics to define mature T-cell entities excluding the T lymphoblastic leukemia/lymphoma which is part of the precursor lymphoid neoplasms (Table 2., [262]).

WHO 2008: the mature T-cell and NK-cell neoplasms.

T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK-cells*
Aggressive NK cell leukemia
Systemic EBV$^+$ T-cell lymphoproliferative disease of childhood (associated with chronic active EBV infection)
Hydroa vacciniforme-like lymphoma
Adult T-cell leukemia/ lymphoma
Extranodal NK/T cell lymphoma, nasal type
Enteropathy-*associated* T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30$^+$ T-cell lymphoproliferative disorder
    Lymphomatoid papulosis
    Primary cutaneous anaplastic large-cell lymphoma
*Primary cutaneous aggressive epidermotropic CD8$^+$ cytotoxic T-cell lymphoma**
Primary cutaneous gamma-delta T-cell lymphoma
*Primary cutaneous small/medium CD4$^+$ T-cell lymphoma**
Peripheral T-cell lymphoma, not otherwise specified
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma (ALCL), ALK$^+$
*Anaplastic large cell lymphoma (ALCL), ALK$^-$**

*These represent provisional entities or provisional subtypes of other neoplasms.
Diseases shown in italics are newly included in the 2008 WHO classification.

Table 2.Source: *WHO classification of haematopoietic and lymphoid tissue tumours*, 2008

3.4.2 Epidemiology

The SEER (Surveillance Epidemiology and End Results) program survey in the United States indicates an incidence rate per population (100000 persons) per year of 33.65 for all lymphoid neoplasms, with 26.13 for B-cell neoplasms and 1.79 for all T-cell neoplasms [263]. Mature T-cell and NK neoplasms are relatively uncommon, accounting for only 12 percent of all non-Hodgkin's lymphomas [264]. The most common subtypes of mature T-cell lymphomas are peripheral T-cell lymphoma not otherwhise specified (NOS) (25.9%) and angioimmunoblastic T-cell lymphoma (AITL) (18.5%) respectively [265].

Significant variations in incidence are shown in different geographical regions and racial populations. T-cell lymphomas are more frequent in Asia, and one of the major risks is the HTLV-1 virus infection. Other regions like the Caribbean basin have also experienced an increased incidence of HTLV-1-related T-lymphomas. Extra nodal NK/T-cell lymphoma, nasal type, aggressive NK leukaemia, EBV+ T-cell and NK lymphomas are also linked to racial predisposition, as commonly observed in Asians [266]. Infectious agents have been shown to contribute to the development of several types of B/T neoplasms. In particular, viruses such as EBV, HTLV-1, HHV8 and Hepatitis C, or bacteria like H. pylori, have been directly or indirectly associated with lymphoma or leukemagenesis [267-271]. Furthermore, epidemiological studies have implicated herbicide and pesticide use in the development of specific B-cell lymphomas [272]. Finally, a major risk factor for mature lymphoid neoplasms appears to be immunodeficiency or autoimmune diseases (HIV infection, Hashimoto thyroiditis, Sjögren disease, ALP syndrome, celiac disease, etc…) [273-277].

Table 3.Relative frequencies of mature T-cell lymphoma subtypes

Peripheral T-cell lymphoma-NOS	25.9%
Angioimmunoblastic	18.5%
Extranodal natural killer/T-cell lymphoma	10.4%
Adult T-cell leukemia/lymphoma	9.6%
Anaplastic large cell lymphoma, ALK+	6.6%
Anaplastic large cell lymphoma, ALK	5.5%
Enteropathy-type T-cell (including EATL)	4.7%
Primary cutaneous ALCL	1.7%
Hepatosplenic T-cell	1.4%
Subcutaneous panniculitis-like	0.9%
Unclassifiable PTCL	2.5%
Other disorders	12.2%

(Table 3:Source: Vose J., *The International Peripheral T-cell lymphoma Project*, 2008 [265])

AIMS OF THE STUDY

L-HES is a rare disease characterized by symptoms linked with the persistence and proliferation of a CD4+ T-cell clone producing cytokines that induce polyclonal hypereosinophilia. This disease shares several characteristics with allergic diseases (allergic rhinitis, asthma, etc…), that exhibit hypereosinophilia driven by allergen specific CD4+ Th2 cells. The CD3-CD4+ T-cell clones detected in L-HES patients however are not directed to a specific antigen, thereby excluding the possibility of anti-idiotype or tolerization therapy. The work presented in this thesis addresses the question of what mechanisms are deregulated in the CD3-CD4+ T cells that allow them to persist and expand in L-HES patients, putting them at significant risk for the development of a T-cell lymphoma.

1. The first aim of this study was to characterize the phenotypic and immunological traits of the CD3-CD4+ T cell-clone found in the majority of L-HES patients.
   Furthering our understanding of the nature of the underlying abnormal T-cell population provides a basis for improved diagnosis and ultimately tailoring treatment for L-HES patients. This objective was achieved by producing a complete profile of the CD3-CD4+ T-cells, including their immunophenotype, cytokine and chemokine secretion pattern, infectious status and T cell receptor rearrangement.

2. The second aim of this work was to identify the genetic alterations linked with the sustained survival and expansion of the CD3-CD4+ T-cell clone. This goal was accomplished at the chromosomal, molecular and cellular levels by assessing the karyotype, the evolving molecular profiles as well as designing functional experiments to characterize mechanisms underlying the persistent expansion of the L-HES T-cell clones. Among the genetic aberrations detected, our ultimate aim was to identify potential suppressor genes and decipher the role they play in the normal apoptotic and proliferative mechanisms responsible for controlling CD4+ T cell growth. Understanding these events should provide new and improved diagnostic tools and therapeutic targets for L-HES associated T-lymphomas.

RESULTS

Part 1: Phenotypic Characterization of L-HES

The aim of the first part of the results was to extensively characterize the phenotype of the CD3-CD4+ T-cells isolated from the peripheral blood of L-HES patients. The first article and two annexes below present detailed descriptions of the immunophenotype and the immunological properties of L-HES T cell-clones. Special attention was also paid in these three articles to the clinical features and biological signs contributing to the diagnosis of this rare (or underestimated) disease.

Article 1

Defective CD3γ gene transcription is associated with NFATc2 overexpression in the lymphocytic variant of hypereosinophilic syndrome.

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This article provides a detailed analysis of the immunophenotype of the CD3-CD4+ T-cells from two L-HES patients during their disease evolution obtained by flow cytometry. A great similitude with remarkable stability was observed between the surface phenotypes exhibited by the CD4+T-cells from the two patients without any detection by the author of underlying viral infection classically involved in CD3 surface repression. In particular, an aberrant CD2+CD3-CD4+CD5+CD7-CD25-CD28+CD45RO+CD62L+CD69- T-cell memory immunophenotype characterizing the L-HES T-cell clones was observed with variable levels of CD69 expression during the clinical evolution of P1.

Maintenance of the long-term culture of IL2 dependent oligoclonal cell lines derived from the circulating CD3-CD4+ cells of P1 is described. Both surface immunophenotype and specifically TCR beta chain rearrangement were demonstrated by the author to be identical to uncultured CD3-CD4+ T-cells from P1. One cell line, deficient for TCR/CD3 expression, offered the opportunity to verify by northern blot the level of expression of the mRNAs encoded by the respective gene components of the TCR/CD3 complex. This analysis revealed a profound defect of the CD3γ chain gene transcription. Moreover, culturing of this CD3-CD4+ derived cell line allowed to perform electromobility shift assays, demonstrating the increased binding of the inhibitory factor NFATc2 to the CD3γ gene promoter.

ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 33 (2005) 1147–1159

Defective *CD3γ* gene transcription is associated with NFATc2 overexpression in the lymphocytic variant of hypereosinophilic syndrome

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Objective. **Determine the molecular defects underlying the $CD3^-CD4^+$ T-cell phenotype and persistence of this clonal population in patients with hypereosinophilic syndrome.**

Patients and Methods. **Patients in this study suffer from the lymphocytic variant of hypereosinophilic syndrome distinguished by a $CD3^-CD4^+$ T-cell clone that overexpresses Th2 cytokines upon activation and thereby provokes the eosinophilia. Interleukin-2-dependent $CD3^-CD4^+$ T-cell lines were derived from patient blood at various disease stages and used to investigate the molecular modifications correlated with their abnormal phenotype.**

Results. **We demonstrate that the $CD3^-CD4^+$ T cells, characterized by a clonal TCRβ gene rearrangement, maintained the same immunophenotype over the 6-year period of our study, during which one patient progressed from premalignant disease to $CD3^-CD4^+$ T-cell lymphoma. We show that a specific loss of *CD3γ* gene transcripts is responsible for the defect in TCR/CD3 surface expression. In addition, the level of NFATc2 binding to NFAT motifs in the *CD3γ* gene promoter was greatly increased in the abnormal T cells. Our studies indicate that *CD3γ* promoter activity can be positively influenced by NFATc1 plus NF-κB p50 and negatively regulated by NFATc2 containing complexes. We show that in patients' $CD3^-CD4^+$ T cells, an increase in nuclear NFATc2 occurs in parallel with a decrease in NFATc1 and NF-κB gene expression.**

Conclusion. **Hypereosinophilic syndrome joins the growing number of pathological conditions where a defect in surface expression and/or function of the TCR/CD3 complex results from altered regulation of *CD3γ* gene expression.** © **2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

An increasing number of geographically dispersed, epidemiologically unlinked patients with the lymphocytic variant of hypereosinophilic syndrome (HES) can be distinguished by a clonal population of T cells (reviewed in ref. [1]). Although these patient's aberrant T cells occur in a variety of different phenotypes ($CD3^-CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^-CD8^-$), the clones generally have a Th2 cytokine production profile and secrete various combinations of interleukin (IL)-4, IL-5, and IL-13 [2–6]. This clonal T-cell population overproduces Th2 cytokines in response to local costimulatory signals in vivo, leading to the characteristic hyperproduction of immunoglobulin E and hypereosinophilia [7]. Some patients with T cell–mediated HES eventually progress to a malignant hematological disorder [2,4,8,9]. In addition, the abnormal T cells frequently carry an abnormal karyotype at preneoplastic disease stages [3,10,11] and/or demonstrate resistance to apoptosis [12], both potentially important precursors to malignant progression. The molecular mechanisms that underlie the loss of T-cell receptor (TCR/CD3) expression and/or lead to increased production of the eosinophilic cytokines in these abnormal T cells are currently unknown.

The range of clinical conditions where defects in TCR/CD3 expression and function have been reported has expanded considerably in recent years from its initial

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association with various T-cell malignancies (reviewed in ref. [13]). The currently documented wide array of clinical conditions include healthy aged humans [14], chronic fatigue syndrome [15], kidney allografts [16], angioimmunoblastic lymphadenopathy [17,18] and ataxia-telangiectasia [19] leading to the development of T lymphoma, inflammatory diseases such as systemic lupus erythematosus [20] and rheumatoid arthritis [21], and infectious diseases such as mycobacterial [22], human herpesvirus (HHV-6 and HHV-7) [23], human T-cell leukemia virus (HTLV-I and HTLV-II) [24–26], and human immunodeficiency virus (HIV-1 and HIV-2) [27–29] infection. Diminished or defective surface expression in both peripheral blood and tumor-infiltrating T cells (TIL) has also been demonstrated for a wide variety of cancer patients (recently reviewed in ref. [30]) and includes Hodgkin and non-Hodgkin lymphoma, B-cell leukemia, cervical carcinoma, ovarian cancer, prostate cancer, metastatic melanoma, gastric cancer, oral carcinoma, colorectal carcinoma, and renal cell carcinoma. Most of these human studies investigated TCR/CD3 expression by flow cytometry or immunohistochemistry and found CD3ζ protein expression was reduced or lost. However, studies in mice demonstrated that in addition to reduced CD3ζ protein, CD3γ protein [31] or *CD3*γ, *CD3*δ, and *CD3*ζ mRNA [32] were also dramatically reduced in TIL from tumor-bearing mice. Our laboratory has been studying the molecular mechanisms that underlie the specific loss of *CD3*γ gene transcripts leading to a TCR/CD3$^-$ phenotype in CD4$^+$ T cells infected with HIV-1, HIV-2, and HTLV-I [33–37].

The appearance of TCR/CD3 defects in such a diverse group of chronic inflammatory and malignant diseases suggests that loss of TCR/CD3-directed antigen responsiveness also affords a distinct advantage for the survival and progressive transformation of the abnormal clone characteristic of T cell–mediated HES. A small cohort of Belgian patients with chronic idiopathic hypereosinophilia distinguished by their CD3$^-$CD4$^+$ T-cell clone has been followed for more than 6 years, with one patient progressing from a premalignant disease state to T-cell lymphoma during this period. In this patient, the original premalignant CD3$^-$CD4$^+$ T-cell population, carrying a clonal TCR gene rearrangement, spawned subclones with different chromosomal abnormalities from which a single cytogenetic subclone emerged as the malignant tumor cell (described in ref. [10]). This suggests that loss of surface TCR/CD3 complexes is an early event in malignant progression favoring the persistence, growth, and accumulation of additional aberrations in these abnormal T cells. We have investigated the molecular defect underlying the abnormal TCR/CD3 phenotype and other changes associated with this abnormal T-cell clone. Once again a specific loss of *CD3*γ gene transcripts is responsible for the TCR/CD3 surface defect. In addition, nuclear expression of the NFAT family of transcription factors, important in the induction of cytokine gene expression, was substantially increased in the abnormal CD3$^-$CD4$^+$ T cells. Thus, HES joins the growing number of pathological conditions where a defect in surface expression and/or function of the TCR/CD3 complex results from altered regulation of *CD3*γ gene transcription.

Material and methods

Patients

The clinical characteristics of the patients analyzed in this study have been previously described in detail [2]. Briefly, patient 1 (P1) is a 20-year-old female who exhibited severe cutaneous manifestations at diagnosis with the CD3$^-$CD4$^+$ T cells representing 69% of her peripheral blood mononuclear cells (PBMC). Her recent follow-up was marked by clinical symptoms that continued to worsen until her diagnosis with peripheral diffuse CD3$^-$CD4$^+$ T lymphoma of small to medium lymphocytes, type 4 (class II, REAL/peripheral T lymphoma, WHO) 6 years after the clone was first detected (detailed in ref. [10]). P1 maintained 65 to 70% CD3$^-$CD4$^+$ T cells in her PBMC until she was successfully treated by allogeneic familial stem cell transplantation. Patient 2 (P2) is a 21-year-old female who exhibited cutaneous symptoms and a population of CD3$^-$CD4$^+$ T cells representing 63% of her PBMC at initial diagnosis. P2 has responded well to continuous low-dose cortisone therapy, which reduced the CD3$^-$CD4$^+$ T-cell population to 4% of PBMC, the level still maintained today (detailed in ref. [10]).

Written informed consent for the collection and analysis of PBMCs was obtained from all patients and controls. This study was approved by the Ethics Committee of the Faculty of Medicine at the Université Libre de Bruxelles.

Antibodies

The following antibodies were used: 1) for cell surface labeling or magnetic bead separation: CD2(Leu5b), CD3(Leu 4), CD4(Leu3a), CD5(Leu1), CD7(Leu9), CD8(Leu2b), CD45RO(UCHL1), CD62L(Leu8), CD69(Leu23; all from BD Biosciences, Erembodegen, Belgium), CD25(H108; kindly provided by Dr. L. Moretta, Genoa, Italy), and CD28 [CLB-CD28/1; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands]; 2) for cell stimulation: CD28 (CLB-CD28/1) plus two CD2 antibodies (CLB-T11.1/1 and CLB-T11.2/1; all from CLB); and 3) for the electrophoretic mobility shift assay (EMSA) supershift assay: NFATc1(SC-7294X), NFATc2(SC-7295X), NF-κB p50 (SC-1190X) and NF-κB p65 (SC-109X; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell purification

Circulating leukocytes were obtained from patients either by venipuncture in 60-mL heparinized syringes or by cytapheresis. PBMC were isolated by density gradient centrifugation prior to separation of the abnormal CD3$^-$CD4$^+$ and normal CD3$^+$CD4$^+$ T cells by magnetic beads or flow cytometry as previously described [2,10]. The purity of these populations was checked after separation by flow cytometry and consistently determined to be $>95\%$ pure.

Cell cultures

The HES patient-derived $CD4^+$ T-cell lines were initially cultured in RPMI 1640 supplemented with 20% fetal bovine serum (FBS), 1.25 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-aspargine (complete RPMI) in 24-well plates. At successive intervals, 100 units per milliliter of recombinant human IL-2 (Cetus Corp, Emeryville, CA, USA) was added to individual cultures. To artificially activate the cells, cultures were treated for 18 hours with either a combination of anti-CD28 antibody plus two anti-CD2 antibodies or phorbol-12-myristate-13-acetate (PMA) (30 ng/mL; Calbiochem, VWR, Leuven, Belgium).

The WE17/10 cell line is an IL-2-dependent $CD4^+$ T-cell line established in our laboratory and maintained in complete RPMI containing 100 IU/mL of recombinant human IL-2 [33,38]. The human B lymphocyte line Raji was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% FBS.

Northern blot hybridization

RNA was extracted from 1 to 5 $\times$ 10^7 cells by the single-step method of isolation by acid guanidium-thiocyanate-phenol-chloroform extraction [39]. Poly$(A)^+$ RNA was selected on oligo (dT)-cellulose columns, treated with glyoxal, and 1.0 μg per sample was migrated in an agarose gel before transfer to Hybond N^+ membranes (Amersham, Belgium). The membranes were hybridized for 16 hours under stringent hybridization conditions using 3 $\times$ 10^6 cpm/mL of the labeled probe for each of the six TCR/CD3 chains in 3$\times$ SSC containing medium (1$\times$ SSC contains 0.15 M NaCl, 0.015 M Na citrate). The TCRα and TCRβ chain probes [40,41] were kindly provided by T. Mak; the CD3ε chain probe by B. Alarcon; the CD3γ chain probe by M.J. Crumpton (pJ6T3γ-2) [42]; the CD3ζ chain probe by A. Weissman; the CD3δ chain probe (pPGBC9) [43] was obtained from the American Type Culture Collection; and the G3PDH probe as described in ref. [44]. Plasmid inserts were labeled to approximately 10^9 cpm/μg with [α-^{32}P] dCTP (3000 Ci/mM) by the random prime technique (Amersham).

EMSA

Nuclear extracts were prepared from 10^7 cells and EMSAs were performed as previously described [36]. Oligonucleotides probes encoding wild-type and mutated NFAT binding motifs in the 5′ upstream region of the human *CD3γ* gene are as follows:

5′-TCCTTAACGGAAAAACAAAA-3′ ($NFAT_{\gamma 1wt}$)
5′-TCCTTAACCCTTAAACAAAA-3′ ($NFAT_{\gamma 1mut}$)
5′-GAGGTGGCTTTCCATTTGGA-3′, ($NFAT_{\gamma 2wt}$)
5′-GAGGTGGCTAAGGATTTGGA-3′ ($NFAT_{\gamma 2mut}$)

In the supershift assay, antibodies directed to NFAT or NF-κB proteins were preincubated with nuclear extracts for 1 hour on ice prior to the addition of the radiolabeled probe.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using the kit from Upstate Biotechnology generally following the manufacturer's protocol. P1-derived $CD3^-CD4^+$ T-cell lines were fixed with 1.5% formaldehyde for 10 minutes at 37°C. Chromatin was isolated, sheared using a Bioruptor (Diagenode, Liege, Belgium) and immunoprecipitated with antibodies directed to NFATc4 (SC-1153X, Santa Cruz Biotechnology), NFATc2 (67.1; kindly provided by A. Rao [45]) or control rabbit immunoglobulin G (Upstate Biotechnology). The presence of the $NFAT_{\gamma 2}$ sequence from the human *CD3γ* gene promoter [36,46] in the immunoprecipitated DNA was assessed by polymerase chain reaction (PCR) using the following primer pair to amplify a 200-bp product containing $NFAT_{\gamma 2}$: forward: 5′-CAGCCTGGGCAA CAAGT-3′; reverse: 5′-GTTGTTAGGATCGTAGCCAGTTG-3′.

The amplified ^{32}P-labeled PCR product was separated on a 6% acrylamide gel and detected by autoradiography.

Gene expression arrays

Flow cytometry was used to purify the $CD3^-CD4^+$ T-cell population, described in ref. [10] from P1 year 6 (T-lymphoma disease stage) where the $CD4^+$ T cells represented 85% of total lymphocytes, which were composed of 85% of $CD3^-CD4^+$ T cells and P2 year 0 (initial diagnosis) where $CD4^+$ T cells represented 87% of total lymphocytes, which were composed of 83% of $CD3^-CD4^+$ T cells [7]. Normal $CD3^+CD4^+$ T cells from P2 year 4 were used as a control (chronic disease phase where only 7% of P2's PBMC were $CD3^-CD4^+$ T cells). Total RNA was extracted from sorted T cells and hybridized on U133A Genechips (Affymetrix, Santa Clara, CA, USA) containing 22,263 probe sets as described in ref. [10]. Biological replicate experiments were performed for each purified T-cell population. Gene expression values from the CEL files were processed using robust multi-array analysis (RMA) [47], and the data interpreted by sorting for robust changes in pairwise comparisons as described in the GeneChip expression analysis manuals (Affymetrix).

Results

Phenotypic stability of the $CD3^-CD4^+$ T cell clone from HES patients

T-cell markers on the surface of abnormal $CD3^-CD4^+$ T cells purified from the blood of HES P1 (90% of $CD4^+$ T cells) and P2 (84% of $CD4^+$ T cells) shortly after diagnosis (year 0) are shown in Figure 1, with the pattern and intensity of antibody labeling remarkably similar. In addition to their $CD3^-CD4^+$ phenotype [2,7,48], both patients' abnormal T cells exhibited the same surface density of CD2, CD5, and CD28. Alternatively, CD7, present on more than 90% of peripheral blood T cells, and the T-cell activation marker CD69 were both negative. The IL-2 receptor α chain, CD25, is expressed at very low levels on some of P2's cells, and at this blood sampling was essentially negative on P1's cells. The CD45RO but not the CD45RA and L-selectin (CD62L) are expressed on both patients abnormal T cells suggesting a central memory phenotype [49]. We followed the expression of these receptors on P1's abnormal $CD3^-CD4^+$ T-cell clone over a period of 6 years, extending from initial HES diagnosis to lymphoma, and found that overall their expression did not fluctuate significantly, either quantitatively or qualitatively (Fig. 2A, C, and D). Minor modifications included a low level of CD25 upregulation on some of the cells over time. CD62L expression decreased from year 0 to year 4,

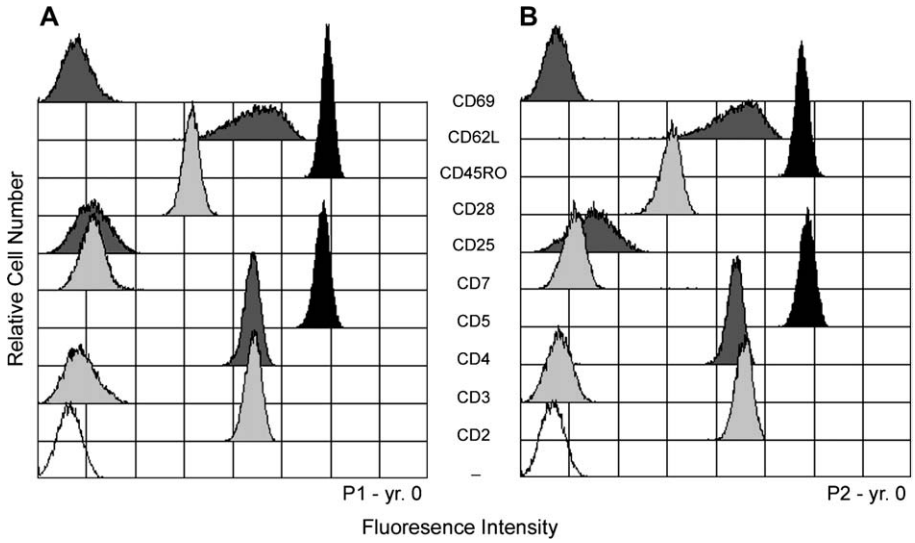

Figure 1. Histogram overlays showing the distribution of antibody labeling to various surface receptors on purified $CD3^-CD4^+$ T cells from P1 and P2's blood at diagnosis. Surface labeling with a mixture of isotype controls is shown on the bottom line of each graph.

returning to a mixed population containing $CD62L^{high}$ and $CD62L^{low}$ expressing cells in year 6. P2 was followed for more than 4 years with no significant changes in the immunophenotype of her abnormal T cells (data not shown).

In a recently published study, we employed oligonucleotide microarrays (HG-U133A arrays; Affymetrix) to examine gene expression in relation to cytogenetic changes in the abnormal T cells [10]. This experiment compared purified $CD3^-CD4^+$ T cells from P1 (lymphoma) and P2 (chronic lymphproliferation) with normal $CD3^+CD4^+$ T cells purified from P2 and used as a control. An examination of the arrays for surface receptor gene expression revealed that the stable CD2 and CD28 immunophenotype (Figs. 1 and 2) was reflected in mRNA transcript levels comparable with normal $CD4^+$ T cells (Table 1). CD4 transcripts but not surface expression were elevated in P1 and P2, whereas both CD5 transcripts and surface expression were increased in comparison with the normal $CD4^+$ T cells. The CD7- and CD25-negative phenotype is mirrored by significant reductions in their gene transcripts. Alternatively, fluctuations in CD62L do not appear to result from changes in transcripts, because this gene's expression was normal. CD69 gene expression was similar to normal $CD4^+$ T cells in P1 and decreased threefold in P2, both of whom are surface-negative for this marker. As expected, decreases in some of the genes encoding the different subunits of the TCR/CD3 receptor were detected and will be discussed in detail below. The CD45 gene was not present on the arrays. Overall, these studies suggest that the phenotype of the abnormal $CD3^-CD4^+$ T cells is extremely stable even during the process of malignant transformation.

Establishment and characterization of IL-2-dependent $CD3^-CD4^+$ T-cell lines

We established a series of IL-2-dependent $CD3^-CD4^+$ T-cell lines from P1 that were derived from her peripheral blood drawn at year 0, year 3, year 4, and year 6. Isolated PBMCs were seeded in 24-well plates and grown in vitro without IL-2. Each week, starting 4 weeks after the initiation of the culture, IL-2 was added to an individual well. During the initial IL-2-deprived period, most of the normal T cells progressively died while a proportion of the abnormal $CD3^-CD4^+$ T cells remained viable but nondividing. By adding IL-2 at progressive intervals, we were able to empirically determine the correct time for initiating growth of the abnormal clone without extensive overgrowth of any remaining normal T cells, particularly $CD8^+$ T cells. The proliferating $CD3^-CD4^+$ T cells upregulated CD25 expression and grew continuously for ± 20 weeks with IL-2 alone. When the cells doubling time eventually slowed (>20 weeks), they could be induced for further IL-2-dependent growth by stimulation with a combination of anti-CD28 plus PMA or anti-CD2.

A number of different parameters were compared between the patient-derived cell lines and $CD3^-CD4^+$ T cells freshly isolated from their blood. The immunophenotype of the cell lines was remarkably similar to the original abnormal T-cell population from which they were derived [P1 PBMC (Fig. 2A) and cell line (Fig. 2B)]. The only exceptions are an upregulation of CD25 in the presence of IL-2, fluctuation in CD62L in vitro as observed in vivo, and a slight upregulation of CD69 likely reflecting the more activated state of the cells grown in vitro. The cell lines were analyzed for their lymphokine secretion profile and found to secrete IL-4, IL-5, and IL-13

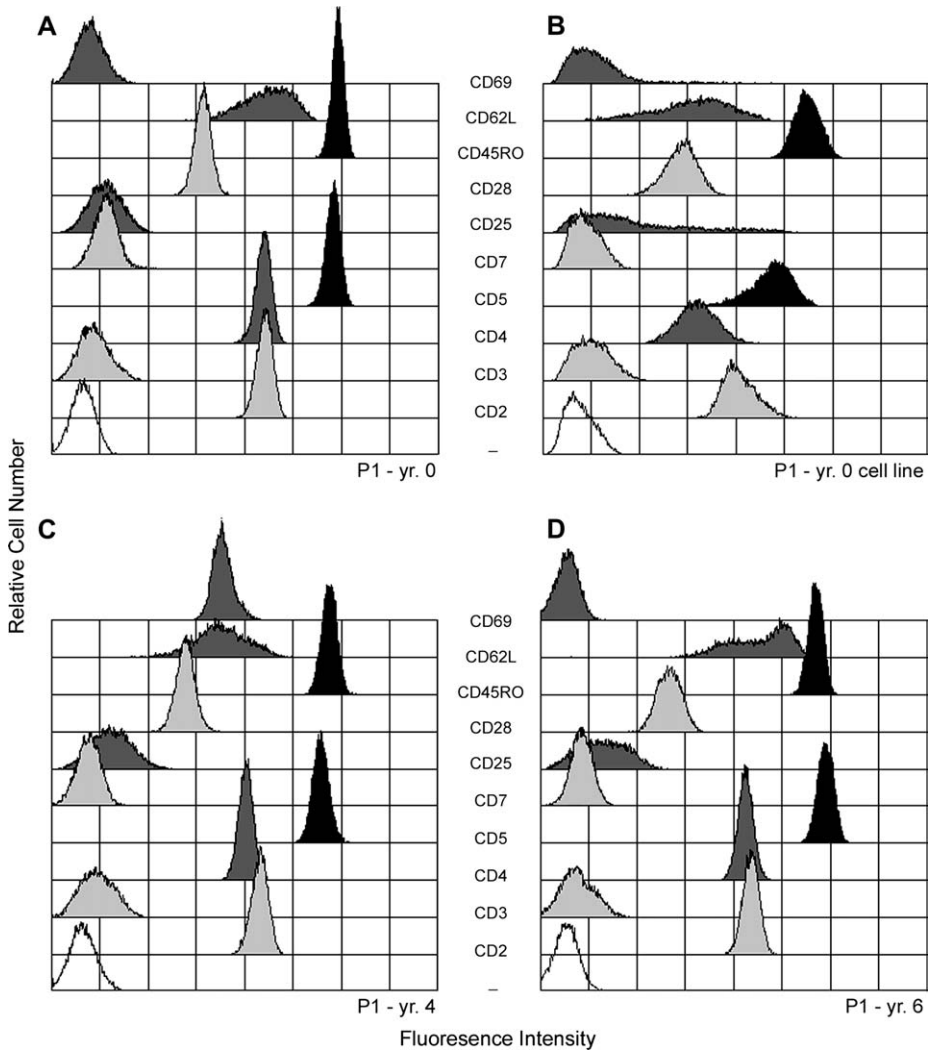

Figure 2. Histogram overlays showing the distribution of antibody labeling to various surface receptors on $CD3^-CD4^+$ T cells purified from P1 blood at different times during the evolution of her disease and a P1-derived $CD3^-CD4^+$ T-cell line. Surface labeling with a mixture of isotype controls is shown on the bottom line of each graft.

upon activation, similar to the freshly isolated cells from which they were derived (data not shown; the experiments were performed as described in ref. [2]). They were also used to establish the clonality of the TCR gene rearrangements as well as to identify and characterize 6q- and/or 10p-deleted chromosomal subclones within the $CD3^-CD4^+$ T-cell clone, enabling their subsequent detection in the patient's blood [10].

Overall, these experiments show that the $CD3^-CD4^+$ T-cell population, derived from a single clonal T cell, maintains a relatively stable immunophenotype and lymphokine secretion profile throughout disease progression even as it accumulates genetic aberrations in the process of malignant transformation. Interestingly, we consistently observed the presence of a small but increasing percentage of cells with an elongated cellular morphology that is highly reminiscent of HHV-8 infected Kaposi's cells in cell lines derived from P1 at years 0, 3, 4, and 6 (Fig. 3). These semiadherent cells were determined to be phenotypically identical ($CD1^-$ $\underline{CD3}^-$ $\underline{CD4}^+$ $CD8^-$ $CD20^-$ $CD45RO^+$ $CD56^-$ and $MAC387^-$) to the suspension cells, including both those growing individually and in large aggregates in the same cultures. We searched for the presence of viruses known to infect human $CD4^+$ T cells and provoke a loss of TCR/CD3 surface expression in the HES patient's PBMCs, isolated $CD3^-CD4^+$ T cells and T-cell lines, and all were negative for HIV-1, HIV-2, HTLV-I, HTLV-II, HHV-7, and HHV-8 (performed as a control). However, while quantitative PCR analysis revealed that both patients' abnormal T cells were positive for HHV-6 DNA, the level of viral DNA did not increase in relation to the number of $CD3^-CD4^+$ T cells in their blood (data not shown). Small quantities of the elongated cells were isolated and nested PCR once again revealed the presence of HHV-6; however, based on two

Table 1. Changes in CD antigen mRNA expression in the abnormal $CD3^{-}CD4^{+}$ T cells

Gene symbol	Accession number	Probe ID	Gene description	P1 year 6,* $CD3^{-}CD4^{+}$ (lymphoma)	P2 year 0,* $CD3^{-}CD4^{+}$ (lymphoprofileration)
CD2	NM_001767	205831_at	CD2 antigen	NC	NC
CD3D	NM_000732	213539_at	CD3 antigen, δ chain	NC	NC
CD3E	NM_000733	205456_at	CD3 antigen, ε chain	NC	NC
CD3G	NM_000073	206804_at	CD3 antigen, γ chain	−1.97	−1.04
CD3Z	J04132	210031	CD3 antigen, ζ chain	−2.69	−2.64
TRA@	AW873544	215796	T-cell receptor α locus	NC	NC
TRB@	M15564	210915_x_at	T-cell receptor β locus	NC	−14.17
TRD@	AW007751	217143_s_at	T-cell receptor δ locus	A	A
TRG@	M16768	209813_x_at	T-cell receptor γ locus	5.37	A
CD4	U47924	203547_at	CD4 antigen	1.90	2.56
CD5	NM_014207	206485_at	CD5 antigen	4.07	4.84
CD7	NM_006137	214049_x_at	CD7 antigen	−187.40	−88.95
IL2RA	K03122	206341_at	Interleukin 2 receptor α (CD25 antigen)	−5.37	−6.17
CD28	NM_006139	206545_at	CD28 antigen	NC	NC
SELL	NM_000655	204563_at	Selectin L (CD62L antigen)	NC	NC
CD69	L07555	209795_at	CD69 antigen (early T-cell activation)	NC	−3.03

*Mean fold change of all probe sets coding for a specific gene in purified $CD3^{-}CD4^{+}$ T cells versus purified $CD3^{+}CD4^{+}$ T cells from P2 year 4. The results shown are an average of duplicate experiments. NC, no change; A, absent.

patients it is difficult to determine whether this is higher than latent HHV-6 prevalence in the normal adult population. Unfortunately, due to the very low numbers of the elongated cells present in these cultures, it was not possible to determine whether they represent a subclone with a specific 6q and/or 10p deletion.

Identification of the molecular defect underlying the loss of TCR/CD3 expression

Loss of TCR/CD3 surface expression appears to be an important early event for the persistence and expansion of the abnormal $CD3^{-}CD4^{+}$ T-cell clone in HES. High-quality mRNA prepared from P1's T-cell lines shows that

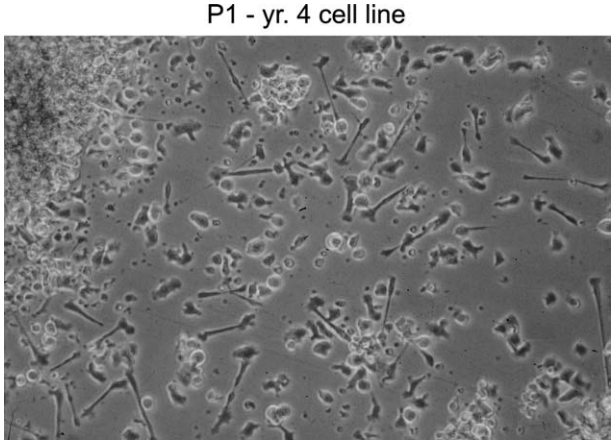

Figure 3. Typical morphological changes observed by phase contrast microscopy in P1 $CD3^{-}CD4^{+}$ T cell lines cultured on plastic. Magnification ×1000.

*CD3*γ chain gene transcripts are specifically lost in the $CD3^{-}CD4^{+}$ T cells (Fig. 4). Transcripts of the other TCR/CD3 chains (TCRα, TCRβ, *CD3*δ, *CD3*ε, and *CD3*ζ) were present in the same mRNA preparations, although some quantitative variation was detected in comparison with the controls, likely due to a change in the turnover of the other chains [50–52] in the absence of *CD3*γ transcripts. G3PDH was used to control that the relative amount of mRNA loaded on the gels was similar.

Previously, we used RNA dot blots to examine a small amount of total RNA available from a previous HES patient's frozen PBMCs collected during the final stage of T-cell lymphoma (the clinical history of this patient is described in refs. [48] and [4]). We found that *CD3*γ transcripts were decreased relative to *CD3*δ transcripts in the patient sample but not a normal control (data not shown). In retrospect, this result suggests that the TCR/CD3 defect in this individual's $CD3^{-}CD4^{+}$ T-cell clone was also due to a loss of *CD3*γ gene transcripts. The remaining patients in our cohort are responding well to their individual therapies and thus maintaining only a very low percentage of $CD3^{-}CD4^{+}$ T cells in their blood, which have all proven refractory to expansion in vitro. Due to the limited quantity of material available from these patients, we were unable to perform Northern or dot blots to identify the defect underlying the $CD3^{-}CD4^{+}$ phenotype. However, sufficient quantities of P2's $CD3^{-}CD4^{+}$ T cells (frozen at diagnosis) were available for microarray analysis. This experiment revealed unaltered expression of the *CD3*δ and *CD3*ε genes and a decrease in *CD3*γ and *CD3*ζ gene transcripts in both patients' abnormal T cells (Table 1).

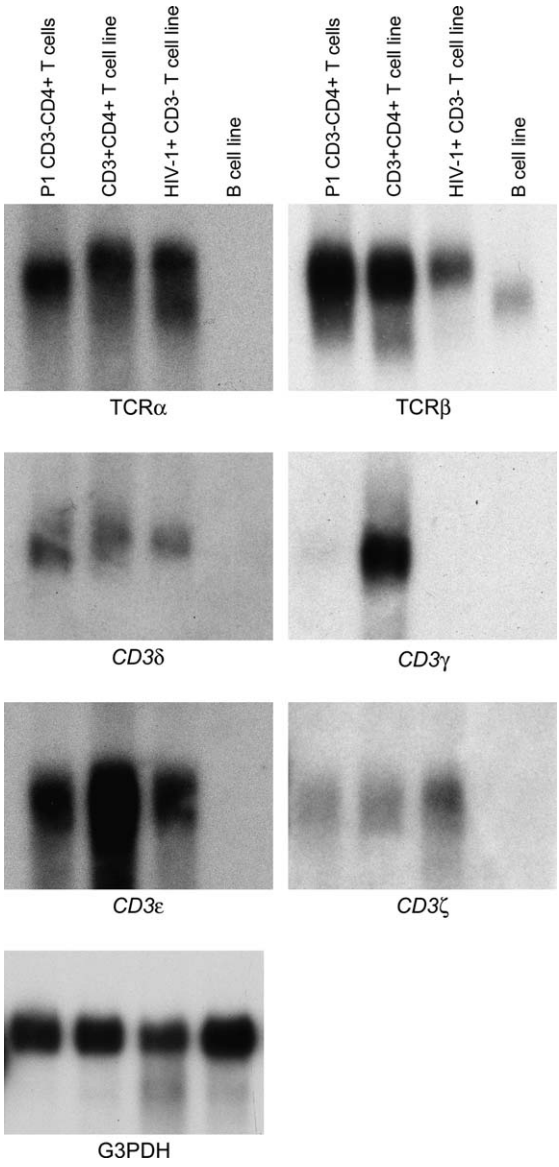

Figure 4. Expression of messages for TCRα, TCRβ, *CD3*δ, *CD3*γ, *CD3*ε, *CD3*ζ, and G3PDH in P1's $CD3^{-}CD4^{+}$ T-cell line compared with the $CD3^{+}CD4^{+}$ cell line WE17/10, the $CD3^{-}(CD3\gamma^{-})CD4^{+}$ HIV-infected WE17/10-cell line and the B-cell line Raji (negative control) as determined by Northern blots. Individual blots were prepared from the same mRNA preparation and exposed for 2 days (TCRα, TCRβ, *CD3*δ, *CD3*ε, and G3PDH) or 7 days (*CD3*γ and *CD3*ζ).

A decrease in expression of the TCRβ locus was also detected in P2 and an increase in expression of the TCRγ locus in P1 (both patients abnormal clones express TCRαβ receptors [10]). This data suggests that a loss of *CD3*γ, *CD3*ζ, and/or TCRβ transcripts potentially underlies the loss of TCR/CD3 surface expression in P2's $CD3^{-}CD4^{+}$ T cells. However, in a parallel microarray experiment, $CD4^{+}$ T cells infected with HIV-1 or HTLV-I (shown to be uniquely $CD3\gamma^{-}$ by Northern blots and quantitative competitive reverse transcriptase (RT)-PCR [33,36,37]) provided quantitatively similar results for expression of the CD3 genes (K.W.G., unpublished data). Thus, cross-reactivity among the CD3 array probes due to the high homology between the mammalian CD3 genes [53] could affect the accuracy of these results. More precise assessment of CD3 gene transcripts in our patients awaits a real-time RT-PCR assay for their individual quantification, which is currently under development in our laboratory.

Nuclear NFAT and NF-κB binding to the NFAT motifs in the human CD3γ gene

Our studies of the elements controlling expression of the human *CD3*γ gene have identified two important NFAT consensus sequences in the proximal promoter region of the human *CD3*γ gene: $NFAT_{\gamma 1}$, which binds either NFATc1 plus NF-κB p50 or NFATc2-containing complexes, and $NFAT_{\gamma 2}$, which binds NFATc2-containing complexes only [36]. We found that the quantity of nuclear NFATc2 binding to the $NFAT_{\gamma 1}$ and $NFAT_{\gamma 2}$ motifs increases in parallel with a decrease in *CD3*γ gene transcripts and surface TCR/CD3 complexes after HIV-1 infection [36]. Furthermore, NFATc2 binding to the $NFAT_{\gamma 2}$ site negatively influences *CD3*γ promoter activity, whereas the $NFAT_{\gamma 1}$ motif can positively or negatively influence its activity depending upon whether NFATc1 plus NF-κB p50 or NFATc2-containing complexes are bound [46]. EMSA experiments were performed to investigate NFAT binding to the *CD3*γ promoter in P1's unstimulated and stimulated $CD3^{-}CD4^{+}$ T cells from fresh blood as well as the IL-2-dependent $CD3^{-}CD4^{+}$ T-cell lines. In the exposure shown, the weaker NFATc1/NF-κB p50 band A is overshadowed by the intense band B migrating just below; however, this gel reveals that in all of the patient samples the quantity of the complexes bound is significantly increased in comparison with the induced $CD4^{+}$ T-cell line, WE17/10. The quantity of complexes bound to the $NFAT_{\gamma 1}$ probe is specific because similar amounts of the constitutively expressed Oct-1 protein bound to an Oct-1 sequence-specific probe from each extract (data not shown). Furthermore, there are differences in the amount of the B and C complexes, with a quantitative shift from the higher molecular weight (B) to the lower molecular weight (C) complex found both in P1's stimulated T cells and T-cell lines compared with the unstimulated T cells. Potentially this lower molecular weight band lacks an additional cellular component induced upon activation to bind in the higher molecular weight complex. The binding of the B and C complexes to the $NFAT_{\gamma 2}$ probe was similar to the $NFAT_{\gamma 1}$ probe for all samples (data not shown).

We investigated whether the complexes from the patient-derived nuclear extracts bound to the CD3γ NFAT motifs also contained NFATc2 or NFATc1 plus NF-κB p50 as previously shown for stimulated and HIV-1-infected WE17/10 cells [36]. EMSA supershift experiments confirmed that NFATc2 and NFATc1 plus NF-κB p50-containing complexes bind to the $NFAT_{\gamma 1}$ probe (Fig. 5B) whereas

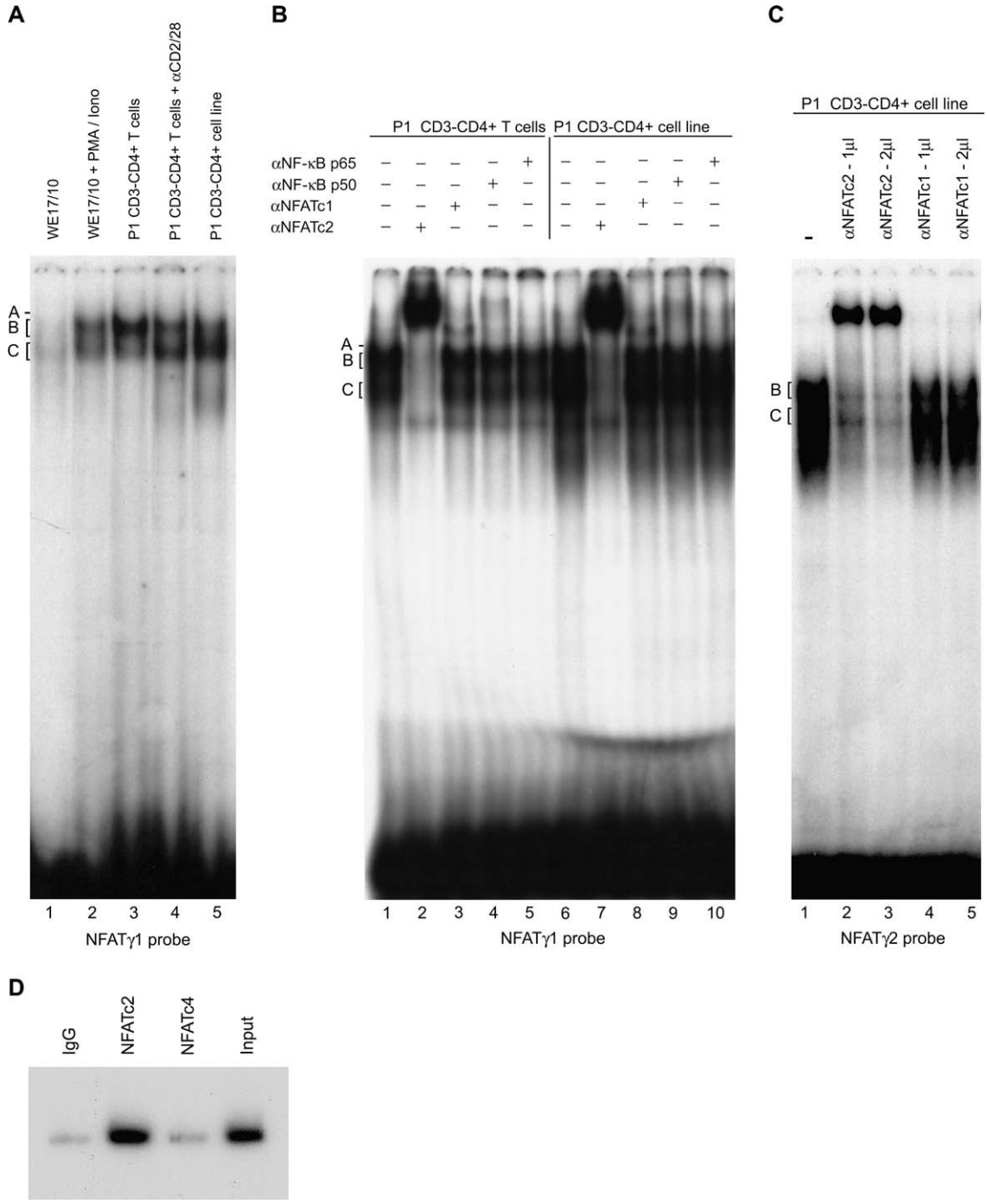

Figure 5. **(A):** EMSAs were performed using the ^{32}P-labeled NFAT$_{\gamma 1}$ probe and nuclear extracts from unstimulated 100% TCR/CD3^{hi} (lane 1) or PMA/ionophore stimulated 100% TCR/CD3^{lo} (lane 2) WE17/10 cells show the three protein:DNA complexes containing NFATc1 plus NF-κB p50 (band A) or NFATc2 (bands B and C) that are induced to bind to the NFAT$_{\gamma 1}$ probe [36] in comparison with unstimulated (lane 3) or anti-CD2/anti-CD28 stimulated (lane 4) purified CD3$^-$CD4$^+$ T cells from P1, and a CD3$^-$CD4$^+$ P1-derived cell line (lane 5). **(B):** Binding to the NFAT$_{\gamma 1}$ probe was examined in a supershift assay using nuclear extracts from purified CD3$^-$CD4$^+$ T cells from P1's blood (lanes 1 to 5) and a CD3$^-$CD4$^+$ P1-derived cell line (lanes 6 to 10) in the absence of antibody (lanes 1 and 6) or in the presence of anti-NFATc2 (*lanes 2* and *7*), anti-NFATc1 (*lanes 3* and *8*), anti-NF-κB p50 (Lanes 4 and 9), and anti-NF-κB p65 (lanes 5 and 10). **(C):** The ^{32}P-labeled NFAT$_{\gamma 2}$ was used in a supershift assay with nuclear extracts from the IL-2-dependent P1-derived T-cell lines in the absence of antibody (lane 1) or in the presence of anti-NFATc2 (lane 2), anti-NFATc1 (lane 3), anti-NF-κB p50 (lane 4), and anti-NF-κB p65 (lane 5). **(D):** ChIP assay showing specific in situ binding of NFATc2 to the NFAT$_{\gamma 2}$ motif in CD3$^-$(CD3γ$^-$)CD4$^+$ P1-derived cell lines.

NFATc2 alone binds to the $NFAT_{\gamma 2}$ probe (Fig. 5C) in nuclear extracts from both purified $CD3^-CD4^+$ T cells as well as patient-derived cell lines. This supershift experiment revealed that once again the A complex contains NFATc1 plus NF-κB p50 while the B and C complexes contain NFATc2. NF-κB p65 does not bind to either probe. The ChIP assay further demonstrates that NFATc2-containing negative regulatory complexes are bound in situ to the *CD3*γ promoter in P1's $CD3^-CD4^+$ T cells (Fig. 5D). An examination of the NFAT and NF-κB gene expression in the microarray experiment revealed a decrease in both NFAT (c1 and c3) and NF-κB (p50, p52, RelB) transcripts in both P1's and P2's purified $CD3^-CD4^+$ T cells compared with normal $CD3^+CD4^+$ T cells (Table 2). Unfortunately, a probe set for NFATc2 was not present on the U133A array. However, expression of calcineurin, the calcium-dependent serine phosphatase responsible for dephosphorylating cytoplasmic NFAT proteins leading to their activation and nuclear translocation [54], was increased more than twofold in both patients. A member of the IκB family (IκBβ) whose association with NF-κB proteins is responsible for their retention in the cytoplasm was also increased two- to threefold in the patient's abnormal T cells.

Discussion

Viewed from their surface, the abnormal $CD3^-CD4^+$ T cells from P1 and P2 maintain an incredibly stable phenotype, exemplified by a lack of significant variation in surface markers between the two patients as well as during their long-term follow up (Figs. 1 and 2). The $CD3^-CD4^+$ T cells from P1 and P2 were previously shown to have a Th2 phenotype based on their lymphokine secretion profile after activation [2,7]. These abnormal T cells are unable to spontaneously secrete cytokines in vitro in contrast to their demonstrably persistent expansion and cytokine production in vivo. A previous study established that TCR/CD3-independent activation-driven cytokine production by the $CD3^-CD4^+$ T cells is contingent upon an autocrine IL-2-dependent pathway both in vitro and in vivo [7]. Activation-dependent cytokine production is additionally supported by a lack of detectable IL-4, IL-5, and IL-13 gene expression in the microarrays performed on unstimulated $CD3^-CD4^+$ T cells from both patients (data not shown). Interestingly, long-term in vitro growth of the $CD3^-CD4^+$ T cells from P1 was achieved using exogenous IL-2 alone. Our ability to extensively grow the abnormal T cells from P1 but not the other patients in our cohort most likely reflects a more advanced stage in their evolution to malignancy, allowing them to proliferate in response to IL-2 alone. This suggests that additional stimulatory signals are critical for expansion and cytokine production by the $CD3^-CD4^+$ T cells during the early chronic HES disease phase in vivo.

The low level of CD25, the lack of CD69, and the $CD62L^{high}$ expression on $CD3^-CD4^+$ T cells freshly isolated from patient blood suggests that in the absence of activation they maintain a resting T-cell phenotype in vivo. Studies have shown that memory T cells are heterogeneous in their expression of CD62L and thus define the $CD62L^{high}$ memory subset as a central memory cell and the $CD62L^{low}$ memory subset as a functional effector cell [55]. This T-cell activation marker did fluctuate on P1's abnormal T cells over time, although there was no variation in CD62L levels between the samples from P1 and P2 that were analyzed in parallel. The expression of $CD62L^{high}$ combined with CD45RO, CCR5, and CXCR4 (the latter two were previously shown to be expressed on their unstimulated cells [56]) suggest that the $CD3^-CD4^+$ T-cell clones from both patients may be derived from a central memory T cell, which can be polarized to produce Th2 lymphokines upon activation [49].

Our ability to expand the $CD3^-CD4^+$ T cells from P1 in vitro allowed us to investigate the molecular changes underlying the abnormal phenotype. The TCR clonality of the $CD3^-CD4^+$ T cells was initially determined in the cell lines by Southern blotting and PCR analysis of the TCRβ and TCRγ chain genes, respectively (data shown in ref. [10]). The same TCR clone was subsequently detected as the major clonotype in P1's blood. The cell lines were also used to identify and characterize subclones with deleted chromosomes within the $CD3^-CD4^+$ T-cell clone. The P1 (year 0) cell line included subclones containing either: 1) a normal karyotype; 2) a deletion 6q13-q22.1; 3) a deletion 10p11.1-p13; or 4) the same deletion 10p11.1-p13 with a deletion 6q11.1-q23.1 (described in detail in ref. [10]). The identification of these chromosomal abnormalities in the patient-derived cell lines provided the basis for their subsequent identification in fresh blood samples. However, the 6q and 10p deletions were not detectable in her PBMC until more than 3 years later despite the high percentage of $CD3^-CD4^+$ T cells, suggesting that these abnormalities provided an advantage for growth in vitro and eventually their expansion in vivo. Interestingly, the frequency of the 6q13-q22.1 subclone was found to increase progressively with P1's disease evolution, with 91% of the cells containing this deletion in concert with her $CD3^-CD4^+$ T-cell lymphoma, and a similar but not identical 6q13-q22.1 deletion has been detected in 25% of P2's abnormal T cells in the chronic disease phase [10].

The $CD3^-CD4^+$ T cells have a clonal TCRβ gene rearrangement and a stable immunophenotype; however, there are progressive chromosomal changes occurring in different cellular subclones, which can be detected both in vitro and in vivo. This suggested that some unidentified intracellular events could be driving changes that favored the persistence and conferred a growth advantage to certain subclones, eventually leading to malignant disease in vivo. We searched for the presence of viruses known to infect

Table 2. Changes in transcription factor gene expression in the abnormal $CD3^{-}CD4^{+}$ T cells

Gene symbol	Accession number	Probe ID	Gene description	P1,* $CD3^{-}CD4^{+}$ (lymphoma stage)	P2,* $CD3^{-}CD4^{+}$ (lymphoproliferation)
NFATC1	U80918	211105_s_at	Nuclear factor of activated T cells, cytoplasmic calcineurin-dependent 1 (NFATcl)	−2.51	−1.68
NFATC3	NM_004555	210556_at	Nuclear factor of activated T cells, cytoplasmic calcineurin-dependent 3 (NFATc3)	−1.15	−2.10
NFATC4	NM_004554	205897_at	Nuclear factor of activated T cells, cytoplasmic calcineurin-dependent 4 (NFATc4)	A	A
PPP3CA	NM_021132	202432_at	Calcineurin A β	2.30	2.0
NFKB1	M55643	209239_at	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (p105/p50)	−1.90	−2.26
NFKB2	NM_002502	207535_s_at	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (pl00/p52)	−2.59	−3.03
RELA	M62399	209878_s_at	Nuclear factor of kappa light polypeptide gene enhancer in B cells 3 (RelA = p65)	NC	NC
RELB	NM_006509	205205_at	Nuclear factor of kappa light polypeptide gene enhancer in B cells 3 (RelB)	−1.62	−2.18
IKBKB	AU153366	209341_s_at	Inhibitor of kappa light polypeptide gene enhancer in B cells kinase β (IκBβ)	3.24	2.26

*Mean fold change of all probe sets coding for a specific gene in purified $CD3^{-}CD4^{+}$ T cells versus purified $CD3^{+}CD4^{+}$ T cells from P2 year 4. The results shown are an average of duplicate experiments. NC, no change; A, absent.

human $CD4^{+}$ T cells and cause a loss of TCR/CD3 surface expression, and although these patients were positive for HHV-6, its presence could not be correlated with the percentage of $CD3^{-}CD4^{+}$ T cells nor the emergence of the leukemic 6q-deleted subclone in P1. HHV-6 principally infects human $CD4^{+}$ T cells, and although infection is widespread early in life, viral load decreases over time in healthy humans. HHV-6 reactivation has been associated with T-cell disorders and viral gene expression has been detected in a growing number of human lymphomas. Though we were unable to establish a direct link with HHV-6 in our patients, we cannot exclude the possibility that its presence does make a contribution to their disease.

The increasing number of reports citing TCR/CD3 defects in clinical pathologies underlines the functional importance of maintaining the mechanisms that correctly regulate the surface expression of this critical immune activation pathway. We found that the abnormal $CD3^{-}CD4^{+}$ T cells from P1 and a previously reported patient [48] have a specific defect in *CD3*γ gene transcripts, and on the basis of the microarray data that *CD3*γ transcripts might also be decreased in P2's abnormal T cells. Targeting the *CD3*γ gene in abnormal T cells is not without precedent. We have shown that a specific defect in *CD3*γ transcripts occurs after HIV-1, HIV-2, and HTLV-I infection of $CD4^{+}$ T cells in vitro [33]. Furthermore, although *CD3*ζ transcripts in the infected cells were not significantly reduced after infection, we found that the progressive loss of *CD3*γ mRNA triggers a subsequent reduction in CD3ζ protein (K.W.G., unpublished data, 2004), likely resulting from its increased turnover [57] in the absence of receptor formation. Numerous clinical studies have demonstrated that CD3ζ protein is reduced in tumor-infiltrating T cells [30] and HIV-1-infected individuals [28]. The microarray experiment revealed a decrease in both *CD3*ζ and *CD3*γ gene transcripts in P1 and P2's $CD3^{-}CD4^{+}$ T cells, although the decrease in *CD3*ζ was not substantiated in the Northern blots of P1's cells expanded in vitro. These data suggest that the molecular mechanism underlying the decrease in CD3ζ protein expression observed in many clinical T-cell abnormalities could be provoked by a defect in *CD3*γ mRNA transcripts.

Due to the limited knowledge of *CD3*γ transcriptional control, we initiated a study to identify and characterize the elements involved in its regulation and their potential parallels with HIV gene expression. We have shown that the *CD3*γ gene is transcribed from a weak, lymphoid-specific TATA-less promoter that functions via an initiator element and two upstream Sp binding sites [46]. We further identified two NFAT motifs and found that they positively ($NFAT_{\gamma 1}$) or negatively ($NFAT_{\gamma 1}$ and $NFAT_{\gamma 2}$) regulate expression of the *CD3*γ gene by their differential binding of NFATc1 plus NF-κB p50 or NFATc2-containing complexes, respectively [36]. We found that an increase in the binding (both in vitro and in situ) of nuclear NFATc2 to these motifs is correlated with a progressive loss of *CD3*γ transcripts after HIV-1 infection [46]. The experiments shown in this study, comparing $CD3\gamma^{+}$-uninfected and $CD3\gamma^{-}$ HIV-1-infected $CD4^{+}$ T cells with $CD3\gamma^{-}$ $CD4^{+}$ T cells from P1, revealed that extremely high levels of NFATc2 are present in the nucleus of P1's abnormal T cells and are bound to the $NFAT_{\gamma 2}$ motif in situ. The nuclear translocation of NFAT family proteins is dependent upon

the serine phosphatase calcineurin [58], and we detected increased expression of the calcineurin gene in parallel with nuclear NFATc2. Furthermore, transcripts for NFATc1 and NF-κB p50, components of the positive regulatory complex along with the NF-κB inhibitory protein IκBβ, were decreased in both patients' abnormal T cells.

The NFAT family of transcription factors plays critical roles in the immune system, and the in vivo functions of the individual NFAT proteins have been elucidated by gene disruption in mice. NFATc1-deficient mice revealed its important role in the development of the Th2 response and in particular IL-4-driven responses [59,60]. NFATc2-deficient mice are characterized by a hyperproliferation of lymphocytes leading to a marked increase in the immune response and a defect in IL-4 production in response to stimulation with anti-CD3 [61,62]. These data indicate that NFATc2 can exert an important negative regulatory role particularly in Th2-based immune responses. NFATc3-deficient mice have a T-cell developmental defect due to the increased apoptosis of double positive thymocytes but maintain normal cytokine production [63]. Thus, NFATc1 and NFATc2 are key regulators of Th2 cytokine production, as exemplified by their regulation of IL-4 gene expression [64]. Although NFATc2 is abundant in the cytoplasm, ready to receive the calcium signal for translocation to the nucleus, expression of the *Nfatc1* gene is regulated by the level of nuclear NFATc2 protein [65]. This suggests that the relatively high constitutive levels of NFATc2 in P1's unstimulated $CD3^{-}CD4^{+}$T cells, with even higher levels induced upon activation, can play both a positive and negative role in Th2 cytokine expression in T cell–mediated HES. NFATc1 is often referred to as the major positive Th2 transcription factor, and the underexpression we detected in the $CD3^{-}CD4^{+}$ T cells may underlie their lack of Th2 lymphokine expression in the absence of activation. In addition, increased nuclear NFATc2 could negatively regulate *CD3*γ gene expression and play a role in prohibiting TCR/CD3 surface expression. Thus, changes in the balance of these important immune regulatory factors may affect the expansion and the accumulation of aberrations within the $CD3^{-}CD4^{+}$ T-cell clone, ultimately leading to malignancy in some HES patients.

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Annex 1

T-cell receptor-independent activation of clonal Th2 cells associated with chronic hypereosinophilia. F. Roufosse, L. Schandené, C. Sibille, B. Kennes, A. Efira, E. Cogan, M. Goldman.

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Through a collaborative study with the Department of Immunology and of Internal Medicine (ULB) and with the Laboratory of Experimental Hematology of the Institute Jules Bordet (ULB), the autor's laboratory participated in the selection of a cohort of L-HES patients since 1997. In particular, the molecular analysis provided evidence for T-cell clonality of the L-HES CD3-CD4 + T-cells by showing the persistence of a monoclonal gene rearrangement of TCRβ and/or γ chains in these aberrant cells.

Ex-vivo, CD3-CD4+ T-cells do not spontaneously secrete IL-5 or Th2-specific lymphokines. Therefore, this article focuses on the Th2 cytokine production of CD3-CD4+ T cells *in vitro* upon an antibody to CD2 or phorbol ester plus calcium ionophore stimulation. In addition, the reported data demonstrates the initiation in CD3-CD4+ T-cells of an autocrine IL-2/IL-2R activation loop mediated independently from anti-CD3 triggering pathway by dendritic cell co-stimulation. Therefore, it was suggested for the first time that co-stimulatory CD2/CD28 pathways could provide growth stimulation to CD3-CD4+ T-cells *in vivo* in the absence of surface TCR/CD3, thereby potentially contributing to L-HES pathogenesis.

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T-Cell Receptor-Independent Activation of Clonal Th2 Cells Associated With Chronic Hypereosinophilia

Florence Roufosse, Liliane Schandené, Catherine Sibille, Bernard Kennes, André Efira, Elie Cogan and Michel Goldman

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T-Cell Receptor-Independent Activation of Clonal Th2 Cells Associated With Chronic Hypereosinophilia

By Florence Roufosse, Liliane Schandené, Catherine Sibille, Bernard Kennes, André Efira, Elie Cogan, and Michel Goldman

We recently observed a clonal expansion of CD3$^-$CD4$^+$ T cells secreting Th2-type cytokines in patients presenting chronic hypereosinophilia. As clonal T cells isolated from such patients did not spontaneously secrete cytokines in vitro, we reasoned that costimulatory signals delivered by antigen-presenting cells might be required to induce their full activation. To address this question, we investigated in two such patients the responses of CD3$^-$CD4$^+$ T cells to dendritic cells (DC). DC elicited proliferation and production of interleukin-5 (IL-5) and IL-13 by clonal cells from patient 1 and upregulated their expression of CD25 (IL-2R-α). These effects were abolished when blocking monoclonal antibodies (MoAbs) against IL-2R-α and IL-2 were added to cocultures, indicating critical involvement of an autocrine IL-2/IL-2R pathway. Cells from patient 2 were stimulated by DC to produce Th2 cytokines only when rIL-2 or rIL-15 was added to cocultures. In both patients, addition of inhibitory MoAbs against B7-1/B7-2 or CD2 to cocultures resulted in dramatic reduction of cytokine production and inhibited CD25 upregulation. Thus, TCR/CD3-independent activation of clonal Th2 cells by DC is an IL-2–dependent process, which requires signaling through CD2 and CD28.

THE IDIOPATHIC hypereosinophilic syndrome (HES) is defined as blood eosinophilia of unknown origin exceeding 1,500 cells/μL and persisting for over 6 consecutive months. Organ damage and dysfunction in HES is due to infiltration of tissues by eosinophils followed by local release of their toxic content.[1-3] The different modes of presentation and disease courses already observed in initial reports suggest that HES represents a heterogenous group of disorders. Furthermore, it is well established that a proportion of patients with HES eventually develop a malignant hematologic disorder after the initial diagnosis of HES, including chronic or acute myeloid leukemia, granulocytic sarcoma, acute lymphoblastic leukemia, and T-cell lymphoma.[2] Recent studies have shed some light on the nature of the primary disorders leading to expansion of the eosinophilic lineage observed in HES patients, explaining the clinical heterogeneity of this disease. On one hand, demonstration of the clonal nature of eosinophils in some HES patients has provided clear evidence that a primitive disorder of the eosinophilic lineage, reminiscent of other myeloproliferative syndromes, can account for hypereosinophilia.[4-7] On the other hand, a subgroup of patients with chronic hypereosinophilia presenting a monoclonal expansion of T cells has been identified.[8-12] The profile of cytokines produced by the clonal T cells suggests their direct involvement in the pathogenesis of hypereosinophilia. Indeed, we and others have demonstrated that these cells consistently produce high levels of interleukin-5 (IL-5), a potent eosinophilopoietic cytokine that also contributes to peripheral activation of eosinophils and promotes their survival through inhibition of apoptosis.[13-16] In some cases, the clonal T cells also secrete IL-4,[8-10] which may favor tissue homing of eosinophils through upregulation of vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells.[17]

In our own series of hypereosinophilic patients with an underlying T-cell disorder, the clonal CD4$^+$ T cells were first detected because of absent expression of the T-cell receptor (TCR)/CD3 complex on their membrane.[8,9] On in vitro activation by phorbol 12-myristate 13-acetate (PMA) and A23187 calcium ionophore, the CD3$^-$CD4$^+$ cells displayed a Th2 phenotype,[18] as indicated by their inability to produce interferon-γ (IFN-γ) and their synthesis of high levels of IL-4, IL-5, and IL-13, together with variable amounts of IL-2.[19] Although the chronic hypereosinophilia observed in these patients and the clonality of the T cells suggested their persistent activation and expansion in vivo, these cells were unable to produce cytokines or proliferate spontaneously in vitro, indicating that they remain dependent on exogenous activating factors. Because of their unique surface phenotype, these clonal T cells represent an ideal tool to investigate alternative activation pathways of Th2-type cells. In this study, we sought to determine whether costimulatory signals provided by antigen-presenting cells (APC) could lead to activation of the clonal Th2 cells in the absence of signaling through the TCR/CD3 complex. To this end, we cocultured highly purified CD3$^-$CD4$^+$ T cells from two hypereosinophilic patients with autologous or allogeneic dendritic cells (DC) in a series of mixed leukocyte cultures (MLC). We observed that DC did indeed stimulate CD3$^-$CD4$^+$ cells to proliferate, to upregulate their expression of the IL-2R-α chain (CD25), and to produce Th2 cytokines. We then investigated the nature of the stimulatory signals delivered by DC, paying particular attention to B7/CD28 and lymphocyte function-associated antigen (LFA)-3/CD2 interactions. Finally, we also

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specified the role of an autocrine IL-2/IL-2R pathway in this model.

MATERIALS AND METHODS

Patients. We recently evaluated seven patients fulfilling the diagnostic criteria of HES with blood eosinophil levels above 1,500/μL. In three of these patients, flow cytometric immunophenotyping of circulating leukocytes showed the existence of an abnormal $CD3^-CD4^+$ lymphocyte subset. Two of them were available for the present study. Patient 1 (P1) was a 20-year-old woman presenting with severe pruritis, eczema, and tenosynovitis of the right ankle. At presentation, circulating leukocyte count was 16,900/μL, including 8,923 eosinophils and 4,630 lymphocytes. $CD4^+$ T cells represented 87% of total lymphocytes and were composed of 88% $CD3^-CD4^+$ cells (3,545 $CD3^-CD4^+$ cells per μL) and 12% $CD3^+CD4^+$ cells. Serum IgE and IgM levels were 340 U/mL (normal, <20) and 310 mg/dL (normal, 40 to 250), respectively, soluble CD25 level was 180 U/mL, and serum IL-5 was 10 pg/mL by enzyme-linked immunosorbent assay (ELISA) (Endogen, Woburn, MA). Five-year follow-up has been characterized by progressive increase in absolute count of the aberrant circulating population of helper T cells (reaching 4,800/μL) and persistence of marked hypereosinophilia (reaching 17,082/μL). Patient 2 (P2) was a 21-year-old woman also presenting with severe pruritis and eczema, as well as cyclic angioedema. Circulating leukocyte count was 14,800/μL, including 9,102 eosinophils and 3,419 lymphocytes. $CD4^+$ T cells represented 87% of total lymphocytes and were composed of 83% $CD3^-CD4^+$ cells (2,469 $CD3^-CD4^+$ cells/μL) and 17% $CD3^+CD4^+$ cells. Serum IgE and IgM levels reached 15,640 U/mL and 1,253 mg/dL, respectively, soluble CD25 level was 175 U/mL and serum IL-5 was below detection threshold (2 pg/mL). The clinical and biological findings were consistent with the diagnosis of Gleich's syndrome.[20] Two-year follow-up has been characterized by good response to glucocorticoid treatment, as evidenced by normalization of eosinophil levels. Although the $CD3^-CD4^+$ cell population had decreased significantly (662/μL) over this period, tapering of steroid dosage was quickly followed by recurrence of hypereosinophilia and clinical manifestations. Neither patient presented clinical evidence of lymphoma, such as enlarged lymph nodes or hepatosplenomegaly. Bone marrow aspiration showed abundant eosinophil precursors and absence of blastic cells. P1 had received glucocorticoids and IFN-α, but was out of treatment for 7 months at the time of the study, while cells from P2 were collected before initiation of glucocorticoid therapy.

Cell purification. Circulating leukocytes were obtained from both patients by cytapheresis, after informed consent, and from buffy coats of healthy blood donors. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. After washing with Hanks' balanced salt solution (HBSS) (GIBCO, Life Technologies, Paisley, UK), PBMC were resuspended in cold-conservation medium composed of 80% RPMI 1640 (Bio Whittaker/Boehringer Ingelheim, Verviers, Belgium), 10% dimethyl sulfoxide (Sigma Chemical Co, St Louis, MO) and 10% heat-inactivated fetal calf serum (FCS) (Bio Whittaker/Boehringer Ingelheim). Cells were stored in liquid nitrogen until tested.

Purified $CD3^-CD4^+$ cells from patients and $CD3^+CD4^+$ cells from healthy subjects were obtained by negative selection. PBMC were thawed and treated with Lymphokwik T (One Lambda, Los Angeles, CA) according to the manufacturer's instructions. The remaining cells were resuspended in culture medium (RPMI 1640 supplemented with 10% FCS and 40 μg/mL gentamicin (Schering-Plough, Kennelworth, NJ) at 10×10^6/mL, and incubated with mouse monoclonal antibodies (MoAb) against CD8, CD14, CD19, and CD56 (Becton Dickinson, Mountain View, CA), as well as against CD3 for patients, for 30 minutes at 4°C. After washing with HBSS, cells were resuspended in culture medium and incubated with sheep anti-mouse IgG-coated magnetic Dynabeads (Dynal, Oslo, Norway) for 45 minutes at 4°C. Coated cells were removed with a magnet, leaving purified $CD3^-CD4^+$ (patients) or $CD3^+CD4^+$ (healthy subjects) cells in suspension. No contaminating B cells, monocytes, or natural killer (NK) cells were detected. The $CD3^-CD4^+$ cell preparations contained less than 0.5% CD3-positive cells and more than 98.5% CD4-positive cells, as assessed by flow cytometry. The monoclonality of the purified $CD3^-CD4^+$ cells was established by Southern blotting and polymerase chain reaction (PCR) analysis for TCR genes (not shown).

Flow cytometry. Flow cytometric analysis of surface phenotype was performed by two- and three-color immunofluorescence using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and peridinin-chlorophyll-a-protein (PerCP)-conjugated MoAbs. Surface antigens of T cells were stained with MoAbs against TCR-α/β, CD3, CD4, CD8, CD7, CD27, CD25, CD2, CD28, CD80, CD40L, and CD45RO from Becton Dickinson and CD86 from Pharmingen (San Diego, CA). Surface antigens of DC were stained with MoAbs against HLA-DR, CD14, CD80 (Becton Dickinson), CD86 (Pharmingen), CD83 (Immunotech, Marseille, France), CD40 (Biosource, Camarillo, CA), CD1a (Dako, Glostrup, Denmark) and the corresponding isotype-matched irrelevant MoAbs. Data were collected on 10,000 viable cells using a FACScan flow cytometer (Becton Dickinson).

The percentage of apoptotic lymphocytes was determined by two methods. At the end of cell cultures, the $CD3^-CD4^+$ cells were directly analyzed by flow cytometry. Apoptotic cells could be distinguished from surviving lymphocytes by their decreased forward scatter and increased side scatter. Secondly, cells from each culture condition were stained with FITC-conjugated Annexin-V (Pharmingen) and propidium iodide (PI) (Pharmingen) according to the manufacturer's instructions, before fluorescence-activated cell sorting (FACS) analysis. Comparison of the two methods showed that all surviving cells according to forward and side scatter parameters were Annexin-V–negative and excluded PI, whereas the apoptotic cells were mostly positive for both markers with a minute proportion of Annexin-V–positive PI-negative cells.

Flow cytometry was also used for the detection of intracytoplasmic cytokine expression in lymphocyte subsets. To this end, total T cells ($CD4^+$ and $CD8^+$) were isolated from PBMC of both patients and healthy control subjects using the same procedure as described above, except that only MoAbs against CD14, CD19, and CD56 were used. These cells were incubated at 10^6/mL with Brefeldine A (Sigma Chemical Co) at 10 μg/mL, alone or combined with 50 ng/mL PMA (Sigma Chemical Co) and 0.1 μg/mL calcium ionophore A23187 (Calbiochem-Behring, San Diego, CA) for 6 hours in culture medium (37°C, 5% CO_2). Surface antigens were stained on aliquots of 2×10^5 cells with FITC- or PE-conjugated anti-CD8 MoAb, and PerCP-conjugated anti-CD3 MoAb. Staining of CD8 was preferred to CD4 because of important downregulation of surface CD4 expression on T cells after in vitro stimulation. Cells were fixed with FACS Lysing Solution (Becton Dickinson) for 10 minutes at room temperature in the dark, washed, and then permeabilized with 0.5 mL FACS Permeabilizing Solution (Becton Dickinson) in the same conditions. Intracellular cytokines were stained with PE- or FITC-conjugated MoAbs against IL-2, IFN-γ, IL-4 (Becton Dickinson), and IL-5 (Pharmingen). Negative controls for cytokine expression were provided by unstimulated cells treated only with Brefeldine A and by intracellular staining with isotype-matched irrelevant PE- or FITC-conjugated MoAbs. Triple-stain flow cytometry permitted distinct analysis of cytokine expression in gated $CD3^+CD8^-$ (equivalent to $CD3^+CD4^+$) and $CD3^-CD8^-$ (equivalent to $CD3^-CD4^+$) cells.

Stimulation of $CD4^+$ cells with mitogenic agents in vitro. Purified $CD3^-CD4^+$ cells from patients or $CD3^+CD4^+$ cells from healthy subjects (5×10^5/mL) were stimulated using 1 ng/mL PMA alone or combined with either 0.1 μg/mL A23187 or 1 μg/mL anti-CD28 MoAb (clone CD28.2) (Immunotech). In addition, cells were also incubated in

wells coated with the anti-CD3 MoAb OKT3 (Orthoclone OKT3, Cilag, Switzerland) in the presence of soluble anti-CD28 MoAb (1 μg/mL). All culture supernatants were harvested after 48 hours for measurement of cytokine concentrations.

Determination of cytokine levels in culture supernatants. Commercial ELISA kits were used to determine concentrations of IL-12 and IL-13 (Biosource). Other cytokine concentrations were measured by two-site sandwich ELISA using antibodies from Genzyme for IL-2, Chromogenix (Mölndal, Sweden) for IFN-γ, Mabtech (Stockholm, Sweden) for IL-4, and Pharmingen for IL-5 and IL-10.

Generation of DC from PBMC. PBMC were isolated from buffy coats obtained from healthy blood donors or from cytapheresis of P1. DC were generated by culturing plastic-adherent PBMC with 800 IU/mL recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Leucomax) and 500 IU/mL rIL-4 (both kindly provided by Schering-Plough) in enriched culture medium (RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine [GIBCO], 1% nonessential amino acids [GIBCO], 50 μmol/L 2-mercapto-ethanol [GIBCO], and 40 μg/mL gentamycin), as previously described by Romani et al.[21] After 6 days, DC were harvested, washed, and incubated at 4×10^5/mL in the presence of 1 μg/mL LPS from *Escherichia coli* (strain 0128:B12) (Sigma, Bornem, Belgium) in 24-well flat-bottom culture plates for a further 24 hours. The resulting DC displayed a mature phenotype, as assessed by high levels of CD40, CD80, and CD86 expression, and positivity for surface CD83 by flow cytometry (not shown). For all MLCs, DC were irradiated using 3,000 rads.

MLCs. All MLCs were performed in enriched culture medium. Purified $CD3^-CD4^+$ cells (2×10^5 cells/well) obtained from both patients were cocultured with allogeneic (P1 and P2) or autologous (P1 only) DC (6.7×10^3 cells/well) in 96-well sterile round-bottom microtiter plates, for 5 days, at 37°C and 5% CO_2. In some experiments with P2 cells, rIL-2 (150 U/mL) or rIL-15 (16.7 ng/mL) (Genzyme) was added to the MLC. To determine the nature of the costimulatory molecules involved in activation of the $CD3^-CD4^+$ cells during cocultures, blocking MoAbs against CD2 (IgG1 isotype), CD80 (IgM), CD86 (IgG2b) (10 μg/mL) (Pharmingen), or CD40 (IgG1) (100 ng/mL) (Genzyme) were added to successive MLCs. Isotype-matched irrelevant MoAbs were used as controls at the same concentrations. To further assess the role of B7/CD28 interactions, soluble CTLA4-Ig (Ancell, Bayport, MN) or control mouse IgG2a (10 μg/mL) was also added to MLCs. Finally, cocultures were performed in the presence of inhibitory concentrations (10 μg/mL) of an anti–IL-2R-α MoAb (Genzyme) alone or combined with an anti–IL-2 MoAb (Genzyme) or with corresponding isotype-matched irrelevant MoAbs (IgG2a and IgG1, respectively). After 5 days of MLC, proliferation of $CD3^-CD4^+$ cells was assessed by ^{3}H-thymidine uptake during the following 16 hours, culture supernatants were harvested for measurement of cytokine concentrations, and the remaining cells were resuspended in fresh medium to determine surface expression of CD3, CD4, and CD25, as well as the proportion of apoptotic cells by flow cytometry.

RESULTS

Phenotype and cytokine profile of clonal T cells from two patients with chronic hypereosinophilia. Lymphocyte phenotyping showed a population of $CD3^-CD4^+$ lymphocytes in two patients (P1 and P2) with chronic hypereosinophilia. These aberrant cells, which did not express α/β or γ/δ TCR (not shown), represented 88% and 83% of total $CD4^+$ lymphocytes in P1 and P2, respectively. As shown in Fig 1, the CD3-negative helper T cells from both patients expressed CD2 and CD28, but neither CD80 nor CD86 (not shown). Furthermore, they expressed the CD45RO isoform characteristic of memory T cells, while lacking CD40L, CD7, and CD27. Finally, the α chain of the IL-2 receptor (CD25) was absent on cells from P1, while it was weakly expressed on cells from P2 (Fig 1). Distribution of surface antigens on the CD3-positive helper T-cell population from P1 and P2 was similar to that of $CD4^+$ T cells obtained from normal subjects.

The clonal nature of the $CD3^-CD4^+$ cell population from both patients was established by Southern blot and PCR analysis of the TCR β-chain and γ-chain genes, respectively (not shown). Cytogenetic analysis of initial blood samples from P1 at time of presentation showed a normal karyotype, but clonal T cells obtained for the present study displayed chromosomal abnormalities characterized by partial deletions of chromosomes 6 and 10. In contrast, circulating leukocytes obtained

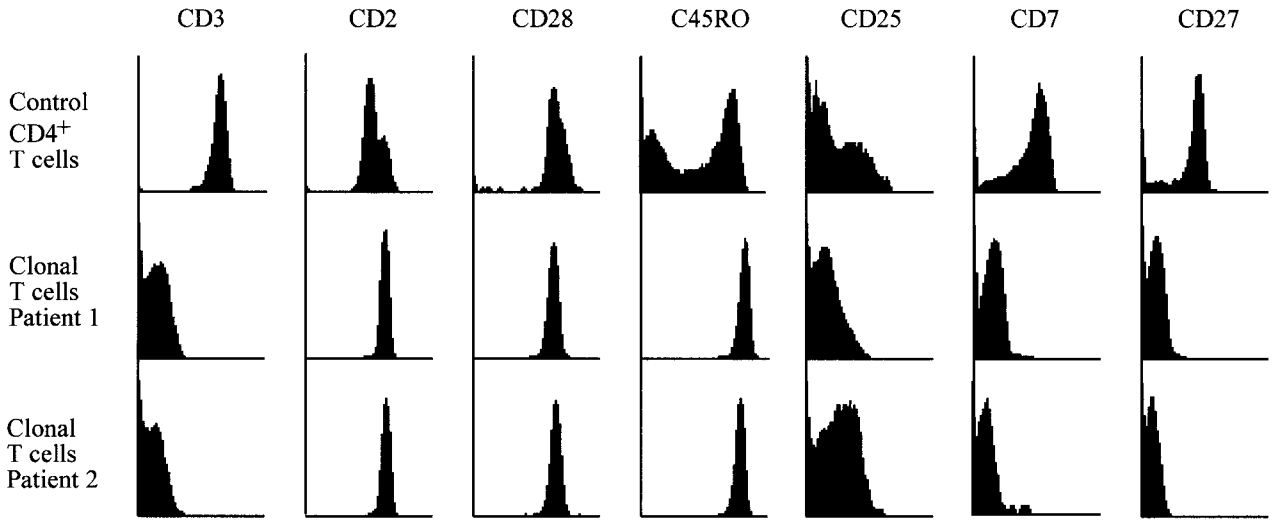

Fig 1. Surface phenotype of clonal T cells from two hypereosinophilic patients. PBMC from two hypereosinophilic patients and from a healthy blood donor were stained with FITC-, PE-, or PerCP-conjugated MoAbs against CD4, CD3, CD2, CD28, CD45RO, CD25, CD7, and CD27 antigens. Flow cytometric determination of surface phenotype is shown after gating on $CD4^+$ (healthy subject) or $CD3^-CD4^+$ (patients) lymphocytes. Data were obtained on more than 10,000 viable cells.

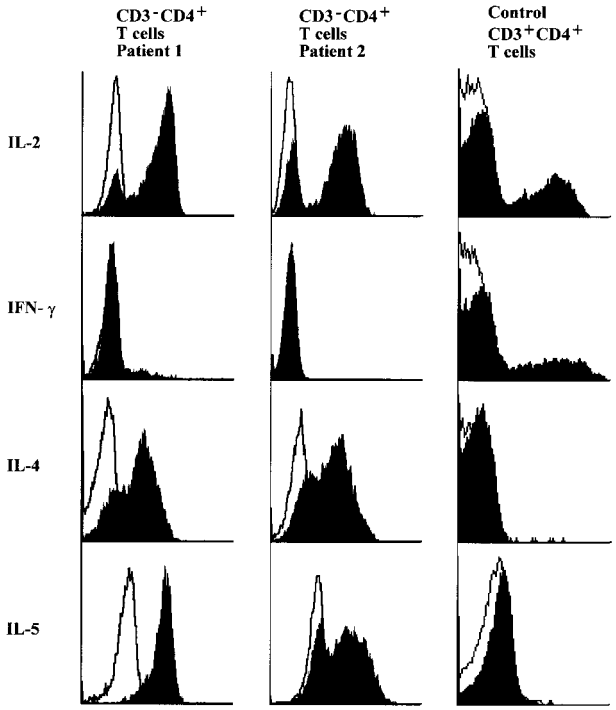

Fig 2. Cytokine profile of clonal T cells from two hypereosinophilic patients. Total T cells ($CD4^+$ and $CD8^+$) were isolated from PBMC obtained from two hypereosinophilic patients and a healthy blood donor by negative selection. After a 6-hour culture (10^6 cells/mL) with Brefeldine A (10 μg/mL) in the absence or in the presence of PMA (50 ng/mL) and A23187 (0.1 μg/mL), cell membranes were stained with FITC- or PE-conjugated anti-CD8 and PerCP-conjugated anti-CD3 MoAbs. The T cells were fixed and permeabilized before staining cytokines with PE- or FITC-conjugated MoAbs against IL-2, IFN-γ, IL-4, or IL-5. Intracytoplasmic expression of cytokines was analyzed by flow cytometry after gating on $CD3^-CD8^-$ (equivalent to $CD3^-CD4^+$) lymphocytes for patients and $CD3^+CD8^-$ (equivalent to $CD3^+CD4^+$) lymphocytes for the control subject. Filled histograms represent staining for cytokines in cells that have been stimulated with PMA and A23187. Negative controls (solid lines) are provided by unstimulated cells that have been incubated in the presence of Brefeldine A alone.

from P2 displayed a normal karyotype at the time of investigation.

The cytokine profile of the $CD3^-CD4^+$ cell population was first determined using flow cytometry after intracellular staining. In the absence of in vitro stimulation, no cytokines were detected. After 6 hours of incubation with PMA and A23187 ionophore, a clearly distinct cytokine profile was observed as compared with control $CD3^+CD4^+$ cells (Fig 2). Indeed, a significant proportion of the $CD3^-CD4^+$ cells expressed IL-4 (77% for P1, 69% for P2) and IL-5 (95% for P1, 69% for P2), whereas IFN-γ was virtually absent. Furthermore, most $CD3^-CD4^+$ cells produced IL-2 (82% for P1, 69% for P2). The cytokine profile of $CD3^+CD4^+$ cells from both patients was comparable to that of control $CD4^+$ cells from normal subjects (percentage of $CD3^+CD4^+$ cells producing IL-2 was 30% and 31% in P1 and P2, respectively, for IFN-γ, 29% and 30%; IL-4, 3% and 6%, and IL-5, 3% and 5%).

Cytokine synthesis by clonal T cells cultured with mitogens. As shown in Table 1, the Th2 cytokine profile of the clonal T cells was confirmed by measurement of IL-5, IL-13, and IFN-γ concentrations in supernatants of purified $CD3^-CD4^+$ lymphocytes incubated for 48 hours with PMA and either A23187 ionophore or anti-CD28 MoAb. IL-4 was also produced, although at lower levels than IL-13, especially after stimulation with PMA and anti-CD28 MoAb. In the following experiments, Th2 activities were therefore assessed by IL-5 and IL-13 measurements. In addition to IL-4, IL-5, and IL-13, $CD3^-CD4^+$ cells from both patients produced high levels of IL-2 in these conditions. As expected by their phenotype, these clonal T cells did not respond to immobilized anti-CD3 MoAb alone or combined with soluble anti-CD28 MoAb, neither in terms of cytokine secretion (levels of IL-5 and IL-13 in 48-hour culture supernatants remained below detection threshold levels for both patients) nor in terms of proliferation (^{3}H-thymidine incorporation after 48 hours of culture: 46 and 128 cpm for patients 1 and 2, respectively, versus 185,348 cpm for normal $CD4^+$ T cells purified from a healthy donor).

DC induce cytokine synthesis by clonal T cells. We sought to determine whether signals provided by DC would lead to activation of clonal T cells despite absence of signaling through the TCR/CD3 complex. Coculture of purified $CD3^-CD4^+$ cells from patient 1 for 5 days with autologous or allogeneic DC indeed resulted in the induction of IL-5 and IL-13 synthesis, whereas IFN-γ remained below detection levels in corresponding supernatants (Table 2). Only low levels of IL-2 (<700 pg/mL) were measured in these conditions, presumably because of reuptake by the clonal T cells during the 5-day culture period (see below). Furthermore, the clonal cells displayed marked proliferation during T-DC cocultures (Table 2). As similar results were obtained with autologous versus allogeneic DC in these experiments (which was consistent with the lack of TCR/CD3 expression by the clonal T cells), allogeneic DC were used in further experiments for the sake of greater availability.

In contrast to patient 1, clonal T cells from patient 2 were not

Table 1. In Vitro Activation of Clonal T Cells From Two Patients With Chronic Hypereosinophilia

	IL-2 (pg/mL)			IL-4 (pg/mL)			IL-5 (pg/mL)			IL-13 (pg/mL)			IFN-γ (U/mL)		
Culture Conditions*	P1	P2	Control	P1	P2	Control	P1	P2	Control	P1	P2	Control	P1	P2	Control
None	<20	<20	<20	<20	<20	<20	<20	<20	<20	53	<10	<10	<2	<2	<2
PMA alone	1,800	<20	<20	<20	<20	<20	91	165	<20	247	630	<10	<2	<2	<2
PMA + A23187	338,500	309,000	366,000	3,184	10,350	114	34,250	18,900	119	16,050	14,750	21	160	4	4,700
PMA + anti-CD28	33,500	98,000	52,000	45	49	<20	8,400	12,950	802	14,050	2,900	195	<2	4	89

*Purified $CD3^-CD4^+$ cells from patient 1 (P1) and patient 2 (P2) or $CD3^+CD4^+$ cells from a healthy blood donor (control) (5×10^5 cells/mL) were stimulated with PMA (1 ng/mL) alone, or PMA + A23187 calcium ionophore (0.1 μg/mL), or PMA + anti-CD28 MoAb (1 μg/mL). After 48 hours, culture supernatants were harvested and cytokine concentrations were determined by ELISA. Data are from 1 of 3 experiments, which gave similar results.

Table 2. Activation of Clonal T Cells From Patient 1 With Autologous or Allogeneic DC

DC Type*	^{3}H-Thymidine Uptake (cpm)		IL-5 (pg/mL)		IL-13 (pg/mL)		IFN-γ (U/mL)	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
None	49	70	57	<20	<10	<10	<2	<2
Autologous DC	27,432	17,638	7,450	>5,000	12,150	3,698	<2	<2
Allogeneic DC	21,146	37,364	4,150	1,621	4,900	4,966	3	<2

*Mixed leukocyte cultures were prepared between purified $CD3^-CD4^+$ cells and mature irradiated DC generated from either autologous or allogeneic PBMC at a DC:T-cell ratio of 1:30. Culture supernatants were harvested after 5 days for cytokine measurements. T-cell proliferation was assessed by measuring ^{3}H-thymidine incorporation during the following 16 hours.

stimulated by DC to secrete cytokines (Table 3). In parallel, we observed a higher degree of apoptosis among P2 clonal cells: after 5-day coculture with DC, mean percentage of apoptotic T cells was 59.1% ± 2.63% in patient 2 (mean ± standard error of mean [SEM], n = 8) versus 36.1% ± 2.79% in patient 1 (n = 9, $P = .0003$ using Mann-Whitney's test). We reasoned that addition of a T-cell survival-promoting factor such as rIL-2 or rIL-15 could influence both their survival and their responses to DC. As shown in Table 3, incubation of clonal P2 cells alone with a high concentration of rIL-2 resulted in enhanced survival and proliferation, but induced the secretion of only low levels of cytokines. Indeed, the presence of both rIL-2 and DC in cocultures was required to induce secretion of high amounts of IL-5 and IL-13 by clonal P2 cells.

Activation signals delivered by DC involve B7/CD28 and CD2 pathways. To investigate the nature of the activation signals delivered by DC to clonal T cells, blocking MoAbs to B7-1, B7-2, CD2, or CD40 were added to cocultures of purified $CD3^-CD4^+$ T cells with DC. For the above-mentioned reason, rIL-2 was added to cultures performed with P2 cells. Addition of anti-CD80 MoAb to T-DC cocultures had no significant effect on IL-5 and IL-13 production. In contrast, addition of anti-CD86 MoAb strongly inhibited IL-5 (Fig 3) and IL-13 production by cells of both P1 and P2 (mean percentages of inhibition in two independent experiments: 77.5% and 83.4% for IL-5, and 88.1% and 80% for IL-13, in P1 and P2, respectively). Blocking both CD86 and CD80 almost completely abolished cytokine production by the clonal T cells (mean percentages of inhibition in two independent experiments: 97.1% and 94.25% for IL-5, and 99.45% and 95.7% for IL-13, in P1 and P2), and this was confirmed using soluble CTLA4-Ig in parallel experiments. Thus, signaling through CD28 appears to play a crucial role in activation of the clonal T cells. Costimulation through CD2 also contributes to this process, as indicated by the major reduction of IL-5 and IL-13 production on addition of anti-CD2 MoAb to T-DC cocultures (mean percentages of inhibition in three independent experiments: 85.6% and 80% for IL-5 and 95.1% and 67% for IL-13 in P1 and P2, respectively). Furthermore, in cocultures prepared with P1 cells, proliferation of clonal cells was inhibited by addition of anti-CD86 MoAb alone (mean percentage of inhibition in two independent experiments: 73.5%) or combined with anti-CD80 MoAb (mean percentage of inhibition in two independent experiments: 99.4%) as well as by addition of anti-CD2 MoAb (mean percentage of inhibition in three independent experiments: 99.5%). In cocultures prepared with P2 cells in which a high dose of exogenous IL-2 sufficient to drive T-cell proliferation was added, such inhibition was not observed. The specificity of these findings was ascertained by control experiments using irrelevant isotype-matched MoAbs. Moreover, the addition of a blocking anti-CD40 MoAb had no significant effect in this system.

Table 3. Activation of Clonal T Cells From Patient 2 by DC Requires Addition of rIL-2

MLC Conditions*	Apoptotic Cells (%)	^{3}H-Thymidine Uptake (cpm)	IL-5 (pg/mL)	IL-13 (pg/mL)	IFN-γ (U/mL)
T cells	54	47	<20	<10	<2
T cells + DC	51	143	<20	<10	<2
T cells + rIL-2	28	22,886	502	427	<2
T cells + DC + rIL-2	24	35,010	32,520	11,100	<2

*Purified $CD3^-CD4^+$ cells from patient 2 were cocultured with mature irradiated DC generated from allogeneic PBMC (DC:T-cell ratio 1:30) in absence or in the presence of rIL-2 (150 U/mL). After 5 days, the percentage of apoptotic cells was determined by flow cytometry using annexin V and PI staining, T-cell proliferation was determined by ^{3}H-thymidine uptake, and cytokine concentrations were measured in supernatants by ELISA. Data are from 1 of 8 experiments, which gave similar results.

Activation of clonal T cells depends on an autocrine IL-2/IL-2R-α pathway. We finally investigated the role of endogenous IL-2 production in the responses of T cells from patient 1 during cocultures with DC. First, we observed that addition of a blocking anti–IL-2R-α (CD25) MoAb to cocultures of P1 cells with DC dramatically decreased their proliferative responses and enhanced their apoptosis rate. In parallel, the secretion of both IL-5 and IL-13 was profoundly inhibited in the presence of the anti-CD25 MoAb. These effects of IL-2R-α blockade were further enhanced by addition of a neutralizing anti–IL-2 MoAb (Table 4). Consistent with these findings, we observed that clonal cells from patient 1 upregulated their membrane expression of CD25 during cocultures with DC, allowing them to respond to their own secretion of IL-2. As shown in Fig 4, this CD25 upregulation depends both on CD28 and CD2 signaling, as it is prevented by the addition of CTLA4-Ig or blocking anti-CD2 MoAb. To investigate the effects of IL-2 on the synthesis of Th2 cytokines independently from its effects on survival and proliferation, cocultures were prepared using clonal cells from P2 in the presence of an alternative T-cell survival promoting factor, rIL-15. In these experiments, anti-IL–2R-α MoAb specifically inhibited cytokine production (99% inhibition for IL-5 and 86% inhibition for IL-13) without affecting T-cell survival or proliferation (Table 5). Taken together, these experiments establish that costimulatory signals delivered by DC initiate an IL-2/IL-2R autocrine loop, which is critically involved in the survival and proliferation of the $CD3^-CD4^+$ cells, as well as in their secretion of cytokines.

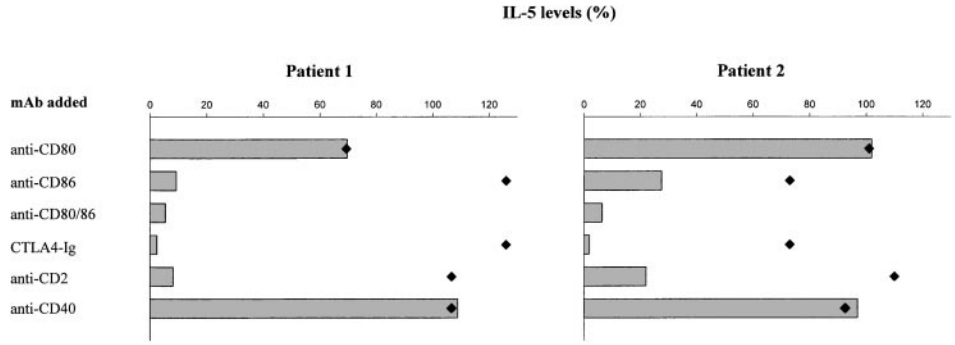

Fig 3. Synthesis of Th2 cytokines by clonal T cells requires signaling through CD28 and CD2 molecules. Purified CD3⁻CD4⁺ cells from hypereosinophilic patients were cocultured with mature allogeneic irradiated DC in the absence (patient 1) or in the presence (patient 2) of 150 U/mL rIL-2 at a DC:T-cell ratio of 1:30. In successive experiments, CTLA4-Ig (10 μg/mL) or blocking MoAbs against B7-1 (CD80), B7-2 (CD86), CD2 (all used at 10 μg/mL), CD40 (100 ng/mL), or isotypic controls at the same concentrations were added to cocultures. After 5 days, supernatants were harvested for measurement of IL-5 concentrations by ELISA. Solid bars represent IL-5 synthesis during cocultures in the presence of the above-mentioned blocking molecules, and diamonds represent IL-5 production in the presence of corresponding isotypic controls. Results are expressed as percentage of IL-5 levels reached in the presence of blocking MoAbs compared with T-DC cocultures prepared in the absence of these MoAbs. Data from one of two experiments, which yielded similar results for each coculture condition, are shown.

DISCUSSION

Absent or low membrane expression of the TCR/CD3 complex on helper T cells has been observed in several pathological settings including (retro-)viral infections,[22-24] chronic antigenic stimulation,[25] and cancer.[26,27] In our hypereosinophilic patients, we have failed so far to show any retroviral sequences and the TCR/CD3-negative phenotype of their clonal T cells was stable throughout T-DC cocultures, as well as after prolonged culture in the presence of rIL-2 (data not shown), indicating that the loss of TCR/CD3 expression did not depend on continuous exposure to a putative exogenous antigen. Whatever the cause of their lack of TCR/CD3 expression, these clonal T cells are likely to be involved in the pathogenesis of hypereosinophilia through secretion of high levels of IL-5.

In this study, we first observed that freshly explanted and purified CD3⁻CD4⁺ cells from our patients did not proliferate and were unable to produce cytokines spontaneously in vitro, as established by flow cytometry showing absence of intracytoplasmic cytokines, and by absence of measurable cytokine release into culture supernatants. Addition of mitogenic factors, which bypass physiological activation pathways such as PMA in combination with either A23187 ionophore or anti-CD28 MoAb, was necessary to elicit cytokine production in vitro. In these conditions, the clonal cells secreted high levels of IL-5, IL-4, and IL-13, but were unable to produce IFN-γ, indicating their Th2 nature.[18,19] Thus, these cells could be incriminated for both hypereosinophilia, through IL-5 production,[28] and high serum IgE levels, through IL-4 and IL-13 production.[29] The absence of spontaneous activation in vitro contrasted with clinical evidence that the clonal T cells were in an activated state in vivo. Indeed, the long-standing hypereosinophilia observed in both patients indicated persistent secretion of Th2-type cytokines, as eosinophils quickly undergo apoptosis in the absence of specific survival-promoting cytokines such as IL-5.[15,16] Furthermore,

Table 4. Evidence for an Autocrine IL-2/IL-2R-α Activation Pathway in Clonal T Cells From Patient 1

MoAb Added to MLC*	Apoptotic Cells (%)	^{3}H-Thymidine Uptake (cpm)	IL-5 (pg/mL)	IL-13 (pg/mL)
None	32.9	21,146	4,150	4,900
Anti–IL-2R-α	74.7	702	109	230
Anti–IL-2R-α + anti-IL-2	78.5	821	83	144
Isotypic ctrl (IgG2a)	39.5	25,080	4,430	2,999
Isotypic ctrl (IgG1)	36.7	25,652	4,430	5,400

*Mixed leukocyte cultures were prepared between purified CD3⁻CD4⁺ cells from P1 and mature irradiated DC generated from allogeneic PBMC at a DC:T cell ratio of 1:30 in the absence or presence of blocking anti–IL-2R-α MoAb (IgG2a) (10 μg/mL) or a combination of anti–IL-2R-α and anti–IL-2 MoAbs (IgG1) (10 μg/mL) or the same concentration of isotypic control (ctrl) MoAbs. After 5 days, the percentage of apoptotic cells, T-cell proliferation, and cytokine levels in culture supernatants were determined as indicated in Table 3. Data are from 1 of 2 experiments, which gave similar results.

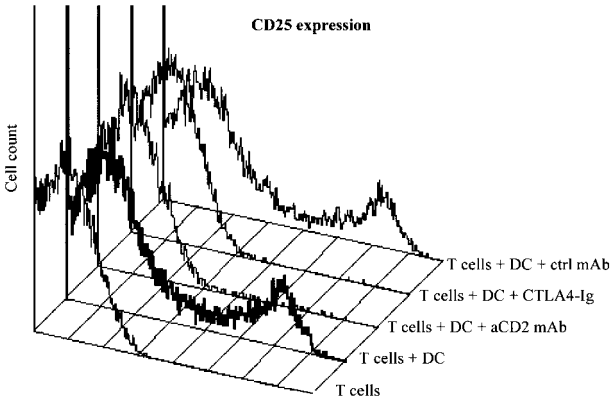

Fig 4. Activation of clonal T cells by DC during cocultures is associated with upregulation of IL-2R-α (CD25) expression. Purified CD3⁻CD4⁺ cells from patient 1 were cultured alone or with mature autologous irradiated DC in the absence or in the presence of CTLA4-Ig (10 μg/mL), blocking MoAb against CD2 (10 μg/mL) or corresponding isotypic controls at a DC:T cell ratio of 1:30. Cells were harvested after 5 days and stained with fluoro-conjugated MoAbs against CD3, CD4, and CD25. Flow cytometric analysis of surface expression of CD25 is shown on at least 10,000 viable cells after gating on CD3⁻CD4⁺ lymphocytes. Data from one of two experiments with similar results for each condition are shown.

Table 5. Role of Endogenous IL-2 in Th2 Cytokine Production by Clonal Cells From Patient 2

MLC Conditions*	Apoptotic Cells (%)	^{3}H-Thymidine Uptake (cpm)	IL-5 (pg/mL)	IL-13 (pg/mL)
T cells	62	388	<20	<10
T cells + rIL-15	39	1,033	<20	<10
T cells + DC	54	704	<20	<10
T cells + DC + rIL-15	37	2,336	13,970	990
T cells + DC + rIL-15 + anti–IL-2R-α	34	2,038	127	142

*MLC were prepared between purified $CD3^-CD4^+$ cells from P2 and mature irradiated DC generated from allogeneic PBMC at a DC:T cell ratio of 1:30 in the absence or presence of rIL-15 (16.7 ng/mL) and blocking anti–IL-2R-α MoAb (10 μg/mL). After 5 days, percentage of apoptotic cells, T-cell proliferation, and cytokine levels in culture supernatants were determined as indicated in Table 3. Data are from 1 of 2 experiments, which gave similar results.

the monoclonality of these cells suggested constant expansion of the cell population. The divergence between in vivo and in vitro behavior of the $CD3^-CD4^+$ cells argues against constitutional activation of signaling pathways in the clonal T cells, as has been described for transformed lymphocytes infected with human T-cell lymphotropic virus type 1 (HTLV-1) in adult T-cell leukemia lymphoma.[30] They suggest on the contrary that the clonal $CD3^-CD4^+$ cell population remains dependent on exogenous signals for both proliferation and cytokine production. In this study, we focused on accessory signals delivered by antigen-presenting cells,[31] by performing cocultures between $CD3^-CD4^+$ cells and DC previously incubated with LPS. Indeed, LPS-induced maturation of DC enhances their T-cell–stimulating properties by upregulating their expression of LFA-3, B7-1, B7-2, and CD40, as well as their secretion of IL-12.[32] We found that DC were able to induce both proliferation and cytokine production by clonal cells obtained from patient 1 in the absence of TCR/CD3 signaling. Despite secretion of IL-12 by DC, the T cells produced IL-5 and IL-13 and remained unable to produce IFN-γ, consistent with recent data demonstrating unresponsiveness of mature Th2 cells to IL-12.[33] Clonal cells obtained from patient 2 differed in that addition of rIL-2 or rIL-15 to cocultures was necessary to observe efficient activation by DC. The different activation requirements of P1 and P2 cells during cocultures probably reflects different thresholds of cytokine-deprivation induced apoptosis in these cells. Indeed, complete blockade of IL-2/IL-2R interactions in P1 cells led to high levels of apoptosis and thus abrogated their responses to DC alone, leading to a situation similar to that observed with P2 cells. Such divergence in survival requirements may be explained by the fact that P1 and P2 cells were obtained at different time points in disease course. Indeed, cells were harvested from P1 several years after initial symptoms and discovery of hypereosinophilia during a treatment-free period characterized by severe eczematous lesions, whereas cells were obtained from P2 at time of diagnosis, only 5 months after first appearance of clinical manifestations. Moreover, P1 cells displayed chromosomal abnormalities, suggestive of progression toward T-cell malignancy, whereas P2 had a normal karyotype. Although not yet capable of autonomous proliferation, clonal cells from P1 may thus be less dependent on growth factors for survival than those from P2. When cocultures using P2 cells were prepared in the presence of rIL-15 to promote T-cell survival instead of rIL-2, addition of anti–IL-2R-α MoAb specifically inhibited Th2 cytokine production without affecting proliferation or apoptosis. Thus, IL-2 was found critical not only to protect clonal T cells from apoptosis, but also to induce their proliferation and synthesis of cytokines.

Data obtained with cells of both patients showed that B7/CD28 and LFA-3/CD2 interactions were critically involved in their TCR-independent activation, as indicated by profound inhibition of proliferation (P1) and cytokine production (both P1 and P2) when cocultures were performed in the presence of blocking antibodies that interfered with these costimulatory pathways. In patient 1, these effects were in part related to CD28/CD2-dependence of CD25 upregulation on clonal T cells. Several investigators have reported that simultaneous ligation of CD2 and CD28 molecules with specific MoAbs in vitro induces prolonged T-cell proliferation compared with stimulation with anti-CD3 and anti-CD28 MoAb.[34-36] Ligation of CD2 and CD28 on human T cells was previously shown to induce IL-2 production, as well as upregulation of the IL-2R-α and -β chains, resulting in autocrine IL-2–dependent stimulation.[34,36-39] Our data obtained in patient 1 are consistent with such a role for CD2 and CD28 pathways in TCR-independent activation of T cells.

The abnormal T cells from our patients share some features with Sezary cells that are found in peripheral blood of patients with advanced stages of cutaneous T-cell lymphoma (CTCL), including their mature $CD4^+CD45RO^+CD7^-CD25^-$ phenotype,[40,41] a Th2-type cytokine profile,[42] and their clonal nature[43] in most cases. The importance of the CD28-signaling pathway in the activation of Sezary cells has been demonstrated in a recent report by McCusker et al.[44] As with our $CD3^-CD4^+$ cells, Sezary cells have a paradoxical proliferative defect in that, although they are malignant lymphomatous cells, they do not proliferate spontaneously in vitro, nor do they respond to the combined action of anti-CD3 and anti-CD28 MoAbs. However, cocultures of purified $CD4^+CD7^-$ cells with allogeneic growth-inactivated PBMC in the presence of anti-CD3 MoAb induced proliferation of Sezary cells, and addition of CTLA4-Ig or of combined anti-B7 MoAbs to these cultures led to substantial inhibition of cell growth. These results indicate that although signaling through CD28 is critical for Sezary cell growth following engagement of the TCR/CD3 complex, another as of yet unidentified signal provided by APC is required for activation of Sezary cells. Similarly, we observed that stimulation of clonal T cells from patient 1 with a combination of anti-CD2 and anti-CD28 MoAbs was not sufficient to elicit optimal cytokine synthesis (data not shown), suggesting that additional costimulatory signals delivered by DC were indeed operative in our coculture experiments.

It has been suggested that the nature of signals leading to T-cell activation can skew the subsequent cytokine profile. Activation of mature T cells through the alternative CD2-dependent pathway has been shown to induce IL-4 production more efficiently than engagement of CD3,[45] and signaling through CD28 was involved in the differentiation of naive $CD4^+$ T cells toward Th2 cytokine-producing cells.[46,47] The specific involvement of the CD28 ligands B7-1 and B7-2 in functional differentiation of target cells remains a controversial

issue.[48] Several studies suggest that ligation of CD28 by CD80 preferentially induces Th1 cytokines, whereas CD86 preferentially induces Th2 cytokines.[49,50] However, the fact that anti-CD86 MoAb often inhibited Th2 cytokine production more efficiently than anti-CD80 MoAb as in our own study could simply reflect different levels and/or kinetics of expression of these molecules on APC.[51,52]

Clinical observations indicate that chronic hypereosinophilia is sometimes associated with a premalignant lymphoproliferative condition as some HES patients will eventually develop a full-blown T-cell lymphoma.[53] Identification of a monoclonal population of $CD3^-$ helper T cells could be a predictive marker of malignant transformation.[54-56] Understanding the activation requirements of such cells could lead to more efficient therapeutic approaches both for the control of eosinophilia and for prevention of malignant evolution. Our observations that TCR-independent activation of the $CD3^-CD4^+$ cells can be provided by APC through an IL-2–dependent process, which requires signaling through CD2 and CD28 molecules may be useful for defining new therapeutic strategies for hypereosinophilic patients with a profile similar to ours.

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Annex 2

Clonal Th2 lymphocytes in patients with the idiopathic hypereosinophilic syndrome. Roufosse F., Schandené L., Sibille C., Kennes B., Efira A., Cogan E., Goldman M.

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This article describes the inclusion of five additional patients in the established L-HES cohort following strict clinical and biological diagnostic criteria. Besides sustained hypereosinophilia, IL-5 and hyper-IgE secretion, the biological diagnosis mainly involved the search for the aberrant CD3-CD4+ immunophenotype associated with a clonal TCR rearrangement. In some cases, TCR rearrangement clonality was detectable only on the purified abnormal T-cell population, suggesting that use of adequate molecular and immunological techniques is mandatory for an accurate L-HES diagnosis. Observation of the clinical features revealed a major contribution of cutaneous signs associated with hypereosinophilia with reduced life-threatening complications in the short-term course of the L-HES disease. However, in this report, one of our L-HES patients was shown to develop an anaplastic null cell T-lymphoma, thus overshadowing the long term prognosis of L-HES confirmed by the following studies.

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Clonal Th2 lymphocytes in patients with the idiopathic hypereosinophilic syndrome

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Summary. Idiopathic hypereosinophilic syndrome (HES) and Gleich's syndrome are related disorders characterized by persistent or recurrent hypereosinophilia of unknown origin. Elevated IgE levels and polyclonal hypergammaglobulinaemia are considered as markers of benign outcome in this setting as they are generally associated with predominant cutaneous manifestations and favourable response to glucocorticoid therapy. In a previous study, we identified a clonal population of $CD3^{-}CD4^{+}$ Th2-like lymphocytes secreting interleukin (IL)-5 and IL-4 in peripheral blood of a patient fulfilling the diagnostic criteria of HES with associated serum hyper-IgE. We now extend this observation by describing identical findings in three additional patients, and we compare their clinical and biological parameters with five other patients with HES. Chromosomal abnormalities were detected in purified $CD3^{-}CD4^{+}$ Th2 cells from three patients, among whom one developed anaplastic null cell lymphoma. We therefore suggest that a careful search for T-lymphocyte clonality and cytogenetic changes should be included in the work-up of HES for adequate management.

Keywords: hypereosinophilic syndrome, lymphoma, IL-5, IgE, $CD3^{-}CD4^{+}$.

Idiopathic hypereosinophilic syndrome is defined as persistent hypereosinophilia of unknown origin complicated by organ damage or dysfunction (Fauci *et al*, 1982; Weller & Bubley, 1994). Although this entity was introduced in an attempt to standardize management of such patients (Chusid *et al*, 1975), the heterogeneous nature of hypereosinophilic syndrome rapidly emerged with regard to clinical and biological parameters, disease course and response to therapy. Patients with clinical and biological markers reminiscent of myeloproliferative disorders generally responded poorly to glucocorticoids and presented severe cardiac complications of hypereosinophilia. In contrast, high IgE levels and polyclonal hypergammaglobulinaemia were considered to be markers of good prognosis as they were frequently associated with restriction of clinical manifestations to the skin and favourable response to corticosteroid therapy (Weller & Bubley, 1994). A similar profile is observed in patients with Gleich's syndrome (Gleich *et al*, 1984), a distinct entity in which chronic idiopathic hypereosinophilia is associated with episodic angioedema.

Interleukin (IL)-5 is a highly specific eosinophilopoietic cytokine which increases eosinophil levels both by stimulating their differentiation from bone marrow precursors and by inhibiting peripheral apoptosis (Her *et al*, 1991; Wardlaw, 1994; Simon & Blaser, 1995; Walsh, 1997). IL-5 overproduction by Th2-type lymphocytes has been demonstrated in a variety of hypereosinophilic disorders, including parasitic or allergic diseases and Omenn's syndrome (Sanderson, 1992; Schandené *et al*, 1993; Romagnani, 1994, 1996). Th2-type cells also secrete IL-4 and IL-13, which are responsible for the hyperproduction of IgE often associated with hypereosinophilia (Punnonen *et al*, 1997).

We and others have demonstrated the presence of circulating T cells overproducing IL-5 in some patients with idiopathic hypereosinophilia (Cogan *et al*, 1994; Brugnoni *et al*, 1996; Kitano *et al*, 1996; Schandené *et al*, 1996; Simon *et al*, 1996, 1999; Bank *et al*, 1998). T-cell

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involvement in the pathogenesis of this disorder is further suggested by the fact that long-term evolution is occasionally complicated by the development of T-cell lymphoma (Weller & Bubley, 1994). This led us to systematically perform T-cell immunophenotyping, a search for T-cell clonality and analysis of cytokine production by peripheral blood T cells in a consecutive series of nine patients with persistent idiopathic hypereosinophilia.

MATERIALS AND METHODS

Patients. Patients with eosinophil levels exceeding $1{\cdot}5 \times 10^9$/l were recruited in several Belgian hospitals. Thorough evaluation of hypereosinophilia was performed by local medical teams and medical records were then consulted by our group. Among these patients, nine presented persistent idiopathic hypereosinophilia and were thus selected for further testing in our laboratory. At time of evaluation, patient 5 was under treatment (methylprednisolone 32 mg/d) because of rapid deterioration of heart function and patient 6 had interrupted corticosteroid therapy 1 week before hospitalization. All other patients were free from treatment.

Cell purification. Circulating leucocytes were obtained from patients either by venepuncture in 60-ml heparinized syringes or by cytapheresis (patients 1, 3 and 4) after informed consent and from buffy coats of healthy blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. After washing with Hank's balanced salt solution (HBSS) (Gibco, Life Technologies, Paisley, UK), PBMCs were resuspended either in normal culture medium composed of RPMI-1640 (Bio Whittaker/Boehringer Ingelheim, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Bio Whittaker/Boehringer Ingelheim) and 40 μg/ml gentamicin (Schering-Plough, Kennelworth, NJ, USA) for immediate testing or in cold-conservation medium composed of RPMI-1640 supplemented with 10% dimethylsulphoxide (Sigma Chemical, St. Louis, MO, USA) and 10% FCS for storage in liquid nitrogen before testing.

Purified $CD3^-CD4^+$ cells were obtained from patients 1, 3, 4 and 9 and $CD3^+CD4^+$ cells were obtained from healthy donors by negative selection. PBMCs were thawed and treated with Lymphokwik T (One Lambda, Los Angeles, CA, USA) according to the manufacturer's instructions. The remaining cells were resuspended in culture medium at 10×10^6/ml and were incubated with mouse monoclonal antibodies (mAbs) against CD14, CD19, CD56 and CD8 with or without anti-CD3 (Becton Dickinson, Mountain View, CA, USA) for 30 min at 4°C. After washing with HBSS, cells were resuspended in culture medium and incubated with sheep anti-mouse IgG-coated magnetic Dynabeads (Dynal, Oslo, Norway) for 45 min at 4°C. Coated cells were removed with a magnet, leaving purified $CD3^-CD4^+$ or $CD3^+CD4^+$ cells in suspension. $CD3^+CD4^+$ cell preparations were $>95\%$ pure, and $CD3^-CD4^+$ cell preparations contained less than 0·5% CD3-positive cells and $>95\%$ $CD4^+$ cells, as assessed by flow cytometry.

Flow cytometry. Flow cytometric analysis of the surface phenotype was performed by two- and three-colour immunofluorescence using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and peridinin chlorophyll-a protein (PerCP)-conjugated mAbs. Surface antigens of T cells were stained with mAbs against T-cell receptor (TCR)-α/β, TCR-γ/δ, CD3, CD4, CD8, CD7, CD27, CD2, CD28, HLA-DR, CD95 and CD45RO from Becton Dickinson. Data were collected on at least 10 000 viable cells using a FACScan flow cytometer and CELLQUEST software (Becton Dickinson).

Flow cytometry was also used for the detection of intracytoplasmic cytokine expression in lymphocyte subsets. To this end, total T cells ($CD4^+$ and $CD8^+$) were isolated from PBMCs of all patients except patient 1 and from healthy control subjects using the same procedure as described above, except that only mAbs against CD14, CD19 and CD56 were used. These cells were incubated at 10^6/ml with Brefeldine A (Sigma Chemical) at 10 μg/ml, alone or combined with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical) and 0·1 μg/ml calcium ionophore A23187 (Calbiochem-Behring, San Diego, CA, USA) for 6 h in culture medium (37°C, 5% CO_2). Surface antigens were stained on aliquots of 2×10^5 cells with FITC- or PE-conjugated anti-CD8 mAb and PerCP-conjugated anti-CD3 mAb. Staining of CD8 was preferred to CD4 because of important down-regulation of surface CD4 expression on T cells after *in vitro* stimulation. Cells were fixed with FACS Lysing Solution (Becton Dickinson) for 10 min at room temperature in the dark, washed and then permeabilized with 0·5 ml FACS Permeabilizing Solution (Becton Dickinson) under the same conditions. Intracellular cytokines were stained with PE- or FITC-conjugated mAbs against IL-4, IL-13, γ-interferon (IFN-γ) (Becton Dickinson) and IL-5 (Pharmingen, San Diego, USA). Negative controls for cytokine expression were provided by unstimulated cells treated only with Brefeldine A and by intracellular staining of stimulated cells with isotype-matched irrelevant PE- or FITC-conjugated mAbs. Triple stain flow cytometry permitted distinct analysis of cytokine expression in gated $CD3^+CD8^-$ (equivalent to $CD3^+CD4^+$) and $CD3^-CD8^-$ (equivalent to $CD3^-CD4^+$) cells.

Intracellular expression of CD3-ε and TCR-α/β in $CD3^-CD4^+$ cells from patients 1, 3, 4 and 9 was demonstrated by flow cytometry. PBMCs were incubated with FITC/anti-CD4 mAb and PerCP/anti-CD3 mAb. Cells were washed, fixed and permeabilized as above, and then stained with PE-conjugated anti-CD3 or anti-TCR-α/β mAb. Intracytoplasmic expression of CD3-ε and TCR-α/β was determined after gating on surface $CD3^-CD4^+$ viable lymphocytes.

Immunocytochemical staining of intracytoplasmic CD3-ε antigen. After purification, $CD3^-CD4^+$ cells from patients 1, 3 and 4 were cytospun, fixed and stained with anti-CD3-ε mAb (Dako, Glostrup, Denmark), revealed by the alkaline phosphatase antialkaline phosphatase (APAAP) technique (Dako), before and after permeabilization with acetone at 4°C for 10 min. Stained cells were then visualized by light microscopy.

Evaluation of T-cell clonality. Rearrangement of the β-chain of the TCR was performed by Southern blot analysis using a

DNA probe corresponding to the second constant region of the gene, as previously described (Cogan *et al*, 1994).

TCR γ-chain gene rearrangement was studied by multiplex polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis using primers according to the method described previously by Theodorou *et al* (1995).

Stimulation of CD4+ T cells with mitogenic agents in vitro. Purified $CD4^+$ T cells from healthy blood donors and $CD3^-CD4^+$ cells from patients 1, 3, 4 and 9 (5×10^5/ml) were stimulated using 1 ng/ml PMA and 0·1 μg/ml calcium ionophore A23187 in culture medium. All culture supernatants were harvested after 48 h at 37°C under 5% CO_2 for measurement of cytokine concentrations.

Determination of cytokine levels and soluble CD25 levels in serum and culture supernatants. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to determine concentrations of IL-13 and soluble IL-2R-α (sCD25) (Biosource, Camarillo, CA, USA). Other cytokine concentrations were measured by two-site sandwich ELISA using antibodies from Genzyme (Cambridge, MA, USA) for IL-2, Chromogenix (Mölndal, Sweden) for IFN-γ, Mabtech (Stockholm, Sweden) for IL-4 and Pharmingen for IL-5.

RESULTS

Clinical profile and laboratory findings in nine patients with chronic idiopathic hypereosinophilia

In our consecutive series of nine patients with chronic idiopathic hypereosinophilia, eight fulfilled the diagnostic criteria of HES, whereas the clinical and biological findings in patient 4 were consistent with the diagnosis of Gleich's syndrome. Their major clinical manifestations and biological findings are summarized in Tables I and II. In all cases, allergy was excluded on the basis of clinical history and negative testing for specific IgE against common allergens and serological testing for infection by the human immunodeficiency virus was negative. Thorough diagnostic work-up including radiological evaluation, radioisotopic imaging and search for blastic cells in blood and bone marrow specimens failed to reveal an underlying malignant haematological disorder at the time of diagnosis. Cytogenetic analysis of blood and bone marrow at time of presentation did not disclose chromosomal abnormalities except for three mitoses with breakpoints on chromosome 1 in a bone marrow specimen from patient 1. This patient developed disseminated anaplastic large null cell K1-positive lymphoma 4 years after diagnosis of idiopathic hypereosinophilic syndrome.

T-cell immunophenotyping in patients with idiopathic hypereosinophilic syndrome

Immunophenotypic analysis of circulating lymphocytes revealed the presence of an abnormal $CD4^+$ subset in four patients (patients 1, 3, 4 and 9) characterized by absent surface expression of the CD3 antigen (Fig 1A). These cells which also stained negatively for α/β and γ/δ TCR (not shown) represented 56%, 90%, 84% and 16% of total $CD4^+$ lymphocytes and reached absolute circulating levels of 0·7, 3·61, 2·5 and $0{\cdot}24 \times 10^9$/l in patients 1, 3, 4 and 9

Table I. Clinical profile of patients with chronic idiopathic hypereosinophilia.

	Age				
Patient	Diagnosis*	Evaluation†	Sex	Clinical manifestations	Evolution
1	30	30	M	Prurigo, dyspnoea, weight loss, digital thrombotic vasculitis	Initial response to combined GC and IFN-α; anaplastic null cell lymphoma (after 4 years)→dead
2	62	65	M	Pruritus, diarrhoea, cholestasis, weight loss	Favourable response to combined hydroxyurea and GC
3	16	20	F	Eczema, subcutaneous nodules‡, tenosynovitis, weight loss	No effect of GC or IFN-α on $CD3^-CD4^+$ subset; favourable response to fludarabine
4	21	21	F	Eczema, urticaria, episodic angioedema	Favourable clinical response and regression of $CD3^-CD4^+$ subset with GC (prednisolone 10 mg/d)
5	36	36	F	Diffuse pulmonary infiltrate, grade 4/4 mitral valve regurgitation, weight loss	Valve replacement → embolic stroke (died 4 months after diagnosis)
6	20	30	F	Eczema, urticaria	Favourable clinical and biological response to GC
7	27	28	M	Mitral valve regurgitation due to chordal rupture, weight loss	Favourable clinical and biological response to combined hydroxyurea and GC
8	34	34	M	Dyspnoea, splenomegaly, weight loss	Initiation of hydroxyurea
9	47	58	F	Urticaria	No treatment

* Age at appearance of symptoms and discovery of hypereosinophilia.
† Age at time of immunological evaluation.
‡ Small nodules in the parotid region.
GC, glucocorticoids.

Table II. Major laboratory findings in hypereosinophilic patients.

	Patient								
	1	2	3	4	5	6	7	8	9
Total WBC (10^9/l)	16·76	26·8	16·9	14·8	18·7	8	18·8	16·4	7·8
Eosinophils	6·12	16·62	8·92	9·1	3·99	3·66	9·15	11·7	2·97
Lymphocytes	1·68	2·57	4·63	3·42	1·32	2	1·91	2·66	2·24
IgE (kU/l)	2505	67	340	15640	< 3·5	327	4	20	478
IgG (g/l)	15·5	6·3	7	10·7	6·3	14·2	11·1	12·2	19·2
IgM (g/l)	72	3·8	3·1	12·5	2·1	2·95	1·5	1·6	2·36
Vitamin B12 (ng/l)	228	287	380	424	975	1043	> 2000	> 2000	1450

Normal values: IgE, < 100 kU/l ; IgG, 6·5–15 g/l ; IgM, 0·4–2·5 g/l ; vitamin B12, 180–700 ng/l.

respectively. Furthermore, the $CD3^-CD4^+$ cells expressed CD2, CD28 and the CD45RO isoform characteristic of memory T cells while lacking CD7 and CD27 (Fig 1B). They displayed an activated phenotype, assessed by expression of HLA-DR, and stained positively for CD95/Fas receptor (Fas-R). Intracytoplasmic expression of the CD3-ε and TCR-α/β chains in $CD3^-CD4^+$ cells from patients 1, 3, 4 and 9 was demonstrated by flow cytometry and by immunocytochemical staining using the APAAP method (not shown) after permeabilization of the cells. Distribution of surface antigens on the $CD3^+CD4^+$ T-cell population from all four patients was similar to that of $CD4^+$ T cells obtained from normal subjects shown in Fig 1B. Immunophenotyping of lymphocytes from patients 2, 5, 6, 7 and 8 was normal except for a CD4/CD8 ratio of 0·6 in patient 2 (Table III). Furthermore, their $CD4^+$ T cells displayed normal expression of the above-mentioned surface markers (Fig 1B).

T-cell clonality in patients with idiopathic hypereosinophilic syndrome

Clonality of circulating T cells was examined on whole blood by Southern blot and PCR analysis of the TCR β- and γ-chain genes respectively. A monoclonal rearrangement pattern was found for three of the patients with an aberrant $CD3^-CD4^+$ T-cell population (1, 3 and 4), as well as for patient 6 (see Table III and Fig 2). A germline configuration was observed in peripheral blood obtained from the remaining patients (not shown).

The $CD3^-CD4^+$ T-cell subset is monoclonal and displays a Th2-type profile of cytokine production

The T-cell clonality detected in peripheral blood of patients 1, 3 and 4 was shown to be restricted to the $CD3^-CD4^+$ subset, as demonstrated after purification (see Cogan *et al*, 1994 for patient 1 and Fig 2A for patients 3 and 4). Furthermore, although the search for T-cell clonality performed on whole blood was negative for patient 9, a clonal rearrangement pattern was clearly observed after purification of the $CD3^-CD4^+$ population (Fig 2C).

The cytokine profile of isolated $CD3^-CD4^+$ cells was investigated by measuring cytokine concentrations in culture supernatants after 48 h stimulation with PMA

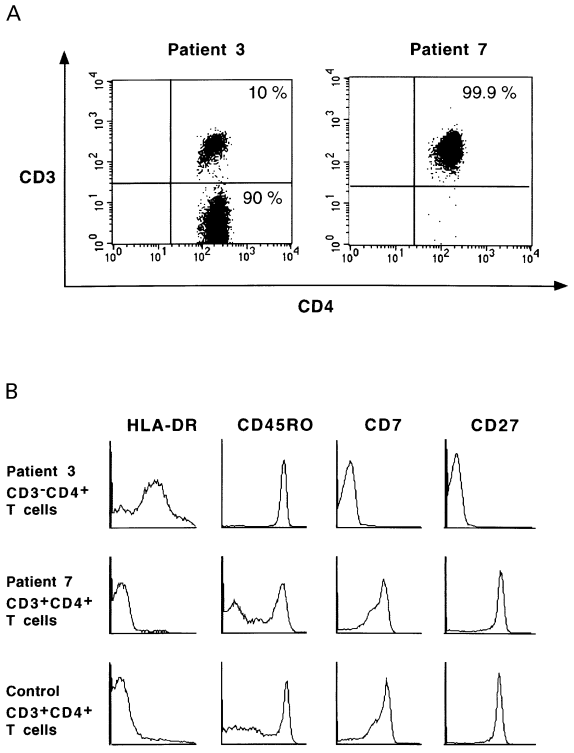

Fig 1. Identification of a subset of $CD3^-CD4^+$ T cells in four patients with chronic idiopathic hypereosinophilia. PBMCs from hypereosinophilic patients and healthy blood donors were stained with FITC-, PE- and PerCP-conjugated mAbs against CD3, CD4, CD7, CD27, CD45RO and HLA-DR and the surface phenotype was analysed by flow cytometry. Results for patients 1, 3, 4 and 9 are illustrated by patient 3, and results for patients 2, 5, 6, 7 and 8 are illustrated by patient 7. After gating on $CD4^+$ lymphocytes (A), flow cytometric analysis revealed a subset of cells lacking expression of CD3 in patient 3, whereas in patient 7 all $CD4^+$ cells stained positively for CD3. Surface phenotype of gated $CD3^-CD4^+$ T cells from patient 3 is compared with that of gated $CD3^+CD4^+$ T cells from patient 7 and from a healthy blood donor (B).

Table III. Overview of T-cell investigations in patients with chronic idiopathic hypereosinophilia.

Patient	Serum IgE (kU/l)	Serum sCD25* (pg/ml)	Serum IL-5† (pg/ml)	Lymphocyte phenotyping	TCR rearrangement	Cytogenetic analysis
1	2505	128	320	56% $CD3^-CD4^+$ cells	C	Chromosome 1‡
3	340	180	50	90% $CD3^-CD4^+$ cells	C	Chromosomes 6, 10§
4	15640	175	< 20	84% $CD3^-CD4^+$ cells	C	Chromosomes 6, 10§
9	478	nd	< 20	16% $CD3^-CD4^+$ cells	C	Normal
2	67	< 16	< 20	CD4/8 ratio 0·6	GL	Normal
5	< 3·5	< 16	< 20	Normal	GL	Normal
6	327	< 16	< 20	Normal	C	Normal
7	4	2506	< 20	Normal	GL	Normal
8	20	nd	< 20	Normal	GL	Normal

* Normal value < 16 pg/ml.
† Normal value < 20 pg/ml.
‡ Bone marrow: three mitoses with breakpoints on chromosome 1.
§ Peripheral blood: partial deletions on chromosomes 6 and 10.
C, monoclonal; GL, germline configuration; nd, not done.

and A23187 calcium ionophore. High levels of IL-4 and IL-5 were detected in supernatants compared with $CD3^+CD4^+$ T cells from healthy subjects and IFN-γ was virtually absent (Table IV). These cells also secreted IL-2, although to a lesser extent than purified $CD4^+$ T cells from healthy subjects. Flow cytometric analysis of intracytoplasmic cytokine expression after brief stimulation using PMA and A23187 confirmed the Th2-like profile of $CD3^-CD4^+$ cells. Indeed, a significant proportion of these cells expressed IL-4 (77% for patient 3, 69% for patient 4, 83% for patient 9), IL-13 (64% for patient 3, 49% for patient 4, 80% for patient 9) and IL-5 (95% for patient 3, 69% for patient 4, 25% for patient 9). These results contrasted with the minute fractions of IL-4-, IL-13- and IL-5-producing cells among normal $CD3^+CD4^+$ lymphocytes from healthy subjects (1·46 ± 0·2% for IL-4, 0·76 ± 0·13% for IL-13 and 0·4 ± 0·14% for IL-5; means ± s.e.m. of cytokine-positive cells in 11 healthy subjects). Patient 9 differed from patients 1, 3 and 4 in that IFN-γ was barely detectable in the $CD3^-CD4^+$ cells from these patients, whereas it was

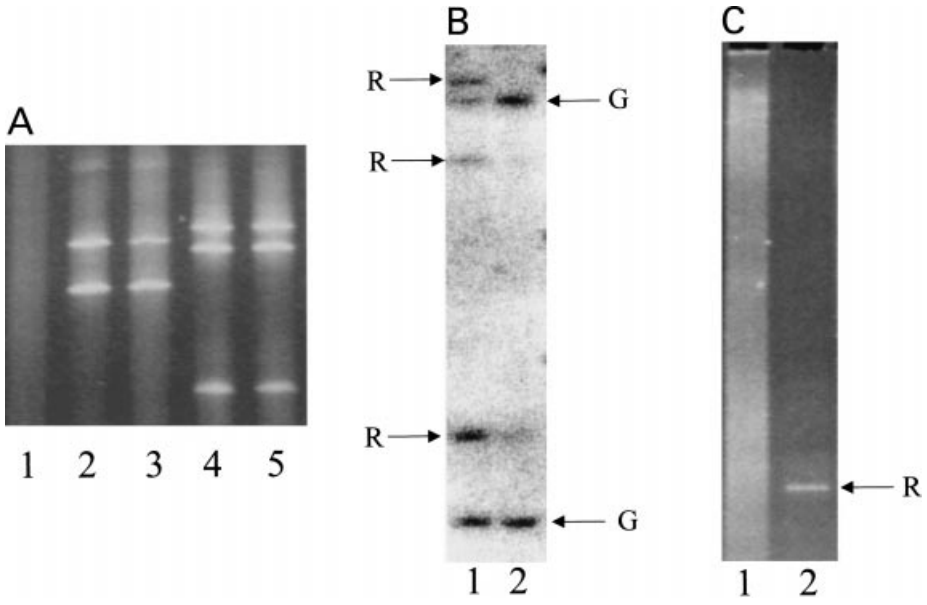

Fig 2. T-cell clonality in peripheral blood of a subgroup of patients with persistent idiopathic hypereosinophilia. (A) PCR analysis of the TCR γ-chain gene rearrangement pattern performed on whole blood from a healthy control subject (lane 1), patient 4 (lane 2) and patient 3 (lane 4) and performed on purified $CD3^-CD4^+$ T cells from patient 4 (lane 3) and patient 3 (lane 5). (B) Southern blot analysis of the TCR β-chain gene rearrangement pattern in peripheral blood from patient 6 (lane 1) and a healthy control subject (lane 2). (C) PCR analysis of the TCR γ-chain gene rearrangement pattern in purified $CD3^-CD4^+$ cells (lane 2) from patient 9 compared with a negative control (lane 1). G, germinal bands; R, rearranged bands.

Table IV. Cytokine profile of clonal $CD3^-CD4^+$ cells.*

Patient	IL-5 (ng/ml)	IL-4 (ng/ml)	IL-2 (ng/ml)	IFN-γ (U/ml)
1	46·75	54·54	750	< 20
3	14·47	6·38	799	160
4	5·47	2·91	172	< 20
9	24·65	nd	172·3	nd
Control†	0·5 ± 0·07	0·67 ± 0·17	2177 ± 391	1066 ± 289

* Purified $CD3^-CD4^+$ cells from patients 1, 3, 4 and 9 or $CD3^+CD4^+$ cells from three healthy subjects (control) were incubated at 5×10^5/ml with PMA (1 ng/ml) and A23187 calcium ionophore (0·1 μg/ml) for 48 h and culture supernatants were harvested for determination of cytokine concentrations by sandwich ELISA.

† Results are expressed as means of three subjects ± s.e.m.

nd, not done.

produced by 11·4% of $CD3^-CD4^+$ cells from patient 9 (compared with 19·5 ± 2·3% as mean ± s.e.m. of IFN-γ-positive cells among total $CD3^+CD4^+$ cells in 11 healthy subjects). The cytokine profile of $CD3^+CD4^+$ lymphocytes from all patients, including those with a clonal $CD3^-CD4^+$ subset, was similar to that of normal $CD4^+$ T cells from healthy subjects (not shown).

DISCUSSION

We have identified four patients presenting persistent idiopathic hypereosinophilia with an underlying T-cell disorder characterized by monoclonal expansion of an aberrant $CD4^+CD3^-$ T-cell population producing high levels of IL-5, IL-4 and IL-13 indicative of a Th2 profile. We compared the clinical and biological features of these patients with those of five other patients fulfilling the diagnostic criteria of HES in whom no aberrant T-cell subset was detected. We observed a homogeneous profile among those with clonal $CD3^-CD4^+$ Th2 cells consisting in predominance of cutaneous manifestations (including pruritus, eczema and urticaria) with lack of severe end-organ involvement, elevated serum IgE levels and polyclonal hypergammaglobulinaemia involving either IgM or IgG. Although vitamin B12 is classically considered as a marker of the myeloproliferative variant of HES, we observed elevated serum levels in one patient with T-cell clonality. Moreover, serum levels of IL-5 and soluble CD25, two potential markers of T-cell activation, were of no help in the discrimination of patients with an underlying clonal T-cell disorder in our series. In this regard, it is now established that eosinophils also produce IL-5 (Wardlaw, 1994; Lamkhioued *et al*, 1996) and are a potential source of soluble CD25 (Aldebert *et al*, 1994).

The $CD3^-CD4^+$ cells were likely to be involved in the development of hypereosinophilia, through the production of IL-5, and increased serum IgE levels, through the production of IL-4 and IL-13. However, their role in the nature of the clinical manifestations remains unknown. It has been shown that the $CD4^+CD45RO^+CD7^-$ subset of T cells preferentially produces Th2-type cytokines (Autran *et al*, 1995) and it has been suggested that it may represent a population of specialized skin-related memory T cells (Legac *et al*, 1992; Baars *et al*, 1995; Reinhold *et al*, 1996). Thus, the $CD3^-CD4^+(CD45RO^+CD7^-)$ cells may be responsible for the predominant development of cutaneous manifestations in our patients.

Since our first observation (Cogan *et al*, 1994), several groups have identified a circulating clone of IL-5-producing T cells in patients with persistent idiopathic hypereosinophilia (Brugnoni *et al*, 1996; Kitano *et al*, 1996; Simon *et al*, 1996, 1999). Recently, in a series of 60 patients with chronic hypereosinophilic disease, Simon *et al* (1999) reported the occurrence of an underlying T-cell disorder characterized by an aberrant surface phenotype in 16 cases. Our own series confirms that three-colour flow cytometric analysis of surface markers on peripheral blood lymphocytes is a critical step in the identification of hypereosinophilic patients with clonal Th2 cells and indicates that particular attention should be paid to the presence of $CD3^-CD4^+$ T cells. However, Southern blot and PCR analysis of TCR gene rearrangement patterns should also be performed systematically on peripheral blood and bone marrow in order to detect T-cell clonality in patients with a normal flow cytometric profile, as in the case of patient 6. In addition, for patients who do present a lymphocyte population with an aberrant phenotype, these investigations should also be performed after purification of the abnormal cells, especially when they represent only a small proportion of total lymphocytes. Indeed, although a subset of $CD3^-CD4^+$ cells was detected by flow cytometry in peripheral blood from patient 9, a clonal rearrangement pattern of the TCR could only be demonstrated after purification of these cells.

The pathogenesis of T-cell clonality in the setting of chronic hypereosinophilic disease remains unknown. Simon *et al* (1999) showed that the aberrant T-cell population lacked CD95/Fas-R expression in 8/16 patients of their series and demonstrated that deficient Fas-mediated apoptosis was involved in expansion of a $CD3^+CD4^-CD8^-$ IL-5-producing lymphocyte subset in one such patient (Simon *et al*, 1996). However, $CD3^-CD4^+$ cells from our patients expressed Fas-R and underwent apoptosis upon engagement of this receptor (unpublished observations), suggesting that primary events leading to clonal T-cell expansion can differ

among hypereosinophilic patients with an underlying clonal T-cell disorder. The homogeneous profile of the aberrant T cells in our patients suggests a common pathogenic agent. The absence of surface TCR/CD3 expression could be consistent with viral infection of the T cells, as lymphotropic viruses such as human immunodeficiency virus (HIV)-1 (Willard-Gallo *et al*, 1990), human T-cell lymphocytotrophic virus (HTLV)-I (de Waal Malefyt *et al*, 1990) and human herpesvirus (HHV)-6 (Furukawa *et al*, 1994) have been shown to down-regulate transcription of specific CD3 chains. Although HTLV-I and HTLV-II proviral sequences were not detected in clonal $CD3^-CD4^+$ cells from patients 1, 3 and 4 and antibodies to TAX were not detected in their serum, involvement of other lymphotropic viruses is currently under investigation.

Detection of an IL-5-producing clonal T-cell population in patients with persistent idiopathic hypereosinophilia has prognostic implications as several investigators have reported protracted development of T-cell lymphoma in this setting. In some cases, a $CD3^-CD4^+$ lymphocyte population was detected before progression towards lymphoid malignancy (O'Shea *et al*, 1987; Bagot *et al*, 1990; Moraillon *et al*, 1991; Simon *et al*, 1999). Importantly, Simon *et al* (1999) observed that 3/16 hypereosinophilic patients with an aberrant T-cell population developed T-cell lymphoma that conserved the abnormal surface phenotype initially observed, suggesting that the aberrant cells could be the precursors of malignant T cells. In another study, lymphomatous cells from a patient presenting cutaneous T-cell lymphoma associated with hypereosinophilia produced Th2-type cytokines and bore a $CD3^-CD4^+$ phenotype (Brugnoni *et al*, 1997). The malignant potential of the $CD3^-CD4^+$ cell population in our patients is suggested by the development of anaplastic null cell lymphoma in patient 1 4 years after presentation and by the appearance of chromosomal abnormalities consisting in partial deletions on chromosomes 6 and 10 in the $CD3^-CD4^+$ cells from patients 3 and 4 after 4 and 2 years' disease progression respectively. Repeated cytogenetic analysis of blood and marrow specimens may help in the identification of patients with clonal IL-5-producing T cells at risk for the development of a malignant disorder. Interestingly, similar cytogenetic changes have been reported in lymphomatous cells from patients with Sezary syndrome (Limon *et al*, 1995), which shares a number of features with our patients' T-cell disorder (Pancake *et al*, 1995, 1996). Both diseases are characterized by the presence in peripheral blood of clonal T cells (Weinberg *et al*, 1995) that display a mature $CD4^+CD45RO^+CD7^-$ phenotype (Wood *et al*, 1990; Bogen *et al*, 1996) and a Th2-type cytokine profile (Vowels *et al*, 1992). However, weak or absent CD3 expression on Sezary cells is uncommon (Bogen *et al*, 1996; Sano *et al*, 1998).

Finally, the T-cell-mediated nature of this hypereosinophilic disease should be taken into consideration for patient management. Corticosteroids remain useful for patients with an underlying T-cell disorder through their dual suppressive action on eosinophils and T-cell cytokine production (Wallen *et al*, 1991; Schleimer & Bochner, 1994; Umland *et al*, 1997). However, although patients 1 and 3 did respond to corticosteroid treatment in terms of clinical manifestations and eosinophil levels, the aberrant T-cell population remained unaffected. In contrast, the clonal population in patient 4 rapidly declined from 84% to 7% of total $CD4^+$ T cells after initiation of corticosteroid treatment. Although IFN-α has been used with success in the management of patients with idiopathic hypereosinophilic syndrome (Bockenstedt *et al*, 1994 Butterfield & Gleich, 1994), monotherapy with type I IFNs for patients with clonal IL-5-producing T cells could be detrimental despite their suppressive effects both on eosinophils (Aldebert *et al*, 1996; Morita *et al*, 1996) and on IL-5 production by lymphocytes (Schandené *et al*, 1996). Indeed, recent studies in our laboratory have demonstrated a potent antiapoptotic effect of IFN-α on clonal $CD3^-CD4^+$ cells from our patients *in vitro* (unpublished observations). Future proposals for the management of hypereosinophilic patients such as ours should be based on knowledge concerning the activation pathways operating in these cells and should be designed to target both T-cell expansion and Th2 cytokine production.

NOTE ADDED IN PROOF

Since submission of the manuscript, patient 3 has developed a lymphomatous mass in the cervical region consisting of $CD3^-$ $CD4^+$ cells.

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Part 2: Genetic Characterization of L-HES

The aim of the second part of this study was to identify the common chromosomal and molecular characteristics present in the CD3-CD4+ T-cell clones from the L-HES patients at diagnosis. Furthermore, in order to analyze their pathogenic role in L-HES, a correlation has been drawn between the specific cytogenetic and molecular variations observed and the patient's clinical evolution.

2.1. Cytogenetic Characterization of L-HES.

Article 2

6q- is an early and persistent chromosomal aberration in CD3-CD4+ T-cell clones associated with the lymphocytic variant of hypereosinophilic syndrome. M. Ravoet and C. Sibille, F. Roufosse, H. Duvillier, C. Sotiriou, L. Schandené, P. Martiat, M. Goldman, K. E. Willard-Gallo.

Haematologica, 2005, 90, 6, pp: 753-765

This article present the first cytogenetic aberrations detected by karyotyping the oligoclonal cell lines derived from the CD3-CD4+ cells of two L-HES patients (P1, P2). Excluding a cultural artefact, the author retrieved, by testing fresh blood samples from P1 and P2 with conventional karyotype or/and by FISH the same chromosomal abnormalities seen three years before in the cell lines. These chromosomal aberrations were polyclonal for P1 at diagnosis, including three distinct subclones, namely characterized by a small 6q deletion, a large 6q deletion associated with a 10p deletion, and an isolated 10p deletion. However, the only abnormality observed in CD3-CD4+ T-cells from P2 was 6q interstitial deletion involving the same cytobands deleted in the small 6q- subclone of P1. These results were consistent with the importance of a non-random and early 6q loss in the CD3-CD4+ T-cells of some L-HES patients. The timeline progression of these cytogenetic aberrations during the clinical evolution of the two patients was further analyzed. By demonstrating the persistence of the 6q- small deletion for both patients during the chronic phase of L-HES, and the emergence of the 6q- subclone as predominant during the disease progression of P1 in T-lymphoma, the results suggest the critical role of 6q genes in the transforming process. A microarray analysis of middle density comparing gene expression from the CD3-CD4+ T-cells from the two patients with CD3+CD4+ corresponding T-cells also revealed a common repression pattern of specific genes located in the 6q interstitial deletion leading to their preliminary identification as possible tumour suppressor genes.

6q- is an early and persistent chromosomal aberration in CD3⁻CD4⁺ T-cell clones associated with the lymphocytic variant of hypereosinophilic syndrome

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Background and Objectives. The lymphocytic variant of hypereosinophilic syndrome (LV-HES) is an underrated disease defined by the monoclonal proliferation of interleukin-5 secreting T-cells. This disease is distinguished by a period of chronic lymphoproliferation without clinical transformation, which is frequently a precursor to T-cell lymphoma. In this study, LV-HES was used as a model of pre-malignancy to identify specific marker(s) predictive of the potential for malignant transformation.

Design and Methods. The karyotypic abnormalities detected in the abnormal CD3⁻CD4⁺ T cells were further characterized by fluorescent *in situ* hybridization. A multi-step retrospective analysis was performed on successive blood samples during a six-year follow up to correlate the evolution of cytogenetic changes with clinical progression. Expression array analysis was used to investigate the effect of these chromosomal aberrations on gene expression.

Results. A 6q deletion was detected in the two LV-HES patients during their chronic disease phase. An additional 10p deletion was found alone or in association with the 6q defect in one patient prior to the development of a CD3⁻CD4⁺ T-cell lymphoma six years after diagnosis. We show that the 6q but not the 10p deletion is both stable and persistent throughout the chronic disease, finally emerging as the predominant aberration in the lymphoma cells. Six genes mapped to the 6q-deleted region displayed altered gene expression profiles both in the chronic and malignant disease phases.

Interpretations and Conclusions. Our data suggest that the 6q deletion represents an early cytogenetic marker for T-cell transformation.

Key words: hypereosinophilic syndrome, 6q, pre-malignancy, CD3⁻CD4⁺ T-cell lymphoma, expression profile.

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Cytogenetic analysis recurrently detects a 6q deletion in lymphoid malignancies, including both T- and B-cell acute lymphoblastic leukemia (ALL)[1-3] and non-Hodgkin's lymphoma (NHL).[3-5] Interestingly, this abnormality has rarely been reported in myeloid leukemia.[6] A variety of solid tumors such as melanoma,[7] breast carcinoma[8] and prostate cancer[9] have also been shown to exhibit the same defect. The recurrence of a 6q deletion in cancer strongly suggests that this region contains an unidentified tumor-suppressor gene(s) and therefore merits further investigation for its role in the malignant process. This hypothesis is supported by studies showing that tumorigenicity can be suppressed by introducing all or part of a normal chromosome 6 into immortalized fibroblasts, ovarian or breast cancer cell lines.[10,11]

The idiopathic hypereosinophilic syndrome (HES) is a heterogeneous group of diseases defined by a persistent blood hypereosinophilia of unknown etiology leading to tissue damage.[12] Two distinct types of HES have emerged from this pathologic group: the myeloid variant related to chronic eosinophilic leukemia[13] and the lymphocytic variant of hypereosinophilic syndrome (LV-HES). The latter is characterized by a monoclonal expansion of peripheral helper T cells overexpressing Th2-type cytokines, including interleukin (IL)-4 and IL-5, the likely cause of the hypereosinophilia *in vivo*.[14-24] The monoclonal T cells are often distinguished by an aberrant surface immunophenotype, which is most frequently either CD3⁻CD4⁺CD8⁻[14,15,19,21] or CD3⁺CD4⁻CD8⁻.[16,17] Disease progression is usually characterized by a long chronic phase associated with cutaneous manifestations and a favorable prognosis. However, some LV-HES patients subsequently develop a T-cell lymphoma originating from the same abnormal T-cell clone. This suggests that these cells possess a potent pre-malig-

nant nature early in the disease process.[18,20,23] A prognostic marker for the malignant potential of these abnormal T cells has not yet been identified, but cytogenetic analysis is a powerful tool routinely used for the diagnosis and prediction of prognosis in malignant hematologic disorders. We therefore initiated a retrospective analysis of the progressive chromosomal aberrations present in the $CD3^-CD4^+$ T-cell clones from two LV-HES patients (initial case reports previously described).[21] A 6q deletion was detected early and persisted throughout the chronic disease phase in both individuals, emerging as the predominant genetic anomaly in one patient in concert with progression of her disease to T-cell lymphoma. Gene expression array analysis was employed to investigate the impact of this deletion on the expression of 6q located genes, and six of the 88 genes examined were found to be consistently downmodulated in the $CD3^-CD4^+$ T cells from both patients.

Design and Methods

Patients

The initial clinical presentation of patients 1 and 2 has been previously described[21] where they are also indicated as P1 and P2, respectively. Briefly, at initial diagnosis patient #1 (a 20-year old female) exhibited severe cutaneous manifestations (eczema, pruritis, tenosynovitis of the right ankle). The circulating leukocyte count was 16.9×10^9/L with 8.92×10^9/L eosinophils and 4.63×10^9/L lymphocytes, including 3.45×10^9/L $CD3^-CD4^+$ T cells. A four-year follow-up was marked by episodes of severe eczema treated with corticosteroids and/or interferon (IFN)-α. Four years after diagnosis, her circulating leukocyte count had reached 26.2×10^9/L, with 17.08×10^9/L eosinophils and 6.32×10^9/L lymphocytes, including 4.80×10^9/L $CD3^-CD4^+$ T cells, and the clinical manifestations had worsened. Six cycles of fludarabine were administered, after which she achieved clinical remission for a few months characterized by a steadily declining number of aberrant T cells in the peripheral blood. The circulating leukocyte count was 1.2×10^9/L, including 0.2×10^9/L eosinophils and 0.38×10^9/L lymphocytes (0.1×10^9/L $CD3^-CD4^+$ T-cells). However, six years after the initial diagnosis, the patient relapsed. Enlarged lymph nodes were detected in the pre-auricular, cervical, and inguinal regions, and a histological diagnosis of peripheral diffuse T lymphoma of small to medium lymphocytes, type 4 (Class II, REAL/peripheral T lymphoma, WHO) was established. The leukocyte count was 15.1×10^9/L, with 8.61×10^9/L eosinophils and 4.0×10^9/L lymphocytes, including 2.93×10^9/L $CD3^-CD4^+$ T cells. After a six-month course of CHOP-like chemotherapy, and, although lymph node size decreased, $CD3^-CD4^+$ T cells remained detectable and hypereosinophilia persisted. Three months later the patient was successfully treated by allogeneic stem cell transplantation from a family donor and is currently in complete remission.

At initial diagnosis patient 2 (a 21-year old female) exhibited cutaneous symptoms. The total leukocyte count was 14.8×10^9/L, including 9.1×10^9/L eosinophils and 3.42×10^9/L lymphocytes (2.47×10^9/L $CD3^-CD4^+$T cells). A four-year follow-up on continuous low-dose corticosteroid therapy has been characterized by remission of symptoms, decreased eosinophilia (0.6×10^9/L) and a significant decrease in the $CD3^-CD4^+$ T cell population to 0.09×10^9/L (leukocyte count was 7.2×10^9/L, including 0.66×10^9/L eosinophils and 1.42×10^9/L lymphocytes).

Cell purification

Circulating leukocytes were obtained from patients and from healthy female donors (25-30 years old) either by venipuncture in 60-mL heparinized syringes or by cytapheresis. Peripheral blood mononuclear cells (PBMC) were isolated and either used for immediate processing or frozen as described.[21] If necessary, PBMC were thawed and cultured overnight in supplemented RPMI 1640 without stimulation. The next morning, the $CD2^+CD3^-CD4^+$ and $CD2^+CD3^+CD4^+$ T-cell populations were purified by flow cytometry on a Beckman-Coulter Elite. PBMC were resuspended in RPMI 1640 without phenol red supplemented with 5% decomplemented fetal bovine serum and labeled with fluorescein-isothiocyanate (FITC)-coupled anti-CD2 monoclonal antibody (Moab) (Clone Leu-5b, BD Biosciences), phycoerythrin (PE)-coupled anti-CD4 Moab (Clone 13B8.2, Beckman Coulter) and phycoerythrin-cyanin5 (PC5)-coupled anti-CD3 Moab (Clone UCHT1, Beckman Coulter). The purity of sorted T-cell populations was analyzed on a FACS Calibur (BD). Moreover, the abnormal $CD3^-CD4^+$ and normal $CD3^+CD4^+$ T-cells were also purified by magnetic beads. The $CD4^+$ T cells were first isolated from PBMC by column depletion using the MACS $CD4^+$ T-cell isolation kit according to manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The isolated $CD4^+$ T cells were then labeled with anti-CD3 Moab coupled magnetic microbeads (Miltenyi Biotec) and the $CD3^-CD4^+$ and $CD3^+CD4^+$ T-cell populations were differentially separated on a magnetic column (Miltenyi Biotec). The purity of these T-cell populations was checked, as described above.

Metaphase cells, preparation of nuclei and karyotyping

Metaphase cells were prepared from fresh peripheral blood samples or T-cell cultures for standard karyotyping[25] by stimulating them for 72 hours with 20

μg/mL phytohemagglutinin (PHA; Life Technologies, Merelbeke, Belgium) and/or 100U/mL recombinant human IL-2 (Cetus Corp., Emeryville, CA, USA). The metaphase cells used in the FISH experiments were obtained from thawed PBMC that were subsequently stimulated for 5 days with a combination of recombinant human IL-2 (100U/mL), anti-CD28 antibody (1 μg/mL; Clone 28.2, Immunotech) and phorbol-12-myristate-13-acetate (PMA; 1ng/mL; Calbiochem, Leuven, Belgium) and were stopped in metaphase by adding colcemid (25 ng/mL; Life Technologies). The nuclei for interphase FISH were obtained either from unstimulated thawed PBMC or from sorted T-cell populations, as described above.

PAC (P1 derived artificial chromosome) and BAC (bacterial artificial chromosome)

All PAC and BAC clones were selected in the Sequence Maps from the National Center for Biotechnology Information (NCBI) and purchased from the Children's Hospital Oakland (available at URL *http://www.chori.org/bacpac*). The chromosome 6-located PAC clones included: RP1-22I17 (at 6q12), RP1-91B17 (at 6q12), RP1-104A17 (at 6q13), RP3-424L16 (at 6q13), RP1-234P15 (at 6q14.1), RP3-429G5 (at 6q21), RP1-238J17 (at 6q22.1), RP1-136O14 (at 6q22.1), RP3-412I7 (at 6q22.1), RP1-193N13 (at 6q22.31) and RP1-293L8 (at 6q22.32). An additional RP3-324N14 PAC clone (at 6q23.1) was kindly provided by Frédéric Chibon (Institut Curie, Paris, France). The chromosome 10-located BAC clones included: RP11-462L8 (at 10p11.22), RP11-271M1 (at 10p13), RP11-2K17 (at 10p13), RP11-398C13 (at 10p13) and RP11-20J15 (at 10q11.21). The clones were grown and the DNA isolated using standard molecular biology techniques. The identities of PAC and BAC clones were verified using FISH to examine the correct chromosomal location or by DNA sequencing and matching to the sequences in the NCBI database.

Fluorescence in situ hybridization

FISH experiments were performed using previously described protocols.[26] Briefly, PAC/BAC and α-satellite centromeric plasmid D6Z1 and D10Z1, specific for centromeres of chromosomes 6 and 10 (kindly provided by Pr A. Hagemeijer, KUL) were labeled with either tetramethylrhodamine-5-dUTP or fluorescein-12-dUTP (Roche Applied Science, Mannheim, Germany) by nick-translation. The labeled probes were denatured in hybridization buffer containing ultrapure formamide (Life Technologies) and PAC/BAC probes were pre-hybridized for 30 minutes. Hybridization was carried out on RNase-treated nuclei or mitotic slides using 50 ng/μL of PAC/BAC and, if necessary, 4 ng/μL of centromeric probes and hybridization was processed in a moist chamber overnight at 37°C. After a post-hybridization wash carried out at 30°C for 2 minutes in a 0.4× SSC bath, the slides were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in an anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA). Hybridized slides were examined using either a DMRB microscope (Leica) or an axioplan 2 microscope (Zeiss) and images were captured using a Photometrics camera and processed by SmartCapture software (Digital Scientific, UK).

In addition, successive dual color FISH hybridization/slide washing cycles of mitotic slides were carried out as described in[26] with an additional wash in Carnoy's solution for 2 minutes before the hybridization.

In interphase FISH experiments, only dual-color FISH was performed using a FITC-labeled specific PAC/BAC probe with a rhodamine-labeled centromeric probe (either D6Z1 or D10Z1, according to the 6q- or 10p-specific probe, respectively) as an internal control for disomy. The interphase FISH results were obtained by counting >500 intact nuclei per patient slide. The cut-off level for each probe was determined by scoring 200 intact nuclei prepared from PBMC and $CD3^+CD4^+$ T cells purified from five healthy donors used to set the baseline based on the mean plus three standard deviations. The thresholds of detection on unsorted nuclei were 4% for RP1-234P15, RP1-238J17 and RP3-424L16, 5% for RP1-91B17, RP1-136O14, RP3-412I7, RP3-429G5 and RP11-462L8 and 7% for RP1-193N13. The thresholds of detection on sorted cells were 6% for RP11-462L8, 7% for RP1-193N13, 8% for RP1-91B17 and 9% for RP3-429G5.

RNA purification

Total RNA was isolated from sorted T cells using TriPure Isolation Reagent (Roche Applied Science) in a single-step extraction method.[27] The quality of the RNA was assessed using the Agilent Capiler system. If required, samples of equal amounts of total RNA were pooled.

Oligonucleotide microarray

Total RNA (2-3 μg) was labeled using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY, USA) following the manufacturer's standard procedures (Affymetrix, Santa Clara, CA, USA). The labeled cRNA were hybridized on test3arrays (Affymetrix) to ensure the quality of the probes. The probes were recovered and hybridized on U133A Genechips, containing 22,263 probe sets. The hybridization, washing, staining and scanning of the array slides were performed according to standard protocols (Affymetrix). Gene expres-

sion values from the CEL files were normalized using RMA.[28] Biological replicate experiments were performed for each purified T-cell population. The data were interpreted by sorting for robust changes in pair-wise comparisons as described in the GeneChip expression analysis manuals (Affymetrix).

Reverse transcription and quantitative real-time PCR (RQ-PCR)

Standard reverse transcription was performed using 200 ng-1 μg of total RNA, random hexanucleotides (50 μM final concentration, Amersham Pharmacia, Freiburg, Germany), MMLV reverse transcriptase (100 U, Promega, Leiden, The Netherlands) and RNase inhibitor (20 U, Promega). After denaturing the RNA, the RT-PCR reaction was performed for 45 minutes at 42°C followed by an enzyme-inactivation step. Next, 25 ng of cDNA were subjected to a real-time PCR reaction using 2× SYBR Green PCR Master Mix (Applied Biosystems, Lennik, Belgium) and 0.32 μM each of the gene-specific forward and reverse primers (Life Technologies). The following primers were designed using the Primer Expression 1.0 program (Applied Biosystems):

FOXO3A/ex3-F: 5'-CATGGGCCATGAGAAGTTCC-3'
FOXO3A/ex3-R: 5'-CATGTCACATTCCAAGCTCCC-3'
FOXO3A/ex4-F: 5'-TGCATAGGCAAAAGGAGTGGA-3'
FOXO3A/ex4-R: 5'-GATCACCCTGACTCAGAACCG-3'
C6ORF37-F: 5'-ACTGCAATGTGCTGAACTGGG-3'
C6ORF37-R: 5'-TGAATCGGAATGGTCTCGCT-3'
PA26-F: 5'-CCTCGACCACTAGGACAGGG-3'
PA26-R: 5'-GTGCGTCTTCACTCCCCACT-3'
MARCKS-F: 5'-CCACAGATCCCATCTCAAATCAT-3'
MARCKS-R: 5'-AGAGAAACAAGGCAGAGGAAGAAG-3'
CD164-F: 5'-TTGACTGAGCGTTGCGAGC-3'
CD164-R: 5'-AGAGCCGCGACATCGTGT-3'
HMGN3-F: 5'-CCAAAGTAACTAAACAGGAGCCCA-3'
HMGN3-R: 5'-GTTCAGGTTTTGGTGGAGCAG-3'
ORC3L-F: 5'-TCAGTGCTGCCCATGCC-3' x
ORC3L-R: 5'-AAGGATTGTTGAGTGCAGTATGGA-3'
SUSP1-F: 5'-TTTCTTCAAGCAATTCCAGCAGT-3'
SUSP1-R: 5'-CCTTTACAATCATTCCAAACTTTATCC-3'
PROL2-F: 5'-GCCTGAGAAGTGGAGATTGCA-3'
PROL2-R: 5'-TGAGACAGCGTCTGTTACCCAG-3'
BACH2-F: 5'-CAGCAATACTGTTCCGAAGTATCCT-3'
BACH2-R: 5'-GTGTCATCACTGCTGTCTTTCCTT-3'
C6ORF162-F: 5'-AAACAAAGCCTAGCCCTACGGT-3'
C6ORF162-R: 5'-CGGGCCAGGTGCCAG-3'

The *ABL* gene was used as an endogenous control.[29] Standard real-time PCR was performed on an ABI Prism 7900 HT (Applied Biosystems). Dissociation curves were verified and the PCR products were visualized on 1.5% agarose gel to ensure the specificity of the PCR reaction. The amplified fragments were then isolated using the QIAquick Gel Extraction kit (Qiagen) and a series of dilutions were prepared to generate the standard curves for reproducibility and efficiency of the reactions. All real-time PCR reactions were processed in duplicate and differences of more than 1 Ct were rejected. The comparative Ct method was applied for data analysis. For all tested genes, biological duplicates were performed, with a third biological replicate analyzed for the *C6ORF37*, *HMGN3*, *MARCKS*, *PA26*, *RAGD* and *SUSP1* genes.

Results

T-cell receptor rearrangements

Samples from both patients were examined annually for rearrangement of their T-cell receptor (TCR) γ chain gene (*TCRG*) by PCR. A clonal *TCRG* rearrangement was routinely detected in both whole blood and isolated $CD3^-CD4^+$ T cells from patients 1 and 2 (P1 and P2). The aberrant mature T-cell phenotype and cytokine profile associated with the $CD3^-CD4^+$ T-cell clone in both patients[21] (*Willard-Gallo et al., submitted to Exp Hematol*) led us to analyze the *TCRB* locus. Using IL-2-dependent $CD3^-CD4^+$ T-cell lines derived from P1 at diagnosis (*Willard-Gallo et al., submitted to Exp Hematol*), we established that these cells had a clonally rearranged *TCRB* gene using Southern blotting (Figure 1). On the basis of our results with the cell lines, we examined sequential whole blood samples from both patients and found that *TCRB* gene rearrangements became detectable three years after diagnosis for P1 (Figure 1) and two years after diagnosis for P2 (data not shown). The clonal *TCRB* rearrangement detected in the blood of P1 was identical to that found in the P1-derived $CD3^-CD4^+$ T-cell lines, indicating that the cells grown *in vitro* accurately reflected the abnormal $CD3^-CD4^+$ T cells present *in vivo*. The presence of a completely rearranged *TCRB* gene in conjunction with the phenotype and cytokine profile of the $CD3^-CD4^+$ T cells indicates the abnormal clone was derived from a mature T cell.

Karyotype of the $CD3^-CD4^+$ T--cells from patient #1

The abnormal surface phenotype and cytokine profiles characteristic of the $CD3^-CD4^+$ T cells were not reflected by an aberrant cellular morphology in either P1 or P2. We therefore examined these cells for potential cytogenetic abnormalities. At initial diagnosis, fresh blood cells from patient #1 appeared to be normal by conventional karyotyping (G-banding, 72-hour PHA stimulation). Once again we stimulated the cell line from patient #1 using IL-2, and although these cultured lines (100% $CD3^-CD4^+$) contained some cells with a normal karyotype, the large majority had abnormalities easily detected by conventional karyotyping. The most frequent aberration identified was a 6q deletion, present either as a unique abnormality (the $6q^-$ subclone) or in association with a 10p deletion (the $6q^-10p^-$ subclone), with an addi-

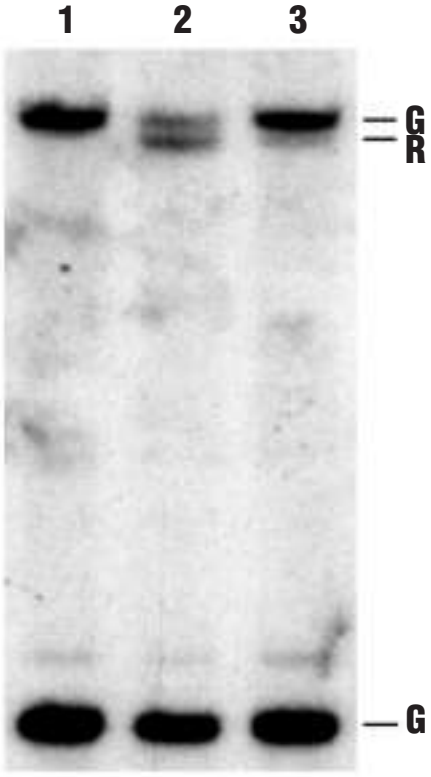

Figure 1. *TCRB* gene clonality. Southern blot analysis of the *TCRB* gene: *lane 1*, a peripheral blood sample from a healthy control; *lane 2*, long-term cultures of CD3$^-$CD4$^+$ T cells from P1 and *lane 3*, a peripheral blood sample from P1. The germline (G) configuration only is detected in the control, whereas in P1's samples an additional rearranged (R) band is present. DNA was digested with *EcoR*I and hybridized with a Jβ2 specific probe as described.[22]

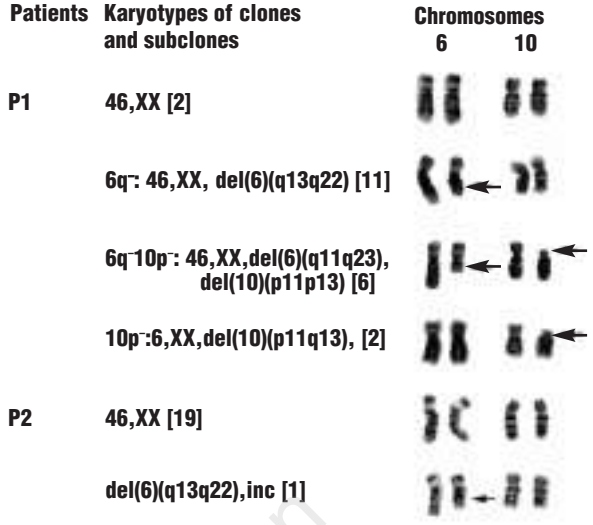

Figure 2. The G-banded karyotypes from P1 and P2 in long-term cultures. The karyotypes obtained from long-term cultures (100% pure CD3$^-$CD4$^+$ T cells) of blood samples drawn at initial diagnosis from P1 and P2. Chromosomes 6 and 10, representative of each clone and subclone, are also shown.

tional subclone possessing the 10p abnormality alone (the 10p$^-$ subclone) (Figure 2). The same cytogenetically abnormal subclones were eventually found to be present in fresh blood from patient #1, but again not until three years after initial diagnosis despite routine annual karyotyping. This suggests that the 6q and 10p abnormalities were not an artifact of *in vitro* cell culture, but instead were undetectable by the standard assays used in routine clinical analysis. Using PHA and IL-2 to stimulate these cells enabled us to consistently detect the presence of the abnormalities in the patient's blood except on a single occasion that was concurrent with fludarabine-induced remission. The *TCRB* and karyotypic analyses demonstrate the usefulness of *in vitro* cell culture as a means of expanding and examining abnormal cell clones that either have a low proliferative capacity or are present at low frequency in the early stages of a given disease.

Karyotype of the CD3$^-$CD4$^+$ T cells from patient 2

The analysis of fresh blood cells from P2 at initial diagnosis once again revealed only a normal karyotype, but after *in vitro* growth using the same conditions as for P1 (*Willard-Gallo et al., submitted to Exp Hematol*), an interstitial 6q deletion was detected in one incomplete metaphase cell (Figure 2). No additional metaphase cells carrying a loss of 6q were detectable in the routine annual analysis of this patient's fresh blood; however, the presence of a clonal 6q deletion was confirmed by interphase FISH using a 6q21-located PAC probe (RP3-429G5) on the CD3$^-$CD4$^+$ T cells purified from the patient's frozen blood drawn at diagnosis (Figure 3). Enrichment of the abnormal T-cell population was essential for the success of these experiments due to the low percentage of CD3$^-$CD4$^+$ T cells and/or 6q$^-$ subclones in blood from P2. In contrast to P1, we did not detect a loss of 10p in the CD3$^-$CD4$^+$ T cells from P2 (*data not shown*). These experiments show that at initial diagnosis the CD3$^-$CD4$^+$ T cells from both patients contained a 6q$^-$ subclone and suggest that a 6q abnormality might be a recurrent abnormality in LV-HES.

Breakpoints of the 6q and 10p deletions detected in the CD3$^-$CD4$^+$ T cells

Using a panel of 6q and 10p-specific PAC and BAC probes (Table 1), the chromosomal breakpoints of the previously detected aberrations were mapped by FISH on metaphase cells from P1 and nuclei from P2. Representative metaphase cells from the 6q$^-$, 10p$^-$ and 6q$^-$10p$^-$ subclones in stimulated PBMC from P1

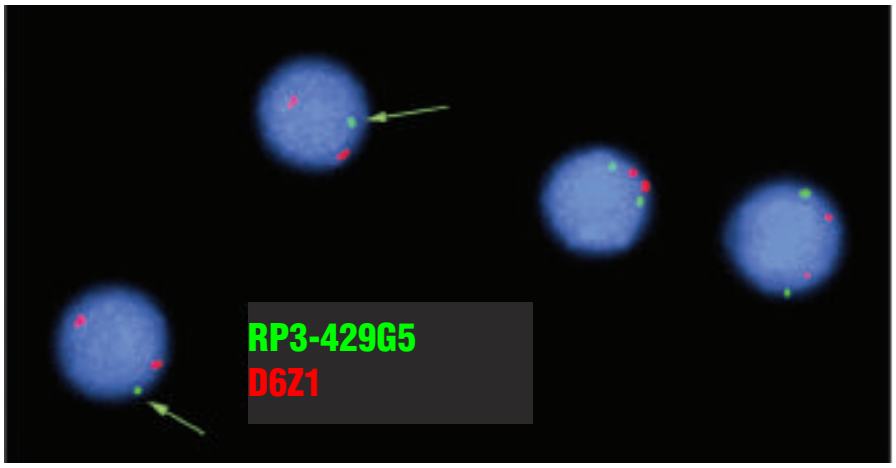

Figure 3. Clonality of the 6q deletion in $CD3^{-}CD4^{+}$ T cells from P2. Interphase dual-color FISH on uncultured $CD3^{-}CD4^{+}$ T cells from P2 purified by flow cytometry (98% pure) from blood at initial diagnosis. Co-hybridization of the FITC-labeled RP3-429G5 probe (at 6q21) and the rhodamine-labeled D6Z1 probe (located at the centromere of chromosome 6 and used as internal control), show one green and two red hybridization signals corresponding to 25% of 6q21-deleted nuclei (green arrows).

are shown in Figure 4. In the $6q^{-}$ subclone, the 6q deletion breakpoints were assigned between band 6q13 (RP1-104A17) and band 6q22.1 (RP1-136O14) (Figure 4A and Table 1A). In the $10p^{-}$ subclone, the interstitial deletion was delineated from band 10p11.1 ($D10Z1^{dim}$) to band 10p13 ($RP11\text{-}2K17^{dim}$) (Figure 4B and Table Ib). The location of the 10p13 breakpoint within the RP11-2K17 probe was confirmed using a set of two overlapping BAC probes (Table 1B). In the $6q^{-}10p^{-}$ subclone, the breakpoints of the 10p deletion are identical to the deletion observed in the $10p^{-}$ subclone (Figure 4C and Table 1B). On the other hand, the 6q deletion in the $6q^{-}10p^{-}$ subclone, delineated from band 6q11.1 (D6Z1) to band 6q23.1 (RP3-324N14), is larger than in the $6q^{-}$ subclone (Figure 4C and Table 1A). These results suggest that the $6q^{-}10p^{-}$ subclone was derived from the $10p^{-}$ subclone and not the $6q^{-}$ subclone, and demonstrate that not only was there heterogeneity in the 6q deletion but that these deletions arose independently. In P2, the 6q deletion was defined from band 6q13 (RP3-424L16) to band 6q22.1 (RP3-412I7) by interphase FISH analysis of the enriched $CD3^{-}CD4^{+}$ T cells (Table 1A). Thus, the three distinct 6q deletions (Table 1A) identify a commonly deleted region lying between 6q14.1 (RP1-234P15) and 6q22.1 (RP1-238J17). This shared deleted region contains approximately 164 genes and is 40Mbp long.

The relationship between cytogenetic changes and the clinical evolution of P1 and P2

The percentage of cells containing the 6q and/or 10p deletion was investigated by interphase FISH on successive blood samples taken at different stages of each patient's disease (Table 2). Blood samples from P1 were tested at diagnosis [P1-yr.0], during the chronic phase controlled by corticotherapy [P1-yr.4] and coincident with the detection of a $CD3^{-}CD4^{+}$ T cell lymphoma [P1-yr.6]. Blood samples from P2 were investigated at diagnosis [P2-yr.0] and after four years of stable clinical remission on corticotherapy (chronic phase) [P2-yr.4]. The $CD3^{-}CD4^{+}$ and

Table 1A. Breakpoint delineation of the 6q deletions in P1 and P2 and identification of the shared minimal deletion.

6q located PAC probes	*Chromosome location*	*P1 6q⁻ subclone*	*P1 6q⁻10p⁻ subclone*	*P2 6q⁻ subclone[a]*
D6Z1	Centr 6	●	●	●
RP1-22I17	6q12	●	○	ND
RP1-91B17	6q12	●	○	ND
RP1-104A17	6q13	●	○	ND
RP3-424L16	6q13	○	○	●
RP1-234P15	6q14.1	○	○	○
RP3-429G5	6q21	○	○	○
RP1-238J17	6q22.1	○	○	○
RP1-136O14	6q22.1	●	○	○
RP3-412I7	6q22.1	ND	ND	●
RP1-193N13	6q22.31	●	○	ND
RP1-293L8	6q22.32	●	○	ND
RP3-324N14	6q23.1	●	●	ND
		del(6) (q13q22.1)	del(6) (q11.1q23.1)	del(6) (q13q22.1)

● *no deletion;* ○ *deletion; ND: not done; boxed area: region of minimal deletion.* [a]*The 6q deletion from P2 was delineated by interphase FISH on nuclei from an enriched $CD3^{-}CD4^{+}$ T-cell population.*

Table 1B. Breakpoint delineation of the 10p deletion in P1.

10p located BAC probes	*Chromosome location*	*P1 $10p^{-}$ and $6q^{-}10p^{-}$ subclones*
RP11-398C13[a]	10p13	●
RP11-2K17[a]	10p13	●
RP11-271M1[a]	10p13	○
RP11-462L8	10p11.22	○
D10Z1	Cent 10	●
RP11-20J15	10q11.21	●
		del(10)(p11.1p13)

● *no deletion;* ○ *deletion;* ● *reduced probe signal.* [a]*These BAC clones are contiguous.*

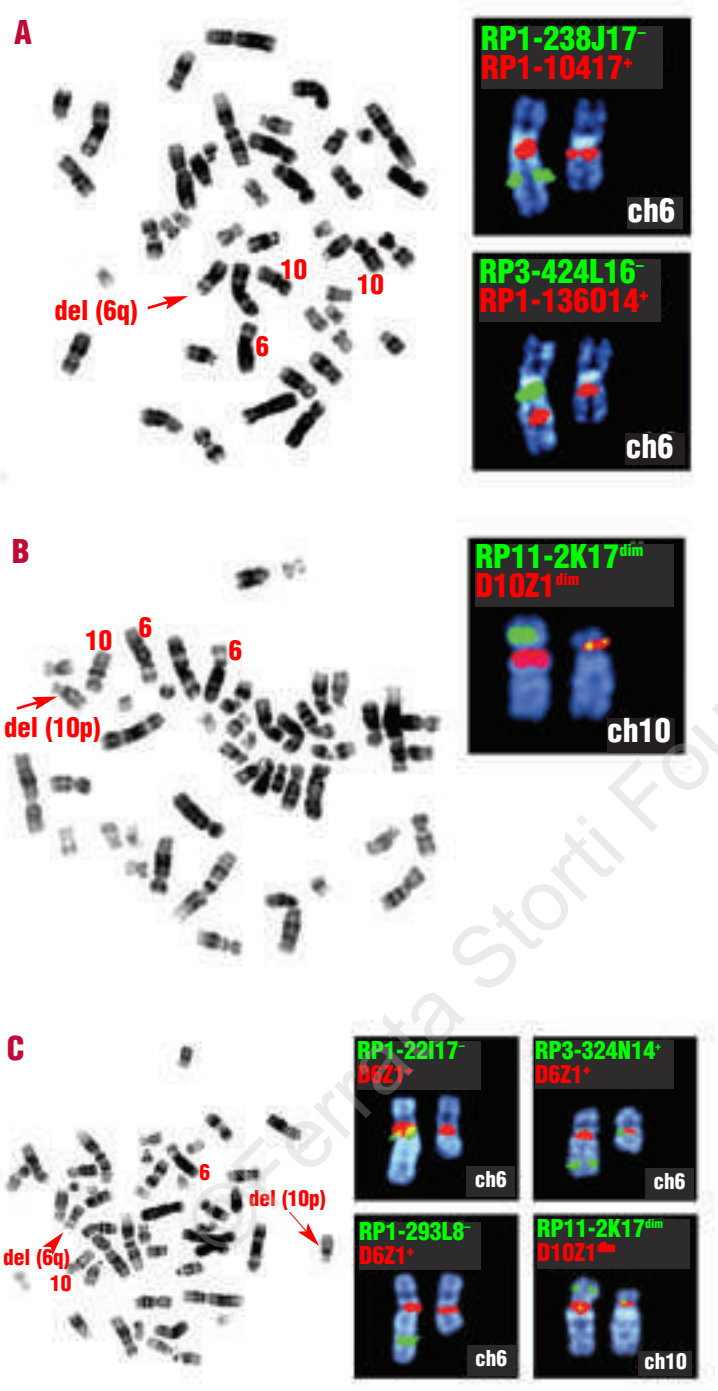

Figure 4. Characterization of the P1 subclones using metaphase FISH. The 6q and 10p deletions in the 6q⁻ subclone (A), 10p: subclone (B) and 6q⁻10p⁻ subclone (C) were delineated using metaphase FISH on PMBC stimulated with IL-2, PMA and anti-CD28. A representative pseudo-G-banded metaphase cell and dual-color FISH of DAPI-stained chromosomes 6 and 10 are shown for each subclone. Probes are indicated in each image (text color corresponding to the FISH detection color) along with the signal detected (+ means retained probe signal; – means lost probe signal; dim means diminished probe signal). The 6q13q22.1 deletion (small) is observed in the 6q⁻ subclone (A): the 6q13 breakpoint is detected by the presence of the RP1-104A17 signal and the absence of the RP3-424L16 signal; the 6q22.1 breakpoint is defined by the presence of the RP1-136O14 signal and the absence of the RP1-238J17 signal. The 10p11.1p13 deletion is identically detected in the 10p⁻ subclone (B) and in the 6q⁻10p⁻ subclone (C) by the co-localization of RP11-2K17 (10p13) and D10Z1 (10p11.1) signals. The 6q11.1q23.1 deletion (long) is observed in the 6q⁻10p⁻ subclone (C): the 6q11.1 breakpoint is defined by the presence of the D6Z1 signal (6q11.1) and the absence of the RP1-22I17 signal (6q12); the 6q23.1 breakpoint is defined by the presence of the RP3-424N14 signal (6q23.1) and the absence of the RP1-293L8 signal (6q22.32).

CD3⁺CD4⁺ T cells from each patient were enriched to greater than 96% purity by flow cytometry prior to assessing the frequency of chromosomal abnormalities in the patients' normal and abnormal T-cell populations. This detailed analysis confirmed that the normal CD3⁺CD4⁺ T cells from both patients lacked even a small 6q21 or 10p11.22 deletion. In the CD3⁻CD4⁺ T-cells from P1, the percentage of 6q21-deleted cells, representing both 6q⁻ subsets (the small and large deletions; Table 1A), consistently increased throughout clinical disease progression, peaking at 91% co-incident with the T-cell lymphoma (Table 2). In contrast, the percentage of 6q12-deleted cells, which reflect the 6q⁻10p⁻ subclone only (Table 1A), decreased in parallel with disease progression until these cells were undetectable in the blood sample taken at year 6. Together, these results suggest that the majority of 6q21-deleted cells detected at the time of the lymphoma were in fact from the 6q⁻ subclone (small 6q deletion). In addition, the percentage

Table 2. Evolution of the 6q and 10p deleted clones (percent of total $CD3^-CD4^+$ T cells) in successive purified blood samples from P1 and P2.

Probe used	*Chromosome location*	*Subclone(s) represented in the probe-deleted population*	*% of probe-deleted nuclei in the total $CD3^-CD4^+$ T-cell population (a)*		
			Chronic phase		*T-cell lymphoma*
			P1-yr.0 (b)	*P1-yr.4*	*P1-yr.6*
RP3-429G5	6q21	$6q^-$ plus $6q^-10p^-$	77% (53%)	80% (80%)	91% (91%)
RP11-462L8	10p11.22	$6q^-10p^-$ plus $10p^-$	54% (37%)	18% (18%)	< threshold
RP1-91B17	6q12	$6q^-10p^-$	33% (23%)	16% (16%)	< threshold
			P2-yr.0	*P2-yr.4*	
RP3-429G5	6q21	$6q^-$	25% (25%)	22% (21%)	

(a) The percentage of probe-deleted cells was normalized to the percentage of $CD3^-CD4^+$ T cells. In brackets: raw percentage of probe-deleted cells in the sample.
(b) This sample was 69% pure $CD3^-CD4^+$ T cells (not purified because of the limited number of available cells).

of 10p-deleted cells, which include both $10p^-$ and $6q^-10p^-$ subclones, also decreased during disease evolution until they were below the threshold of detection at year 6. In P2, the percentage of 6q21-deleted cells in the $CD3^-CD4^+$ T-cell population remained stable in parallel with this patient's chronic but treatment-responsive disease (25% to 21%; Table 2). These studies underline the apparent importance of the 6q deletion, which was present early and persisted throughout the chronic disease phase in two LV-HES patients, finally emerging in a single subclone in parallel with the $CD3^-CD4^+$ T-cell lymphoma in P1.

Expression analysis of 6q-located genes

Whole-genome oligonucleotide microarrays were performed on the patients' samples using HG-U133A chips (Affymetrix). Genes located in the commonly deleted 6q14.1-q22.1 region (88 of these genes are present on the U133A chip) were examined in detail by comparing purified $CD3^-CD4^+$ T cells from P1-yr. 6 ($6q^-$ present in 91% of the $CD3^-CD4^+$ T-cells) and P2-yr.0 ($6q^-$ present in 25% of the $CD3^-CD4^+$ T cells) with $CD3^+CD4^+$ T-cells purified from P2-yr.4 as a control. Genes whose expression decreased more than two-fold in the abnormal T cells from P1-yr.6 and P2-yr.0 relative to the control are shown in Table 3. None of the 6q-deleted genes was upregulated in the abnormal T cells.

These changes in gene expression were confirmed and quantified using quantitative real-time PCR (RQ-PCR), employing normal $CD3^+CD4^+$ T cells purified from P2-yr.4 blood as well as from a pool of three healthy donors as controls (Table 3). In general, the loss of expression detected in the arrays was reflected as a loss of expression in RQ-PCR, however there were some discrepancies that are worth mentioning. In the abnormal T-cells from P1, the microarray detected a lower expression of exon 3 than exon 4 of the *FOXO3A* gene (Table 3). An alternatively spliced variant of the *FOXO3A* gene, *FKHRL1P2* lacks exon 4 and is specifically expressed in T-helper cells,[30] suggesting there could be a specific decrease in this alternatively spliced transcript in the cells from P1. Although both techniques did detect a significant decrease in *FOXO3A* gene expression, a specific loss of *FKHRL1P2* expression in the abnormal T cells fom P1 could not be confirmed by RQ-PCR. The array experiments did detect a significant decrease in *BACH2* transcripts in the abnormal T cells from P1 as well as a slight reduction of this transcript in the abnormal T cells from P2. RQ-PCR analysis confirmed that this gene is significantly downmodulated in both patients' abnormal T-cells (Table 3). *BACH2* has been shown to be expressed in B cells,[31] and thus we compared the level of *BACH2* expression in normal $CD4^+$ T cells with $CD20^+$ B cells (>97% pure) isolated from healthy donors. Interestingly, RQ-PCR revealed that while *BACH2* transcripts are expressed in $CD4^+$ T-cells, transcript levels are 3-fold lower than in B cells but 50-fold higher than in P1's and 7-fold higher than in P2's abnormal T-cells (*data not shown*).

The most interesting conclusion drawn from these studies is that the genes whose expression was downregulated in both the microarray and RQ-PCR analysis of abnormal T cells from P2 (*C6ORF37*, *BACH2*, *HMGN3*, *PA26*, *RAGD* and *MARCKS*) also had the greatest changes in the abnormal T cells from P1 (Table 3). Furthermore, the greater frequency of the 6q deletion in $CD3^-CD4^+$ T cells from P1 (91%) compared to those from P2 (25%) was associated with an increase in the number of 6q downmodulated genes as well as frequently lower levels of expression. These data suggest that the changes observed in this subset of 6q-deleted genes can potentially be correlated with the abnormal phenotype and/or malignant progression.

Table 3. Decreased expression fold change of 6q14.1-q22.1 located genes.

GB Acc. No.	Gene description	Locus	Array	RQ-PCR[a]	RQ-PCR[b]
P1-yr.6					
NM_017633	*C6ORF37* (chromosome 6 open reading frame 37)	6q14.2	-15.2	-37.6±0.3	-25.4±1.2
NM_021813	*BACH2* (BTB and CNC homology 1 basic leucine zipper transcription factor 2)	6q15	-4.8	-16.3±2.2	-17.6±0.5
NM_004242	*HMGN3* (high mobility group nucleosomal binding domain 3)	6q14.1	-2.8	-5.7±0.4	-8.8±0.4
NM_014454	*PA26* (p53 regulated PA26 nuclear protein)	6q21	-2.7	-4.7±0.6	-12.8±1.4
NM_021244	*RAGD* (Rag D protein)	6q15	-3.9	-3.8±0.7	-5.1±0.3
NM_002356	*MARCKS* (myristoylated alanine-rich protein kinase C substrate)	6q21	-3.9	-2.5±0.5	-0.5±0.3
NM_006016	*CD164* (CD164 antigen sialomucin)	6q21	-3.0	-2.5±0.1	-1.8±0.3
NM_015571	*SUSP1* (SUMO-1-specific protease)	6q14.1	-2.1	-2.2±0.1	-3.5±1.6
NM_006813	*PROL2* (proline rich 2)	6q15	-2.2	-2.1±0.7	-1.9±0.1
NM_001455	*FOXO3A* (forkhead box O3A (exon 4))[d]	6q21	-2.3	-2.0±0.1	-2.0±0.5
NM_020425	*C6ORF162* (DKFZp586E1923)	6q15	-2.6	-1.9±0.6	-2.1±0.3
NM_181837	*ORC3L* (origin recognition complex subunit 3-like (yeast))	6q15	-2.8	-1.8±0.1	-1.9±0.1
NM_001455	*FOXO3A* (forkhead box O3A (exon 3))[c]	6q21	-16.0	-1.7±0.1	-1.9±0.1
P2-yr.0					
NM_004242	*HMGN3* (high mobility group nucleosomal binding domain 3)	6q14.1	-5.0	-12.9±0.6	-20.0±1.3
NM_002356	*MARCKS* (myristoylated alanine-rich protein kinase C substrate)[e]	6q21	-9.7	-11.5±4.4	-2.5±1.8
NM_017633	*C6ORF37* (chromosome 6 open reading frame 37)	6q14.2	-3.9	-10.1±0.5	-6.9±0.1
NM_014454	*PA26* (p53 regulated PA26 nuclear protein)	6q21	-2.3	-4.6±0.6	-12.5±1.4
NM_021244	*RAGD* (Rag D protein)	6q15	-2.1	-4.1±0.2	-4.1±0.9
NM_021813	*BACH2* (BTB and CNC homology 1 basic leucine zipper transcription factor 2)	6q15	-1.5	-2.4±0.1	-5.7±0.9

Array: Mean fold change of all probe sets coding for a specific gene in purified $CD3^-CD4^+$ T cells versus purified $CD3^+CD4^+$ T cells from P2-yr.4. RQ-PCR: Mean fold change from two representative and independent experiments analyzed using RQ-PCR on RNA from purified $CD3^-CD4^+$ T cells versus purified $CD3^+CD4^+$ T-cells ([a]) from P2-yr.4 and ([b]) from a pool of 3 healthy donors. ([c])This probe set hybridizes with both FOXO3A and its alternative spliced variant FKHRL1P2 while ([d]) this probe set hybridizes only FOXO3A. ([e])Variations between replicate samples suggest the instability of this transcript.

Discussion

This study has provided evidence that a 6q deletion is recurrent in two LV-HES patients in association with the monoclonal proliferation of their abnormal $CD3^-CD4^+$ T cell clones. Chromosomal abnormalities have been infrequently reported in this discrete pathological entity (summarized in Table 4), because karyotyping was either not performed[20,23] or found to be normal.[15] However, the importance of detecting recurrent chromosomal abnormalities as a diagnostic factor and/or therapeutic target has been illustrated in other types of leukemia emerging from HES.[13] Two major cytogenetic entities have been identified in chronic eosinophilic leukemia: translocations involving the *PDGFRB* gene at 5q33 and a cryptic deletion at 4q12 giving rise to the *FIP1L1-PDGFRA* fusion gene are correlated with a low rate of transformation to acute leukemia and significant clinical responsiveness to tyrosine kinase inhibitors.[32-34] Alternatively, abnormalities involving the 8p11 band (resulting in *FGFR1* gene rearrangements) are frequently detected in pluripotent lymphoid-myeloid stem cells associated with eosinophilia and T-cell lymphoblastic leukemia and correlated with rapid blast transformation and a poor prognosis.[35] Previously published observations indicated that the 6q deletion occurs in only 12-40% of patients with various subtypes of NHL.[5,36] In our study, no unbalanced change on chromosome 6q was detected using interphase FISH to examine the abnormal $CD3^-CD4^+$ T cells from four additional LV-HES patients in our cohort (*data not shown*). The small number of individuals studied is not sufficient to definitively establish the clinical involvement or the prognostic sig-

Table 4. Summary of the cytogenetic changes detected in patients with T-cell-mediated HES.

Study	Sex/ Age	Source of cells	Karyotype
(56)	F/55	PBL[a]	47,XX,add(1)(q44),del(6)(q?),add(7)(p15),+8, t(9;14)(p21;q11),del(14)(q?)[25]/46,XX [5]
	M/40	PBL[a]	47,XY,+5,i(13q),+der(14)t(1.14)(q23;q32), +15,der(17)t(1;17)(q25;q25),-22 [29]/46,XY [19]
(15)	M/70	BM[b]	46,XY
(17)	M/53	$CD3^+CD4^-$ $CD8^-$ T cells[c]	46,XY,-16,+der(16)t(16;?)(q22;?) [10]
(57)	M/20	PBMC[d]	46,XY,+7 [3]
Present study	F/20	PBMC[d]	46,XX,del(10)(p11p13)/ 46,idem,del(6)(q11q23)/46,XX 46,XX,del(6)(q13q22)([e])
	F/21	PBMC[d]	del(6)(q13q22),inc/46,XX([e])

[a]Peripheral blood leukocytes; [b]bone marrow; [c]purified by cytometry; [d]peripheral blood mononuclear cells; [e]the refined breakpoints of these deletions and percentage of each subclone are described in the text.

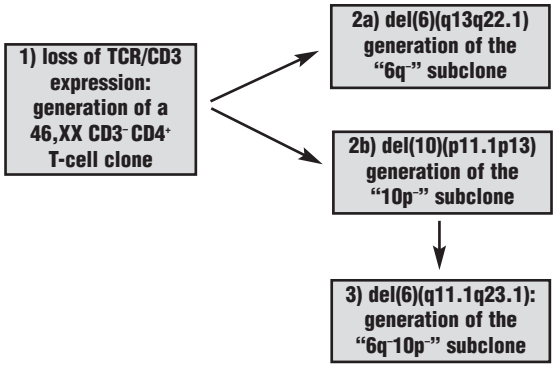

Figure 5. The progressive appearance of phenotypic and genetic abnormalities during disease evolution in P1.

nificance of the 6q deletion in LV-HES. However, this aberration has been correlated with distinct biological and hematologic features in specific subsets of lymphoid malignancies, including chronic lymphocytic leukemia[37] and small lymphocytic NHL.[38] In addition, Offit *et al*. associated the deletion of specific 6q regions with different subtypes of NHL, including a 6q21 deletion with high-grade NHL, a 6q23 deletion with low-grade NHL without t(14;18) and a 6q25-27 deletion with intermediate grade NHL.[5] The recurrent 6q deletion reported in various malignancies provided our rationale for investigating its role in the progressive evolution of LV-HES. However, the difficulty of obtaining abnormal metaphase cells for karyotyping in low-grade lymphoproliferative diseases such as LV-HES likely favors an underestimation of the frequency of chromosomal aberrations, including the 6q and 10p deletions. Routine annual karyotyping of our patients did not detect chromosomal aberrations in blood from either patient during the early lymphoproliferative disease phase, making interphase FISH crucial to our study. In addition, increasing the sensitivity by purifying and/or expanding the abnormal cells *in vitro* were effective approaches.

As a model of pre-malignancy, LV-HES provides us with a unique opportunity to investigate chromosomal abnormalities in the early stages of T-cell lymphomagenesis. These patients are frequently diagnosed at a chronic lymphoproliferative phase based on the presence of an aberrant T-cell clone whose lymphokine expression provokes the hypereosinophilia and related cutaneous symptoms.[20] The disease provides a rare opportunity for a genetic follow-up of patients from a pre-cancerous stage through to full-blown malignancy. We propose a timeline for the progressive appearance of biological defects in these patients based on the sequence of clinical events in P1 (Figure 5). Chromosomal abnormalities were detected exclusively in the $CD3^-CD4^+$ T cells, suggesting that loss of TCR/CD3 surface expression preceded the acquisition of an abnormal karyotype in both patients. Subsequent to TCR/CD3 loss, chromosomal abnormalities acquired in P1 followed a pathway of both genetic diversion and progression: the del(6)(q13q22.1) and the del(10)(p11.1p13) occurred independently in individual 46,XX $CD3^-CD4^+$ T cells, giving rise to the $6q^-$ and the $10p^-$ subclones, respectively. Next, the del(6)(q11.1q23.1) occurred in the $10p^-$ subclone, producing a second-generation $6q^-10p^-$ subclone. Our demonstration that the two 6q deletions were acquired independently underscores the importance of this aberration for the survival of the pre-malignant T cells. The coexistence of two 6q deletions of different sizes has been previously reported for an adult ALL patient; however, this study did not establish the sequential evolution of the karyotype.[39] Based on our data, we postulate that the 6q deletion represents an early and critical genetic event in progression of this lymphoproliferative disease to malignancy.

The 6q deletion has occasionally been reported as the sole aberration in lymphoid malignancies,[38,40] however, genetically unbalanced gains and losses including this defect usually develop secondary to disease-specific translocations.[41] Among the secondary events necessary for clonal development, the 6q defect persists throughout tumor development. It has been suggested that in follicular lymphoma associated with a t(14;18) translocation, the 6q deletion initiates one of several cascades of additional genetic changes leading to malignancy.[42] Another study found that the malignant cells from a $CD30^+$ T-cell lymphoma patient, obtained from the primary tumor and after two relapses, may have originated from a common ancestor clone carrying a 6q defect along with trisomy 9.[43] These studies suggest that the inactivation of tumor suppressor genes present in the 6q region are likely important in the early stages of neoplastic progression.

The role of the 6q deletion in LV-HES progression was further investigated by profiling the expression of genes located in the 6q14.1-q22.1 region using microarrays and RQ-PCR. Six of the 88 genes mapped to the 6q region (6.8%) were significantly downregulated in both patients' abnormal T cells. In contrast, no change in the expression of the same genes was observed in $CD3^-CD4^+$ T cells from another LV-HES patient with a normal karyotype (*data not shown*). Furthermore, only 3.4% of the 22,263 genes on the array showed similarly altered and statistically significant expression changes in both patients. These data suggest that the transcriptional alterations

present during both the chronic and malignant phases of LV-HES are more frequent in genes located in the 6q deletion than in the genome as a whole. This observation reinforces our hypothesis that the 6q deletion plays a critical role in the early rather than the later stages of disease progression. Gene expression profiling in association with a 6q defect has not been frequently reported for lymphoid malignancies. Recently, Haslinger *et al*. used microarrays to correlate 17p, 11q and 6q deletions in B-CLL patients with the recurrent downregulation of several genes located in these regions, suggesting a gene dosage effect in disease development.[44] The downregulation of genes located in the deleted 6q13 to 6q21 region corroborates our data since this is the same region that was lost in the abnormal cells from our patients. However, none of the 6q-linked genes downmodulated in Haslinger's study were the same as those with decreased expression in our patients. While we cannot exclude a technical bias, this suggests that similar 6q deletions might induce different abnormalities in B cells and T cells leading to B-CLL or LV-HES.

The identification of 6q-located tumor suppressor gene(s) can be accomplished by analyzing regions of minimal deletion (RMD) using loss of heterozygosity assays or cytogenetic analysis for large groups of patients with lymphoid malignancies. Several studies have identified more than one distinct 6q RMD in an otherwise homogeneous group of patients,[2,3,5,45,46] which suggest tumor-suppressor genes are putatively located at bands 6q15-q16.1,[47] 6q16.3-q21,[2,3,36] 6q23[38,46] and 6q25-q27.[48] Our chromosomal breakpoint analysis of P1 and P2 delineated a large common region of deletion from bands 6q14.1 to 6q22.1, which spans at least two RMD previously found in other lymphoproliferative diseases.

Potentially, the analysis of genes that are downmodulated in conjunction with a 6q loss could also identify candidate tumor suppressor genes. Our microarray experiments detected at least three tumor suppressor candidate genes worthy of further investigation. *C6ORF37* was appreciably downmodulated in the abnormal T cells from P1 and although the function of this gene is unknown it is widely expressed with sequence conservation across species barriers, suggesting an essential cellular role.[49] BACH2 dimerizes with a Maf protein to function as a transcription factor, and mediates the oxidative stress response by inhibiting MARE-dependent gene expression.[50] Furthermore, the loss of BACH2 expression is thought to be a contributing factor in B-cell lymphomagenesis.[31] Studies reported that BACH2 expression occurs only in neurons and B cells;[51] however, *BACH2* mRNA was later detected in the thymus.[31] We have shown that BACH2 is also expressed in mature human $CD4^+$ T cells, and the coupling of its downmodulation with the 6q deletion suggests that genes governed by BACH2 warrant further investigation. PA26 has been shown to be a potent member of the GADD protein family and the main alternative transcript (*PA26-T2*) is known to be upregulated by p53 after genotoxic stress, suggesting its potential role as a tumor suppressor.[52,53] Surprisingly, this gene was downregulated to the same extent in the abnormal T-cells of both patients, despite their different percentages of 6q-deleted cells. We also detected a 2-fold decrease in *p53* expression in both patients' abnormal T-cells, which could in turn decrease *PA26*. p53 haploinsufficiency may contribute to tumorigenesis by a disproportionate alteration (greater than 2-fold) in its transcriptional activity,[54,55] suggesting that *PA26* trancripts could also be reduced via a 6q deletion-independent mechanism resulting from decreased p53 activity.

We took advantage of an exceptional opportunity to follow the progression of an abnormal T-cell clone *in vivo* (*Willard-Gallo et al., submitted to Exp Hematol*) from its pre-malignant state through to the development of full malignancy. These patients afforded us a unique choice to compare an individual's normal T cells ($CD3^+CD4^+$) with the various developmental stages in the life of the abnormal counterparts ($CD3^-CD4^+$). A natural extension of our studies is to use whole genome microarrays as a means of identifying both the significant molecular changes that are consistently correlated with disease progression as well as for the analysis of abnormal T-cells from other LV-HES patients. These data could provide insight into the expression of specific gene sets that are correlated with precise genetic, biological and/or clinical features.

MR and CS contributed equally to this work. MR, CS and KWG were responsible for designing the study, performing the majority of the experiments, for the analysis and interpretation of the data and for writing the article. FR, LS, and MG were responsible for the clinical analysis and follow-up of the HES patients and for critical revision of the article. HD was responsible for purifying the $CD4^+$ T-cells by flow cytometry. Ch.S contributed his expertise to the microarray experiments and interpretation of these data. PM was involved in successfully treating patient #1's lymphoma by bone marrow transplantation and critical review of the manuscript. All the authors approved the article. The authors declare that they have no potential conflicts of interest.

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2.2. Molecular Characterization of L-HES

Article 3

Molecular profiling of CD3-CD4+ T cells from patients with the lymphocytic variant of hypereosinophilic syndrome reveals targeting of growth control pathways. M. Ravoet, C. Sibille, C. Gu, M. Libin, B. Haibe-Kaines, C. Sotiriou, M. Goldman, F. Roufosse, K.E. Willard-Gallo

Blood , 2009, vol 114, 14, pp: 2969-2983

With the aim of detecting the critical molecular changes occurring in CD3-CD4+ T-cells from L-HES patients, an entire genome expression-array comparative analysis of L-HES CD4+ T-cell clones was performed at diagnosis on three patients relative to the CD3+CD4+ T-cells from five healthy controls. The molecular profiling displayed 850 genes differentially expressed between the three L-HES T-cell clones and the CD3+CD4+ control T-cells. These results, confirmed by quantitative RT-PCR, molecularly established the Th2 memory nature of the CD3-CD4+T-cells and revealed the upregulation of the IL17RB gene expression encoding the IL-25 receptor specific for the Th2 cells. Surface expression of IL17RB and secretion of both IL-5 and IL-13 by CD3-CD4+ T-cells cultured in the presence of rhIL-25 was demonstrated by flow cytometry consistent with a possible activation loop *in vivo* of L-HES T-cell clones by IL-25-secreting eosinophils. The CRTH2 receptor gene was also seen overexpressed compatible with an inflammatory tropism effect on these aberrant Th2 cells at variable sites *in vivo*. Microarrays performed on CD2/CD28 stimulated versus unstimulated CD3-CD4+ T-cells confirmed that Th2 cytokines production was dependent on co-activation. Transcriptional deregulation involved mainly genes related to the TGFβ pathway and to apoptosis/survival cellular mechanisms, thus suggesting an important change in the immune and homeostasic regulation of these cells. In particular, several receptors belonging to the TNF receptor/ligand family were found deregulated. Transcriptomic experiments were also conducted on CD3-CD4+ T-cells from P1 in parallel with the clinical evolution from chronic disease to T-lymphoma and the detected altered expression of target genes associated with transformation and homing.

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Molecular profiling of CD3 CD4+ T cells from patients with the lymphocytic variant of hypereosinophilic syndrome reveals targeting of growth control pathways

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Molecular profiling of $CD3^-CD4^+$ T cells from patients with the lymphocytic variant of hypereosinophilic syndrome reveals targeting of growth control pathways

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The clonal $CD3^-CD4^+$ T-cell population characterizing lymphocytic variant hypereosinophilic syndrome (L-HES) persists for years, with a subgroup of patients ultimately progressing to T lymphoma. The molecular changes associated with the premalignant clone and the emergence of malignant subclones are unknown, precluding the development of targeted therapy for this HES variant. In this study, we used whole genome arrays to examine gene expression in the $CD3^-CD4^+$ T cells and found that 850 genes were differentially regulated during chronic disease compared with $CD3^+CD4^+$ T cells from healthy donors. Changes in the expression of 349 genes were altered in association with the clinical progression from chronic L-HES to T lymphoma in 1 patient, with 87 of 349 genes representing further changes in genes whose expression was altered in all chronic disease patients (87 of 850). Array analysis after CD2/CD28-mediated activation revealed that the major gene expression changes observed in the $CD3^-CD4^+$ T cells do not reflect activation induced alterations but rather pathways involved in T-cell homeostasis, including transforming growth factor-β signaling, apoptosis, and T-cell maturation, signaling, and migration. Examination of microRNA expression in the $CD3^-CD4^+$ T cells from patients with chronic disease identified 23 microRNAs that changed significantly, among which miR-125a further decreased in association with one patient's evolution to T lymphoma. (Blood. 2009;114:2969-2983)

Introduction

Patients with lymphocytic variant hypereosinophilic syndrome (L-HES) are distinguished by the presence of abnormal T-cell populations ($CD3^-CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, or $CD3^+CD4^-CD8^-$) that are frequently monoclonal.[1] These clonal T cells secrete various combinations of interleukin-4 (IL-4), IL-5, and IL-13, resulting in hypereosinophilia and, in many cases, increased serum IgE levels.[2-6] Some patients with L-HES eventually develop peripheral T lymphoma[2,4,7] with detection of an abnormal karyotype[3,8,9] and resistance to apoptosis[10] observed at preneoplastic disease stages. Despite this knowledge, T cell–mediated HES remains a heterogeneous group of diseases lacking definition of the molecular mechanisms underlying the persistence and expansion of the T-cell clone during chronic disease as well as the generation of increasingly abnormal subclones leading to T lymphoma. This contrasts with the discovery of the disease-inducing *FIP1L1/PDGFRA* fusion gene in HES patients with features of myeloproliferative disease, who are now treated with and remarkably responsive to the tyrosine kinase inhibitor imatinib mesylate.[11]

The goal of this study was to establish a molecular profile for $CD3^-CD4^+$ T cell–mediated L-HES by comparing gene expression (both mRNA and microRNA) in the abnormal T cells relative to normal $CD3^+CD4^+$ T cells. These analyses established a comprehensive immunophenotype/genotype that reflects the cells' Th2 nature as well as specific characteristics of polarization, signaling, and function. In patients with chronic disease, significant changes in gene expression were detected in critical growth control pathways of potential clinical relevance, including the IL-25 receptor and genes from the transforming growth factor-β (TGF-β) superfamily. Our previous studies found that the $CD3^-CD4^+$ T cells are dependent on exogenous T-cell receptor (TCR/CD3)–independent activation signals for Th2 cytokine expression.[12] Assessment of gene expression changes associated with CD2/CD28-mediated costimulation revealed that the molecular alterations found in quiescent T cells associated with chronic disease did not simply reflect activation-associated changes in the abnormal T cells. Finally, we explored molecular changes linked with the outgrowth of a 6q-deleted subclone as one patient developed T lymphoma[8] and found that approximately one-third of these genes were also altered in patients during chronic disease, suggesting that they may be of particular interest in terms of conferring a selective survival and growth advantage to the $CD3^-CD4^+$ T cells.

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Methods

Detailed methods are provided in supplemental data (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Patients

Patients with hypereosinophilic syndrome were selected for cohort inclusion based on the presence of a monoclonal $CD3^-CD4^+$ T-cell population in their peripheral blood. The clinical characteristics of all patients analyzed are summarized in supplemental Table 1. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and this study was approved by the ethics committees at Institut Jules Bordet and Hôpital Erasme.

Cell purification

Circulating leukocytes were obtained from the peripheral blood of patients and healthy donors (controls) by venipuncture or cytapheresis, and peripheral blood mononuclear cells (PBMCs) were isolated and frozen as previously described.[12] PBMCs were thawed and resuspended in X-vivo-20 medium (Lonza Braine SA). Patient $CD3^-CD4^+$ and donor $CD3^+CD4^+$ T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec).[8] The isolated cell populations were checked for purity by flow cytometry and were consistently more than 95% pure $CD3^-CD4^+$ (patients) or more than 90% pure $CD3^+CD4^+$ (controls). Thawed and purified T cells were incubated in X-vivo-20 at 37°C/5% CO_2 for 18 hours to eliminate dying cells before RNA extraction. For the costimulation experiments, purified $CD3^-CD4^+$ T cells were cultured for 18 hours in X-vivo-20 supplemented with rhIL-2 (100 U/mL) and anti-CD28 (CLB-CD28/1; 1 μg/mL) plus 2 anti-CD2 antibodies (CLB-T11.1/1 and CLB-T11.2/1; 5 μg/mL each; Sanquin). In the IL-25 experiments, purified $CD3^-CD4^+$ (patient) and $CD3^+CD4^+$ (patient and control) T cells were stimulated for 48 hours with phorbol ester (10 ng/mL) and anti-CD28 in the absence or presence of increasing concentrations of rhIL-25 (R&D Systems).

RNA extraction and gene expression microarray procedures

RNA was extracted by the single-step method of isolation using Trizol (Invitrogen). RNA quantity and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a Bioanalyzer (Agilent); 1.5 μg of total RNA was labeled following the manufacturer's protocols for probe preparation and hybridization (Affymetrix); 15 μg of cRNA was hybridized onto a U133 Plus 2.0 GeneChip.

Statistical analysis of microarray data

Raw data were analyzed using the *SScoreBatch*[13,14] function from the *SScore* package (Version 1.5.1) in the R statistical environment (Version 2.3.0; http://www.r-project.org/; http://www.bioconductor.org). To identify genes consistently deregulated in the 3 patients relative to the 4 controls, we selected genes where each patient's *P* value (generated by S-score algorithm and Z transformation of mean S-score values) was inferior to .05 in comparison with individual controls (supplemental text and supplemental Table 2). A further filter was applied to probe sets of low significance. Similar analyses were performed to determine the genes deregulated during P1's evolution from chronic disease to T lymphoma (supplemental Table 3).

Flow cytometry

Flow cytometric analysis was performed by labeling 2 to 5×10^5 cells with 5 μL fluorescein isothiocyanate–conjugated anti-CD45RO, 5 μL peridinin chlorophyll protein-conjugated anti-CD3, 5 μL allophycocyanin-conjugated anti-CD4, and 5 μL of the phycoerythrin-conjugated test antibody (BD Biosciences; supplemental Table 4) in 50 μL X-vivo-20. A total of 10 000 viable cells were acquired on a FACS Calibur (BD Biosciences). Measurement of cytokine concentrations in culture supernatants was performed using BD Cytometric Bead Array Flex Sets.

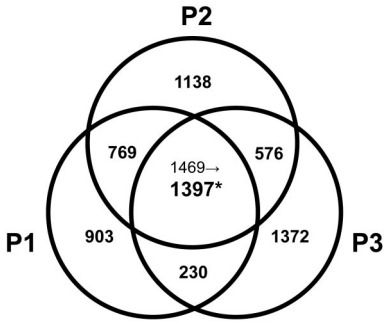

Figure 1. Venn diagram of altered gene probe sets in the L-HES patients (P1-P3) vs controls. Significant changes in the expression of gene probe sets were based on a $P < .05$ after Z transformation of the mean S-score values obtained from all possible 2-chip comparisons between triplicates of P1-yr 0 or duplicates of P2-yr 0 and P3-yr 0 and individual arrays from 4 controls (supplemental data). The number of altered probe sets is shown, and the individual genes are listed in supplemental Table 2. *Among the 1469 commonly changed probe sets detected in the comparison of P1 to P3 vs controls, only 1397 probe sets passed a further filter that was applied to probe sets with a $P > .01$ and/or with fold change < 2 for at least 1 patient.

Real-time quantitative RT-PCR

Total RNA (200 ng to 1 μg) was reverse-transcribed with random hexanucleotides using the SuperScript III First-Strand Synthesis System (Invitrogen) and standard protocols. Primers specific for the *MAP3K8, RUNX1, RUNX2, DIABLO, TGFBR1, TGFBR2, TGFBR3, KIT, SMAD5, SMAD7, NOG, ACVR2A*, and *CYSLTR1* genes were purchased from QIAGEN (QuantiTect Primer Assays). Primers for *ABL* (endogenous control) were kindly provided by Dr J.-L. Vaerman. Quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was performed on a Roche LightCycler 480 (Roche Applied Science). Analyses (supplemental Table 5) were performed using LightCycler Basic software, Version 1.5 (Roche).

MicroRNA quantification

Quantification of mature microRNAs was achieved using stem-loop-mediated RT-PCR with the TaqMan microRNA assay-early access kit or with individual microRNA assay mixes using the manufacturer's protocols (Applied Biosystems; supplemental Table 6). Standard real-time PCR was performed on an ABI Prism 7900HT (Applied Biosystems). Putative microRNA target genes were predicted using MiRanda algorithm-associated MirBase software (http://microrna.sanger.ac.uk).

Results

Comprehensive gene expression analysis of $CD3^-CD4^+$ T cells from L-HES patients

We compared the gene expression profiles of clonal $CD3^-CD4^+$ T cells isolated from L-HES patients (P1-P3; supplemental Table 1) during chronic disease with $CD3^+CD4^+$ T cells from controls. We also evaluated changes in gene expression associated with CD2/CD28 activation of their $CD3^-CD4^+$ T cells in vitro, an antibody combination targeting costimulatory receptors previously shown to mediate their Th2 cytokine production and proliferation.[12] We further analyzed changes in gene expression linked with P1's clinical progression[8,15] by assessing $CD3^-CD4^+$ T cells at diagnosis (yr 0), yr +4 (both premalignant stages of chronic L-HES), and yr +6 (concurrent with T lymphoma diagnosis). After comprehensive and stringent statistical analyses, we detected 850 genes (1397 probe sets) that were differentially regulated in all 3 patients $CD3^-CD4^+$ T cells compared with control $CD3^+CD4^+$ T cells (Figure 1; supplemental Table 2), 312 genes (411 probe sets) that were altered in all 3 patients $CD3^-CD4^+$ T cells after CD2/CD28 costimulation (supplemental Table 2), and 349 genes

(450 probe sets) whose expression was altered in concert with P1's malignant evolution (supplemental Table 3). The original data for all 54 675 probes from each array are provided at Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, under accession number GSE12079.

Gene expression changes in CD3⁻CD4⁺ T cells from L-HES patients compared with CD3⁺CD4⁺ T cells from controls

Immunophenotype/genotype. Based on the microarray data, we compiled a comprehensive phenotype/genotype of the CD3⁻CD4⁺ cells that extends previous characterizations of L-HES (Figure 2, supplemental Table 7). Prior studies of surface receptors on the CD3⁻CD4⁺ T cells[2,5,9,10,12,15-17] validated parallel changes observed in this study, including reduced or lost CD3 (*CD3γ,CD3ζ*), *CD7*, CD27 (*TNFRSF7*), and *CD69* mRNA transcripts and increased *CD5*, CD95 (*FAS*) and HLA class II antigen mRNA transcripts. We determined whether altered mRNA expression corresponded to increased surface protein expression for numerous previously unexplored immunophenotypic markers using flow cytometry (supplemental Table 4) and an enlarged patient cohort (P1-P7; supplemental Table 1). Some gene expression changes can be attributed to the clonal Th2 nature of the patient's CD3⁻CD4⁺ T cells compared with the heterogeneous CD3⁺CD4⁺ T-cell population from controls.[18] These include down-regulation of the Th1 genes *BTLA, CCL5, IL-18R, NOTCH2, JUN, SLAMF7*, and integrin α6 (*ITGA6*) and up-regulation of the established Th2 genes *IL4R, CCR3, CCR8, GATA3*, CRTH2 (CD294, *GPR44*), and *IL17RB* in the abnormal T cells.

IL-17RB, the receptor for IL-25 (*IL17E*), is expressed on memory Th2 cells[19] and as recently shown also on human CD14⁺ cells.[20] IL-25 induces Th2 inflammation in mice and, when bound to human Th2 cells, enhances Th2 cytokine production in response to T cell–stimulating agents.[19] In this study, we detected increased expression of the *IL17RB* gene not only in all patients CD3⁻CD4⁺ T cells during chronic L-HES (Figure 2) but also observed further increases on P1's T lymphoma cells (Table 1) and after CD2/CD28 costimulation of P1 to P3's CD3⁻CD4⁺ T cells (Table 2). Flow cytometry detected a significantly higher proportion of P3's CD3⁻CD4⁺CD45RO⁺ T cells expressing membrane IL-17RB compared with CD3⁺CD4⁺CD45RO⁺ or CD45RO⁻ T cells from controls (Figure 3A). P3's CD3⁻CD4⁺ T cells cultured with rhIL-25 induced a dose-response increase in the production of IL-5 and IL-13 but not interferon-γ and enhanced their proliferation in response to phorbol ester/anti-CD28 (Figure 3B). The further up-regulation of *IL17RB* mRNA observed on P1's T lymphoma cells and after CD2/CD28 costimulation of CD3⁻CD4⁺ T cells from chronic disease suggests that eosinophil-produced IL-25 may facilitate sustained and preferential expansion of the abnormal T-cell clone. Thus, increased *IL17RB* expression may provide a selective advantage to the CD3⁻CD4⁺ T-cell clone (or a subclone), thereby contributing to the maintenance of chronic disease and malignant progression.

Modulation of surface receptor expression is a common mechanism for controlling T cell–mediated immune responses that are characterized by cytokine production and proliferation. mRNA expression for several immunomodulatory genes other than the TCR/CD3 complex were altered in the patient's CD3⁻CD4⁺ T cells (Figure 2, supplemental Table 7), and some with apparent relevance include (1) decreases in the membrane complement regulatory proteins, which have been associated with autoimmune and inflammatory disease as well as the regulation of T-cell activation responses[21]; (2) decreased expression of genes involved in negatively regulating T-cell responses, including *CTLA4* (CD152) and *IL27RA*, both shown to play critical roles in murine Th2 cell homeostasis[22,23] and whose downmodulation potentially contributes to persistence and expansion of the CD3⁻CD4⁺ T-cell clone; and (3) increases in several immunoregulatory receptors, which perhaps provide a more pertinent characterization of costimulatory and regulatory pathways operating in the CD3⁻CD4⁺ T cells, including: (1) SLAMF5 (*CD84*), an inhibitory receptor for the high affinity IgE receptor[24]; (2) DCAL1 (*CLECL1*), a type II transmembrane C-type lectin-like protein expressed on dendritic cells and B cells (no previous reports of T-cell expression) and whose interaction with T cells has been shown to enhance their IL-4 production[25]; (3) *CD99*, a T-cell costimulatory receptor capable of fully activating cells stimulated with a suboptimal TCR/CD3 signal[26]; and (4) *CD200R1*, an inhibitory receptor regulating the activation threshold of inflammatory immune responses, which might also affect CD3⁻CD4⁺ T-cell activation.

The lack of TCR/CD3 expression on the abnormal T-cell surface dictates the potential loss of this important signaling pathway, although the CD3⁻CD4⁺ T cells do remain responsive to costimulatory signals.[12] Some critical TCR/CD3 downstream signals were found decreased in unstimulated CD3⁻CD4⁺ T cells, including inhibitory receptors, PI3K-associated or family proteins, tyrosine and MAP3 kinases, and activation responsive transcription factors (supplemental Table 7). Interestingly, gene expression analysis of the abnormal T cells after CD2/CD28 costimulation revealed that relatively few of the gene expression changes detected in "quiescent" CD3⁻CD4⁺ T cells isolated from patient blood involved genes whose expression was affected by activation (Table 2, supplemental Table 2). Thus, induction of the Th2 cytokine genes *IL5*, *IL13*, and other immune response genes by costimulation in vitro apparently induces transient signals that may be elicited by a sustained stimulus present in local immune microenvironments in vivo.

G-protein–coupled receptors. Numerous G-protein–coupled receptors were altered on the CD3⁻CD4⁺ T cells (Figure 2) and include 2 of particular significance. A 19-fold decrease in cysteinyl leukotriene receptor 1 (*CYSLTR1*) gene expression was observed in the CD3⁻CD4⁺ T cells with the greatest degree of downmodulation detected in P1-yr 0. Decreased CYSLTR1 expression was confirmed by quantitative RT-PCR (P1-P5, P7; Figure 4A, supplemental Table 5) but revealed a disparity in expression between the patients (ie, it is not expressed in P1 and low expression levels were detected in P2, P3, and P5). CYSLTR1 is normally expressed on myeloid cells, including eosinophils, and induced on CD4⁺ T cells by Th2 cytokines and TCR/CD3-mediated activation. The CYSLTR1 ligand, leukotriene D4, is produced by eosinophils and other myeloid cells and plays an active role both in cell survival and leukocyte recruitment to inflamed tissues.[27] In contrast to the decreased expression observed on the CD3⁻CD4⁺ T cells, CYSLTR1 is significantly up-regulated and functional on CD4⁺ T cells from mice carrying an LAT gene mutation. These mice develop a Th2 lymphoproliferative disorder characterized by marked infiltration of CD3^{lo}CD4⁺ T cells in secondary lymphoid organs.[28] Lack of CYSLTR1 on patients CD3⁻CD4⁺ T cells may render them less responsive to some eosinophil-derived survival signals and thereby contribute to the more indolent nature of L-HES.

The prostaglandin D2 receptor CRTH2, a G-protein–coupled receptor selectively expressed by Th2 cells, eosinophils, and basophils, is currently considered the most reliable marker for memory Th2 cells.[29] Two CRTH2 (*GPR44*) gene probes revealed a 5- and 22-fold increase in expression in the patients abnormal T cells, which was confirmed by flow cytometry (supplemental

Symbol	Fold Change P1-P3 vs D
A. Immunophenotype	
HLA-DQA1/2	15.5
DCAL1	15.1
HLA-DQB1	14.9
IL9R	8.8
HLA-DRB1	8.6
HLA-DPB1	4.9
HLA-DPA1	4.7
CD200R1	3.7
HLA-DRA	3.4
CD5	2.8
SPN	2.3
PBEF1	2.3
CD84	2.1
CD58	2.1
TFRC	1.9
ICAM3	1.8
CD99	1.8
ITGB1	1.7
CD82	1.5
IL7R	-1.6
IL27RA	-1.6
IL6ST	-1.7
CLEC2D	-1.9
CCL4	-2.2
CD59	-2.4
CD3G	-2.5
CXCR4	-2.5
CD247	-2.6
CXCR6	-2.8
CRTAM	-2.9
CD69	-2.9
SLAMF1	-3.3
AIF1	-3.4
CR2	-3.7
CTLA4	-3.7
CLEC2B	-3.9
CD55	-3.9
NT5E	-4.5
CD7	-6.0
CCR6	-6.4
ITGA4	-6.6
IL23A	-9.9
CR1	-14.8
CCR7	-15.4
B. Th1/Th2 specific	
GPR44	22.4
IL17RB	22.4
CCR8	11.3
CCR3	6.8
GATA3	3.6
IL4R	3.0
NOTCH2	-1.7
BTLA	-2.8
IL18R1	-4.8
SLAMF7	-5.2
ITGA6	-5.7
IL18RAP	-6.3
CCL5	-26.2
C. Apoptosis	
TNFSF14	3.7
TNFSF10	3.5
RBBP8	3.0
FAS	2.5
TRADD	1.8
TNFRSF10B	-1.7
RBL2	-1.9
CD47	-2.0
ATM	-2.2
CASP10	-2.3
STK17A	-2.6
BNIP3	-4.2
SESN1	-4.3
LATS2	-4.6
RIPK2	-5.9
BACH2	-6.7
TNFRSF7	-42.4
D. Survival	
TNFSF11	10.4
TNFRSF11A	7.5
BIRC4	2.1
DIABLO	1.8
FYN	-2.2
MYC	-2.4
BCL2L11	-2.6
MYB	-2.8
MYBL1	-3.2
TNFRSF10D	-3.4
YES1	-4.9
FAIM3	-5.1
KIT	-5.2
BCL2	-5.2
TNFSF13B	-9.9
LGALS3 /// GALIG	-14.5
IGF1R	-22.8
E. TGF β pathway	
CLU	9.5
SMAD5	4.6
TGFBR3	3.0
ACVR2A	2.1
BMPR1A	1.9
TGFBR1	-2.6
ACVR1C	-4.7
SMAD7	-5.5
TGFBR2	-6.3
NOG	-41.8
F. G-coupled receptors	
GPR137B	5.5
P2RY14	4.6
RGS1	4.2
GPR68	3.0
GPR171	2.5
PTGER4	2.4
P2RY5	2.2
P2RX4	1.7
GPR108	1.7
P2RY8	1.6
RGS10	-2.8
GPR15	-3.5
GPR92	-3.9
GPR160	-4.2
ADRB2	-4.4
GPR18	-4.4
CYSLTR1	-19.1
G. Transcription factors / repressors	
MSC	3.3
LMO4	2.8
BATF	2.7
RUNX2	2.4
ETS1	-1.5
NFATC1	-1.9
LEF1	-2.0
ETS2	-2.1
TCF7	-2.2
JUN	-2.2
KLF7	-2.6
LMO2	-2.7
FOXP1	-3.7
NFKBIZ	-4.6
KLF9	-4.9
SOX4	-9.0
H. Signaling	
PTPRN2	11.9
RAB27B	7.4
DUSP4	6.5
MAP3K8	4.1
JAKMIP1	3.7
NDFIP2	3.6
SOS1	3.2
TNIK	3.1
PPAPDC1B	2.5
RAB11FIP5	2.2
PPP1R16B	2.2
MAP4K1	2.0
INPP4B	2.0
PPIB	1.9
PTPLAD2	1.9
IKBKB	1.8
RHOF	1.8
PPP3CA	1.7
PPP1R15A	1.7
RAB8A	1.7
FKBP2	1.6
TIAM1	1.5
PIK3R5	-1.6
STAT1	-1.7
MAP3K7IP2	-1.7
JAK1	-1.8
RAB15	-2.0
FLT3LG	-2.0
PDE7A	-2.1
RASA2	-2.1
PDE3B	-2.4
PTPN4	-2.5
RASSF3	-2.5
INPP5A	-2.5
RAB43	-2.8
DUSP2	-2.9
IFI44	-3.1
LMO7	-3.2
DUSP1	-3.3
PTPN13	-3.5
PRMT2	-3.7
PIP3-E	-3.8
RAPGEF6	-3.9
RAB34	-3.9
PDE9A	-4.3
PDE4DIP	-4.5
PTPRM	-4.9
PITPNC1	-5.7
RASGRF2	-8.1
RIN3	-8.8
TXK	-24.6
I. Proteolysis	
PSMB7	3.9
PSMD8	1.4
PSMA1	-2.8
PRF1	-3.3
GZMH	-3.4
CST7	-4.2
GZMA	-5.7
GZMK	-15.2
J. Other functions	
HN1	2.0
CBLB	-3.6
RALGPS2	-4.9
SPON1	-7.7
NELL2	-19.6

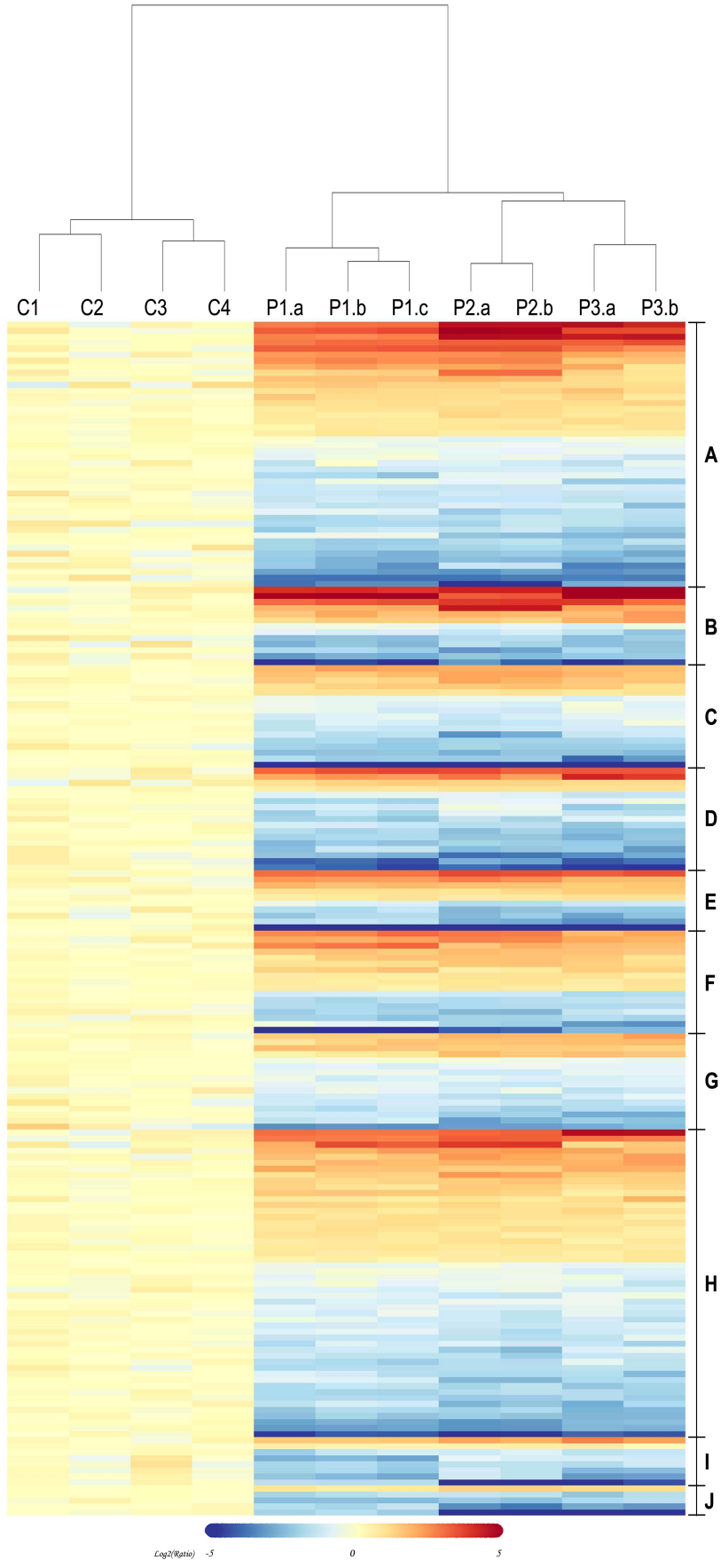

Figure 2. Heat map of selected gene alterations detected in the comparison of L-HES patients (P1-P3) vs controls. A total of 198 of the commonly altered genes detected in all 3 patients were selected from supplemental Table 2 based on their functional relevance (one probe set/gene is shown and represents the greatest absolute fold change in patients relative to controls). The genes are classified in functional groups and listed in numerical order based on fold change with the same order and group numbers maintained in the heat map. Each column in the heat map represents the expression from an individual gene chip and includes the 4 healthy controls (C1-C4), triplicates of P1-yr 0 (a-c), and duplicates of P2 and P3 (a-b). The clustering and the heat map were generated using R 2.5.1. The dendrogram was derived from a group of selected genes using the hierarchical clustering method and shows the relatedness of gene expression patterns in the L-HES patients relative to the controls.

Table 1. Gene expression changes detected in CD3⁻CD4⁺ T cells from chronic L-HES patients relative to controls and in association with P1's evolution to T lymphoma

Symbol	Name	Probe set	P1-P3 vs C[a,b]	P1 yr+6 vs P1-yr0[b,c]
ABLIM1	actin binding LIM protein 1	200965_s_at	-2.26	1.48
		210461_s_at	-2.44	
ANKRD57	ankyrin repeat domain 57	227034_at	3.13	-1.69
		219496_at	2.96	
APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	209870_s_at	-3.81	-1.56
		209871_s_at	-12.51	
AQP3	aquaporin 3 (Gill blood group)	39248_at	-3.07	1.56
		39249_at	-2.16	
		203747_at	-2.14	
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	236796_at	-6.70	-1.78
		221234_s_at	-5.59	
		1556451_at	-4.38	
		236307_at	-4.53	
		227173_s_at	-4.86	
		215907_at	-1.77	
BCAT1	branched chain aminotransferase 1, cytosolic	225285_at	26.07	2.58
		226517_at	27.21	2.52
		214452_at	6.56	3.11
		214390_s_at		1.94
BCL2	B-cell CLL/lymphoma 2	232210_at	-5.23	
		232614_at	-4.83	
		244035_at	-3.84	
		203685_at		-1.64
BEXL1	brain expressed X-linked-like 1	215440_s_at	-2.19	-1.90
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	201849_at	-4.24	-2.27
		201848_s_at	-3.40	
C18orf1	chromosome 18 open reading frame 1	242551_at	-2.90	2.38
		207996_s_at	-2.47	
C9orf40	chromosome 9 open reading frame 40	222781_s_at	1.81	2.01
CCR7	chemokine (C-C motif) receptor 7	206337_at	-15.39	3.13
		243107_at	-3.58	
CCR8	chemokine (C-C motif) receptor 8	208059_at	11.33	1.50
CDCA7	cell division cycle associated 7	230060_at	5.24	2.63
		224428_s_at	6.32	2.61
CEP55	centrosomal protein 55kDa	218542_at	3.07	2.08
ChGn	chondroitin β1,4 N-acetylgalactosaminyltransferase	1569387_at	1.93	
		219049_at		1.36
CLEC2B	C-type lectin domain family 2, member B	209732_at	-3.88	
		1556209_at		-1.59
COTL1	coactosin-like 1 (Dictyostelium)	1556346_at	4.25	1.51
		224583_at	4.28	
		221059_s_at	3.39	
CST7	cystatin F (leukocystatin)	210140_at	-4.21	3.61
CTSC	cathepsin C	201487_at	2.09	1.45
		225647_s_at	2.17	1.43
		225646_at	2.13	
DCAL1	CLECL1, dendritic cell-associated lectin-1	244413_at	15.14	1.56
DIABLO	diablo homolog (Drosophila)	219350_s_at	1.79	2.18
DKFZp761P0423	homolog of rat pragma of Rnd2	235085_at	-6.77	1.49
		240690_at	-9.70	
EMR1	egf-like module containing, mucin-like, 1	207111_at	4.35	1.36
ETFB	electron-transfer-flavoprotein, beta polypeptide	202942_at	2.58	1.76
F5	coagulation factor V (proaccelerin, labile factor)	204713_s_at	-3.53	4.70
		204714_s_at	-3.06	4.90
FAIM3	Fas apoptotic inhibitory molecule 3	221601_s_at	-4.61	-1.62
		221602_s_at	-5.13	
FAM13A1	family with sequence similarity 13, member A1	232628_at	-2.75	2.53
		202973_x_at	-2.43	2.10
		217047_s_at		2.29
FAM50B	family with sequence similarity 50, member B	205775_at	1.82	1.53
FANK1	fibronectin type III and ankyrin repeat domains 1	232968_at	9.90	1.58
FGF9	fibroblast growth factor 9 (glia-activating factor)	206404_at	-3.48	-2.10
FLJ20152	hypothetical protein FLJ20152	218532_s_at	-9.93	-1.91
		218510_x_at	-6.90	
FLJ21272	hypothetical protein FLJ21272	220467_at	-3.35	-1.57
FLJ42957	FLJ42957 protein	237591_at	2.40	2.04
GLIPR1	GLI pathogenesis-related 1 (glioma)	204222_s_at	2.13	
		214085_x_at	2.27	
		204221_x_at	2.24	
		233515_at		-1.89
GLUL	glutamate-ammonia ligase (glutamine synthetase)	215001_s_at	1.97	1.38
		200648_s_at	2.02	
GPR68	G protein-coupled receptor 68	229055_at	3.02	1.50
GTPBP8	GTP-binding protein 8 (putative)	223486_at	2.29	1.93
		221046_s_at	2.19	
GZMA	granzyme A (granzyme 1)	205488_at	-5.70	2.40
HLA-DQA1/2	major histocompatibility complex, class II, DQα1/2	212671_s_at	15.46	3.07
HLA-DQB1	major histocompatibility complex, class II, DQβ1	212998_x_at	14.90	2.49
		211656_x_at	4.23	2.02
		209823_x_at	5.17	2.17
		211654_x_at	3.54	1.60
HLA-DRA	major histocompatibility complex, class II, DRα	208894_at	3.45	3.01
HLA-DRB1	major histocompatibility complex, class II, DRβ1	215193_x_at	8.60	1.64
		208306_x_at	7.51	1.41
		209312_x_at	8.34	1.52
		204670_x_at	7.01	1.52
HN1	hematological and neurological expressed 1	222396_at	2.05	1.36
		217755_at	2.00	
HPCAL4	hippocalcin like 4	219671_at	-4.90	-3.01
HRBL	HIV-1 Rev binding protein-like	222126_at	1.77	
		1554618_at		-2.23
IGSF9B	immunoglobulin superfamily, member 9B	215255_at	2.48	1.64
IL17RB	interleukin 17 receptor B	219255_x_at	22.24	1.55
		224156_x_at	22.37	1.45
		224361_s_at	16.85	1.47
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	244599_at	-6.56	
		213416_at		2.08
		205884_at		2.55
		205885_s_at		2.78
KIAA1199	KIAA1199	212942_s_at	6.10	1.79
KLF9	Kruppel-like factor 9	203543_s_at	-4.86	-2.12
		203542_s_at	-4.19	-2.66
KLHL3	kelch-like 3 (Drosophila)	221221_s_at	-2.64	1.77
LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	212442_s_at	-7.49	2.01
		212446_s_at	-5.80	
LOC283174	hypothetical protein LOC283174	229734_at	2.71	1.71
LOC89944	hypothetical protein BC008326	213713_s_at	2.80	1.77
LYZ /// LILRB1 LYZ	lysozyme (renal amyloidosis) /// leukocyte Ig-like receptor, subfamily B, member 1	213975_s_at	-3.26	10.01
		1555745_a_at		3.41
MAN1C1	mannosidase, alpha, class 1C, member 1	218918_at	-3.14	-1.66
		214180_at	-2.75	
MAP3K8	mitogen-activated protein kinase kinase kinase 8, COT, TPL2	235421_at	2.90	1.90
		205027_s_at	4.10	
MGAT5	mannosyl (alpha-1,6-)-glycoprotein e beta-1,6-N-acetyl-glucosaminyltransferase	241893_at	-2.75	
		215528_at		1.56
MSC	musculin (activated B-cell factor-1)	209928_s_at	3.28	1.95
NELL2	NEL-like 2 (chicken)	203413_at	-19.57	-6.10
P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	204088_at	1.75	1.64
P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	200656_s_at	2.05	1.46
		1564494_s_at	1.81	1.59
		200654_at	2.04	
PBEF1	pre-B-cell colony enhancing factor 1	243296_at	2.07	1.98
		1555167_s_at	1.76	1.82
		217739_s_at	2.33	1.93
		217738_at	2.08	1.88
PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin)	236704_at	-4.48	
		212390_at		-2.59
PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	202457_s_at	1.70	
		1562467_at		-3.53
PRSS21	protease, serine, 21 (testisin)	220051_at	7.10	1.44
PTPLAD2	protein tyrosine phosphatase-like A domain 2	244050_at	1.85	1.34
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	203029_s_at	11.85	2.20
		203030_s_at	9.14	2.49
		211534_x_at	2.68	1.99
RBBP8	retinoblastoma binding protein 8	203344_s_at	3.02	1.73
RGS10	regulator of G-protein signalling 10	204319_s_at	-2.81	1.40
		204316_at	-2.02	
RNF130	ring finger protein 130	217865_at	-3.41	-2.08
RUNX2	runt-related transcription factor 2	232231_at	2.41	1.86
SCML1	sex comb on midleg-like 1 (Drosophila)	235652_at	-11.11	-2.61
		218793_s_at	-12.21	
		222747_s_at	-4.39	
SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	230748_at	2.86	2.10
		207038_at	2.76	
SLC1A4	solute carrier family 1, member 4 (glutamate/neutral amino acid transporter)	212811_x_at	6.71	2.20
		209610_s_at	9.11	2.07
		212810_s_at	6.62	2.18
		244377_at	5.53	2.05
		235875_at	5.03	1.89
		209611_s_at		2.14
SLC2A3	solute carrier family 2, member 3 (facilitated glucose transporter)	202499_s_at	1.62	
		236180_at		2.16
SLC39A10	solute carrier family 39 (zinc transporter), member 10	225295_at	-1.93	1.60
		226444_at	-2.38	
SLC44A2	solute carrier family 44, member 2	224609_at	-2.17	-1.65
		225175_s_at	-2.12	
SOS1	son of sevenless homolog 1 (Drosophila)	212780_at	2.97	1.45
		229261_at	3.08	
		230337_at	3.23	
		232883_at	2.37	
		242018_at	2.53	
		212777_at		1.51
SPON1	spondin 1, extracellular matrix protein	213994_s_at	-7.71	-2.57
		209436_at	-5.41	-2.03
		213993_at	-7.27	
		209437_s_at	-3.37	
TBL1X	transducin (beta)-like 1X-linked	213400_s_at	-2.68	-1.80
		201869_s_at	-2.01	-1.57
		201867_s_at		-2.01
TCEAL4	transcription elongation factor A (SII)-like 4	202371_at	-6.95	-3.00
WWTR1	WW domain containing transcription regulator 1	202133_at	6.17	2.15
ZNF30	zinc finger protein 30	232014_at	-2.61	-1.53
ZNF439	zinc finger protein 439	236562_at	-4.82	-1.80
		237441_at	-3.46	-1.76
ZNF447	zinc finger protein 447	218312_s_at	-7.77	
		217593_at		-1.89

[a]Fold change comparing the mean expression of duplicate arrays from P1, P2, and P3 with the mean expression from 4 controls; data are from supplemental Table 2.
[b]Consistently up-regulated genes are highlighted in dark gray; and consistently down-regulated genes in light gray.
[c]Fold change comparing the mean expression of triplicate arrays from P1-yr 0 and P1-yr +6; data are from supplemental Table 3.

Table 2. Changes in the expression of immune response genes associated with anti-CD2/CD28 activation of the CD3$^-$CD4$^+$ T cells from P1-P3

Symbol	Name	Probe set ID	Mn fold chg: P1-P3 vs C[b]	Mn fold chg: P1 yr+6 vs yr0[c]	Mn fold chg: P1-P3 S vs NS[d,e]
ATF4	activating transcription factor 4	200779_at	nc[f]	nc	1.55
BCL2	B-cell CLL/lymphoma 2	232210_at	-5.23	nc	18.44
		203685_at	nc	-1.64	8.91
BCL2L1	BCL2-like 1	212312_at	nc	nc	1.70
BCL2L11	BCL2-like 11 (apoptosis facilitator)	225606_at	-2.62	nc	nc
CCL5	chemokine (C-C motif) ligand 5 (RANTES)	1405_i_at	-26.17	nc[g]	nc
CCR2	chemokine (C-C motif) receptor 2 (CD192)	206978_at	nc	2.15	2.01
CCR3	chemokine (C-C motif) receptor 3 (CD193)	208304_at	6.79	nc	nc
CCR5	chemokine (C-C motif) receptor 5 (CD195)	206991_s_at	nc	1.80[g]	nc
CCR6	chemokine (C-C motif) receptor 6 (CD196)	206983_at	-6.40	nc	nc
CCR7	chemokine (C-C motif) receptor 7 (CD197)	206337_at	-15.39	3.13	nc
		243107_at	-3.58	nc	nc
CCR8	chemokine (C-C motif) receptor 8 (CD198)	208059_at	11.33	1.50	nc
CCR10	chemokine (C-C motif) receptor 10	220565_at	nc	2.81	nc
CD27	CD27 molecule	206150_at	-42.39	nc	nc
CD3G	CD3g molecule, gamma (TCR/CD3)	206804_at	-2.47	nc	nc
CD47	CD47 molecule	227259_at	-1.99	nc	nc
CD5	CD5 molecule	230489_at	2.78	nc	nc
CD53	CD53 molecule	203416_at	nc	nc	1.38
CD55	CD55 molecule, DAF for complement	243395_at	-3.93	nc	nc
CD58	CD58 molecule	243931_at	2.10	nc	nc
CD59	CD59 molecule, complement regulatory	212463_at	-2.43	nc	nc
CD69	CD69 molecule	209795_at	nc	nc	2.36
		237009_at	-2.88	nc	nc
CD7	CD7 molecule	214551_s_at	-6.03	nc	nc
CD74	CD74 molecule, MHC, class II invariant	209619_at	nc	nc	2.73
CD80	CD80 molecule	1554519_at	nc	nc	3.33
CD82	CD82 molecule	203904_x_at	1.53	nc	nc
CD84	CD84 molecule	205988_at	2.11	nc	nc
CD96	CD96 molecule	1555120_at	nc	1.62	nc
CD99	CD99 molecule	201028_s_at	1.78	nc	nc
CD200R1	CD200 receptor 1	1552875_a_at	3.69	nc	nc
CD247	CD247 molecule	210031_at	-2.56	nc	nc
CIITA	class II, MHC, transactivator	205101_at	nc	nc	2.87
CISH	cytokine inducible SH2-containing protein	223961_s_at	nc	nc	11.83
DUSP1	dual specificity phosphatase 1	201041_s_at	-3.25	nc	nc
DUSP2	dual specificity phosphatase 2	204794_at	-2.86	nc	nc
DUSP4	dual specificity phosphatase 4	204014_at	6.51	nc	nc
DUSP5	dual specificity phosphatase 5	209457_at	nc	nc	5.76
DUSP6	dual specificity phosphatase 6	208891_at	nc	nc	6.93
DUSP16	dual specificity phosphatase 16	224336_s_at	nc	nc	-2.14
EGR1	early growth response 1	227404_s_at	nc	nc	7.82
EGR2	early growth response 2	205249_at	nc	nc	8.02
EGR3	early growth response 3	206115_at	nc	nc	6.54
EPHA4	EPH receptor A4	206114_at	nc	nc	-1.75
EPHB6	EPH receptor B6	204718_at	-2.98	nc	nc
FAIM3	Fas apoptotic inhibitory molecule 3	221601_s_at	-4.61	-1.62	-1.94
		221602_s_at	-5.13	nc	-1.83
FLT3LG	fms-related tyrosine kinase 3 ligand	206980_s_at	-2.03	nc	3.36
		210607_at	nc	nc	4.01
FYB	FYN binding protein (FYB-120/130)	227266_s_at	nc	nc	-1.44
FYN	FYN oncogene related to SRC, FGR, YES	212486_s_at	-2.18	nc	nc
HLA-DMA	MHC, class II, DM alpha	217478_s_at	nc	nc	2.42
HLA-DMB	MHC, class II, DM beta	203932_at	nc	nc	2.31
HLA-DPA1	MHC, class II, DP alpha 1	211990_at	4.68	nc	nc
HLA-DPB1	MHC, class II, DP beta 1	201137_s_at	4.89	nc	nc
HLA-DQA1	MHC, class II, DQ alpha 1	212671_s_at	15.46	3.07	2.49
HLA-DQB1	MHC, class II, DQ beta 1	212998_x_at	14.90	2.49	2.03
HLA-DRA	MHC, class II, DR alpha	208894_at	3.45	3.01	4.17
IFI44	interferon-induced protein 44	214453_s_at	-3.08	nc	nc
IFI6	Interferon, alpha-inducible protein 6	224533_s_at	-22.00	nc	nc
IFITM1	interferon induced transmembrane 1	201601_x_at	nc	1.57	2.17
		214022_s_at	nc	nc	2.10
IFITM2	interferon induced transmembrane 2	201315_x_at	nc	1.59	nc
IL2RA	interleukin 2 receptor, alpha (CD25)	211269_s_at	nc	nc	4.22
IL2RB	interleukin 2 receptor, beta (CD122)	205291_at	nc	nc	1.73
IL4R	interleukin 4 receptor (CD124)	203233_at	2.98	nc	nc
IL5	interleukin 5 (CSF, eosinophil)	207952_at	nc	nc	6.82
IL6ST	Interleukin 6 signal transducer (CD130)	212195_at	-1.71	nc	nc
IL7R	interleukin 7 receptor (CD127)	205798_at	-1.59	nc	-1.50
IL9R	interleukin 9 receptor (CD129)	214950_at	8.81	nc	nc
IL13	interleukin 13	207844_at	nc	nc	7.34
IL17RB	interleukin 17 receptor B	219255_x_at	22.24	1.55	2.97
IL18R1	interleukin 18 receptor 1 (CD218a)	206618_at	-4.85	nc	nc
IL18RAP	interleukin 18 receptor AP (CD218b)	207072_at	-6.27	nc	nc
IL23A	interleukin 23, alpha subunit p19	234377_at	-9.93	nc	nc
IL27RA	interleukin 27 receptor, alpha	222062_at	-1.61	nc	nc
IL32	interleukin 32	203828_s_at	nc	2.50	nc
INPP4B	inositol polyphosphate-4-phosphatase	205376_at	1.99	nc	1.78
IRF4	interferon regulatory factor 4	204562_at	nc	nc	6.12
ISGF3G	interferon-stimulated transcript. factor 3	203882_at	nc	nc	1.83
ITGA4	integrin, alpha 4 (CD49D, VLA-4)	205885_s_at	nc	2.78	nc
		213416_at	nc	nc	2.05
ITGA6	integrin, alpha 6	244599_at	-6.56	nc	nc
ITGB1	integrin, beta 1 (CD29)	215177_s_at	-5.67	nc	nc
LGALS1	lectin, galactoside-binding 1 (galectin 1)	1553678_a_at	1.70	nc	nc
LGALS3	lectin, galactoside-binding 3 (galectin 3)	201105_at	nc	2.40	nc
LIF	leukemia inhibitory factor	208949_s_at	-14.53	nc	nc
LMAN1	lectin, mannose-binding, 1	205266_at	nc	nc	2.13
LMNA	lamin A/C	224629_at	1.49	nc	1.46
LMNB1	lamin B1	1554600_s_at	nc	nc	2.13
LTA	lymphotoxin alpha (TNFSF1)	203276_at	nc	nc	1.76
LTB	lymphotoxin beta (TNFSF3)	206975_at	nc	nc	3.33
MAL	mal, T-cell differentiation protein	207339_s_at	nc	nc	1.58
MAP2K5	mitogen-activated protein kinase kinase 5	204777_s_at	nc	nc	2.19
MAP3K7IP2	mitogen-activated protein 3x kinase 7 IP2	216765_at	nc	-1.75	nc
MAP3K7IP3	mitogen-activated protein 3x kinase 7 IP3	212184_s_at	-1.72	nc	1.68
MAP3K8	mitogen-activated protein 3x kinase 8	227357_at	nc	1.45	nc
		205027_s_at	4.07	nc	nc
MAPKAPK3	mitogen-activated protein kinase-act 3	235421_at	2.91	1.93	nc
MAP4K1	mitogen-activated protein 4x kinase 1	202788_at	nc	nc	1.54
NDFIP2	Nedd4 family interacting protein 2	206296_x_at	2.00	nc	nc
		224802_at	3.62	nc	8.10
NFIA	nuclear factor I/A	224799_at	nc	nc	6.62
NFIL3	nuclear factor, interleukin 3 regulated	224970_at	nc	1.76	nc
NFATC1	NFAT, cytoplasmic 1	203574_at	nc	2.73	2.69
NFKBIZ	NF-kB inhibitor, zeta	211105_s_at	-1.92	nc	nc
OSM	oncostatin M	223218_s_at	-4.56	nc	nc
PAK1IP1	PAK1 interacting protein 1	230170_at	nc	nc	3.52
PBXIP1	pre-B-cell leukemia TF interact. protein 1	218886_at	nc	nc	1.82
PDE3B	phosphodiesterase 3B, cGMP-inhibited	214582_at	nc	nc	-1.62
		222330_at	-2.12	nc	-1.80
PDE4DIP	phosphodiesterase 4D interacting	236704_at	-2.37	nc	nc
		212390_at	-4.48	nc	nc
PDE7A	phosphodiesterase 7A	1552343_s_at	nc	-2.59	nc
		224046_s_at	nc	nc	-1.57
PDE9A	phosphodiesterase 9A	205593_s_at	-2.06	nc	nc
PIM1	pim-1 oncogene	209193_at	-4.32	nc	nc
PIM2	pim-2 oncogene	204269_at	nc	nc	2.22
PMAIP1	PMA-induced protein 1	204285_s_at	nc	nc	2.25
PPIB	peptidylprolyl isomerase B (cyclophilin B)	200968_s_at	nc	nc	5.69
PPIF	peptidylprolyl isomerase F (cyclophilin F)	201490_s_at	1.91	nc	nc
PPIL1	peptidylprolyl isomerase, cyclophilin-like 1	222500_at	nc	nc	1.66
PRDM1	PR domain containing 1, with ZNF domain	228964_at	nc	nc	1.72
PTGER2	prostaglandin E receptor 2 (subtype EP2)	206631_at	nc	2.07	1.98
PTGER4	prostaglandin E receptor 4 (subtype EP4)	204897_at	nc	nc	4.05
RGS1	regulator of G-protein signalling 1	202988_s_at	2.37	nc	nc
RGS10	regulator of G-protein signalling 10	204319_s_at	4.21	nc	nc
RGS10	regulator of G-protein signalling 10	204316_at	-2.81	1.40	nc
RGS16	regulator of G-protein signalling 16	209324_s_at	-2.02	nc	nc
RUNX1	runt-related transcription factor 1	209360_s_at	nc	nc	2.29
RUNX2	runt-related transcription factor 2	232231_at	nc	nc	1.35
SNFT	Jun dimerization protein p21SNFT	220358_at	2.41	1.86	nc
SOCS1	suppressor of cytokine signaling 1	210001_s_at	nc	nc	2.28
SOCS2	suppressor of cytokine signaling 2	203373_at	nc	nc	5.15
SOCS3	suppressor of cytokine signaling 3	227697_at	nc	nc	4.44
SORL1	sortilin-related receptor, L(DLR class)	230707_at	2.07	nc	4.49
SOS1	son of sevenless homolog 1	212780_at	2.97	1.45	-1.85
SOX4	SRY (sex determining region Y)-box 4	201417_at	-9.01	nc	1.65
SOX6	SRY (sex determining region Y)-box 6	235526_at	nc	1.59	nc
STAT1	signal transducer & act. transcription 1	AFFX-HUMISG	-1.67	nc	4.44
		209969_s_at	nc	nc	6.74
STAT3	signal transducer & act. transcription 3	208992_s_at	nc	nc	1.61
TFRC	transferrin receptor (CD71)	208691_at	1.89	nc	1.84
TNF	TNF superfamily, member 2	207113_s_at	nc	nc	5.16
TNFAIP3	TNF, alpha-induced protein 3	202644_s_at	nc	nc	-1.58
TNFRSF4	TNF receptor superfamily, member 4	214228_x_at	nc	1.65	3.30
TNFRSF10B	TNF receptor superfamily, member 10b	209295_at	-1.71	nc	nc
TNFRSF10D	TNF receptor superfamily, member 10d	227345_at	-3.39	nc	nc
TNFRSF11A	TNF, member 11a, NFKB activator	238846_at	7.45	nc	nc
TNFSF4	TNF (ligand) superfamily, member 4	207426_s_at	nc	2.77	nc
TNFSF8	TNF (ligand) superfamily, member 8	235735_at	nc	nc	2.30
TNFSF10	TNF (ligand) superfamily, member 10	214329_x_at	2.92	nc	1.63
		202688_at	3.47	nc	nc
TNFSF11	TNF (ligand) superfamily, member 11	210643_at	10.35	nc	nc
TNFSF13B	TNF (ligand) superfamily, member 13b	223502_s_at	-9.87	nc	nc
TNFSF14	TNF (ligand) superfamily, member 14	207907_at	3.72	nc	nc
TNIK	TRAF2 and NCK interacting kinase	211828_s_at	2.05	nc	-1.62
TNIK	TRAF2 and NCK interacting kinase	213107_at	3.06	nc	nc

[a]One probe (greatest fold change) is shown for each gene, except where differences were detected in individual probes to the same gene.

[b]Fold change comparing the mean expression of duplicate arrays from P1, P2, and P3 nonstimulated with the mean expression from 4 controls; data are from supplemental Table 2.

[c]Fold changes detected in the mean expression from triplicate arrays of P1 nonstimulated CD3$^-$CD4$^+$ T cells from yr +6 compared with yr 0; data are from supplemental Table 3.

[d]Fold change comparing the mean expression of duplicate arrays from stimulated (S) vs nonstimulated (NS) CD3$^-$CD4$^+$ T cells from P1 to P3; data are from supplemental Table 2.

[e]Genes whose expression is similarly altered both after activation and in L-HES patient cells (either chronic disease or T lymphoma) are highlighted in gray.

[f]NC indicates no change.

[g]In P1, CCL5 levels are decreased yr 0 vs C and yr +4 vs yr 0 but then increase in yr +6 vs yr 4 (supplemental Table 3).

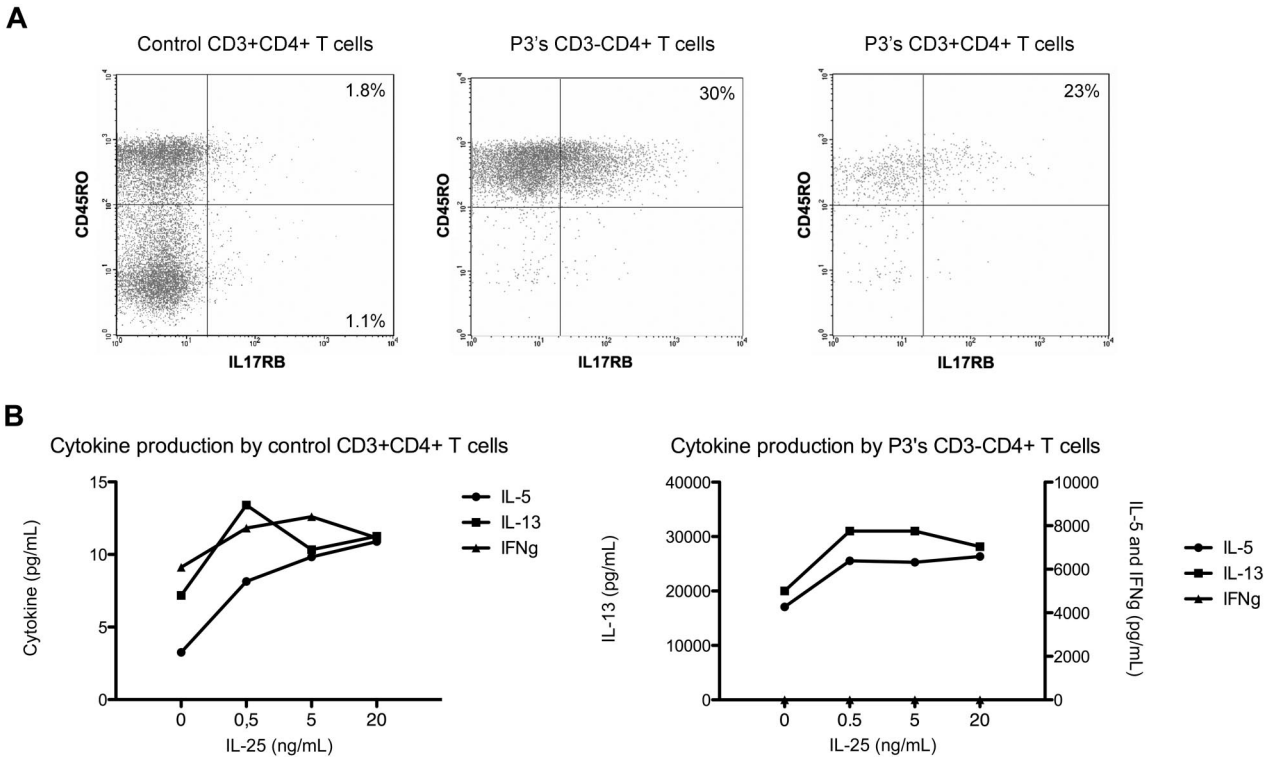

Figure 3. IL-17RB (IL-25 receptor) and cytokine expression by L-HES CD3⁻CD4⁺ T cells. (A) Four-color immunofluorescent labeling of control and P3's PBMCs. The lymphocyte populations were gated on CD4 and CD3 positivity/negativity. (B) Purified CD3⁻CD4⁺ T cells from P3 were cultured for 48 hours with phorbol ester and anti-CD28 in the absence or presence of increasing concentrations of rhIL-25, and cytokine concentrations were determined using BD Cytometric Bead Array Flex Sets. A representative experiment is shown.

Table 4). CRTH2 is involved in Th2 cell migration, GATA3 up-regulation, and induction of Th2 cytokine production.[30] Our experiments found that GATA3 nuclear binding is up-regulated in patients CD3⁻CD4⁺ T cells only after CD2/CD28 costimulation[31]; however, the arrays detected a 3.6-fold increase in *GATA3* transcripts in the patient's quiescent CD3⁻CD4⁺ T cells, which contrasts with the reported lack of differences in a microarray study of quiescent human Th1 and Th2 cells.[32] Taken together, our data suggest that significant levels of GATA3 may be present in the cytoplasm waiting for activation signals that rapidly induce phosphorylation and nuclear translocation of this Th2 transcription factor, followed by cytokine gene up-regulation. The gene expression profiles of unstimulated versus CD2/CD28 costimulated CD3⁻CD4⁺ T cells clearly show that their Th2 cytokine expression is dependent on activation (Table 2). Together with our previous studies,[12] these data suggest that, despite high CRTH2 and GATA3 expression levels, activating signals from local microenvironments in vivo are required to bring the circulating cells out of standby.

Apoptosis. The clonal CD3⁻CD4⁺ T cells persist at relatively stable levels for many years in vivo, suggesting equilibrium between cell proliferation and apoptosis during chronic disease. The death domain containing tumor necrosis factor (TNF) superfamily plays critical roles in controlling the induction and progression of cell death, and the microarrays revealed altered expression of several TNF family member genes in the CD3⁻CD4⁺ T cells, including both proapoptotic genes and antiapoptotic (pro-proliferation) genes (Figure 2, supplemental Table 7); some were confirmed by quantitative RT-PCR (Figure 4A-B). Increased surface expression of FAS and a lack of CD27 in L-HES have been previously described[2,10] and were reconfirmed in this cohort (supplemental Table 4). RANKL (*TNFSF11*; 10-fold increase) augments the costimulatory properties of antigen-presenting cells and thus could be important for CD3⁻CD4⁺ T cell activation in vivo. A 10-fold increase in RANKL mRNA was also detected in microarrays of CD4⁺ T cells from patients with Sezary syndrome.[33] Interestingly, there was no further increase in RANKL expression associated with P1's progression to lymphoma or on costimulation. Further investigation into the functional implications of increased RANKL expression in L-HES is warranted given the ongoing development of humanized monoclonal antibodies for clinical use. The altered expression in specific subsets of genes involved in programmed cell death observed in this study suggests that there is a controlled balance that potentiates the increased survival and persistent expansion of the CD3⁻CD4⁺ T-cell clone.

T-cell homeostasis and the TGF-β family. Altered expression among the TGF-β superfamily (TGF-β, activins, inhibins, growth differentiation factors, and bone morphogenetic proteins [BMP]) has been described for a variety of epithelial-derived solid tumors and hematologic malignancies.[34] A microarray study revealed that TGF-β is the major signaling pathway that constitutively keeps human CD4⁺ T cells in a resting state.[35] In this study, we detected numerous changes in TGF-β family gene expression in the L-HES CD3⁻CD4⁺ T cells during chronic disease, with a subset of these genes changing further during P1's evolution to T lymphoma (Figure 5, supplemental Table 7); in contrast, no additional changes were observed in the patients' abnormal T cells after CD2/CD28 costimulation. Decreased expression of the type I TGF-β receptor genes, TGF-βRI (*TGFBR1*) and *ACVRIC*, and the type II receptor gene TGF-βRII (*TGFBR2*) in the CD3⁻CD4⁺ T cells was confirmed by quantitative RT-PCR (Figure 4A). A previous study of CD4⁺ T-cell lines derived from T lymphoma patients found that decreased TGF-βRI and TGF-βRII expression was related to reduced responsiveness to TGF-β1-mediated growth inhibition,[36]

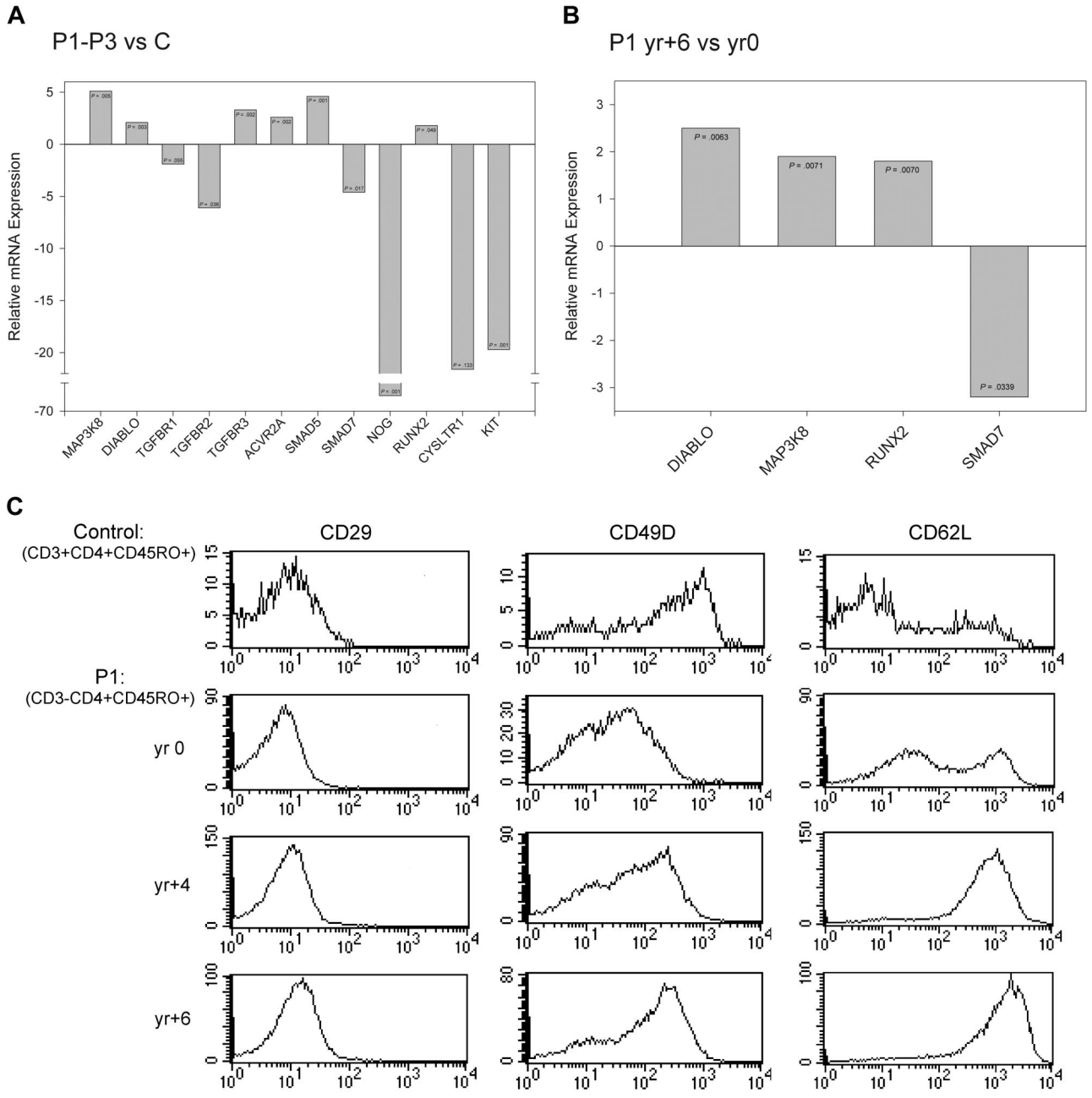

Figure 4. Validation of changes in gene expression using quantitative RT-PCR and flow cytometry. (A-B) Fold change in the expression of selected genes measured by quantitative RT-PCR for (A) patients (P1-P3) relative to controls[4] and (B) P1-yr +6 relative to P1-yr 0. *P* values were calculated based on 3 independent experiments using the Student *t* test and are indicated in the corresponding bar. (C) Histograms showing the surface expression of CD29 (*ITGB1*), CD49D (*ITGA4*), and CD62L (*SELL*) on control $CD3^+CD4^+CD45RO^+$ T cells and P1-yr 0, P1-yr +4, and P1-yr +6 $CD3^-CD4^+CD45RO^+$ T cells. Isotype controls (not shown) for each sample were set between 10^0 and 10^1.

whereas microarrays of Sezary T cells detected *TGFBR2* gene down-regulation.[33]

Studies have shown that a third TGF-β receptor, TGF-βRIII (*TFGBR3*; betaglycan) is frequently down-regulated in solid tumors[34] in contrast to B chronic lymphocytic leukemia where its up-regulation has been reported.[37] We detected increased *TGFBR3* in the $CD3^-CD4^+$ T cells from patients with chronic disease. One study reported that corticosteroids can selectively stimulate TGF-βRIII expression in hepatic stellate cells[38]; and although corticosteroids are standard therapy for symptomatic L-HES patients, this treatment is probably not responsible for the *TGFBR3* up-regulation observed because the fold changes for P2 and P3 (treated; supplemental Table 1) were lower than P1 (untreated).

TGF-βRIII binds all TGF-β isoforms and presents them to TGF-βRII, thereby initiating the recruitment and phosphorylation of TGF-βRI that leads to kinase activation. However, evidence indicates that TGF-βRIII also functions independently from TGF-β ligand presentation by working as a coreceptor with type 2 activin receptors (*ACVR2A* and *ACVR2B*, both genes increased in the $CD3^-CD4^+$ T cells). Activins and inhibins are structurally related members of the TGF-β superfamily that act as antagonists, with the former providing positive and the latter negative intracellular signals. High affinity binding of inhibin by TGF-βRIII is favored in cells coexpressing ACVR2A, thereby inhibiting the activin pathway. We also detected an increase in the BMP type I receptor gene, *BMPRIA*, which can interact with ACVR2A to bind BMPs. Finally,

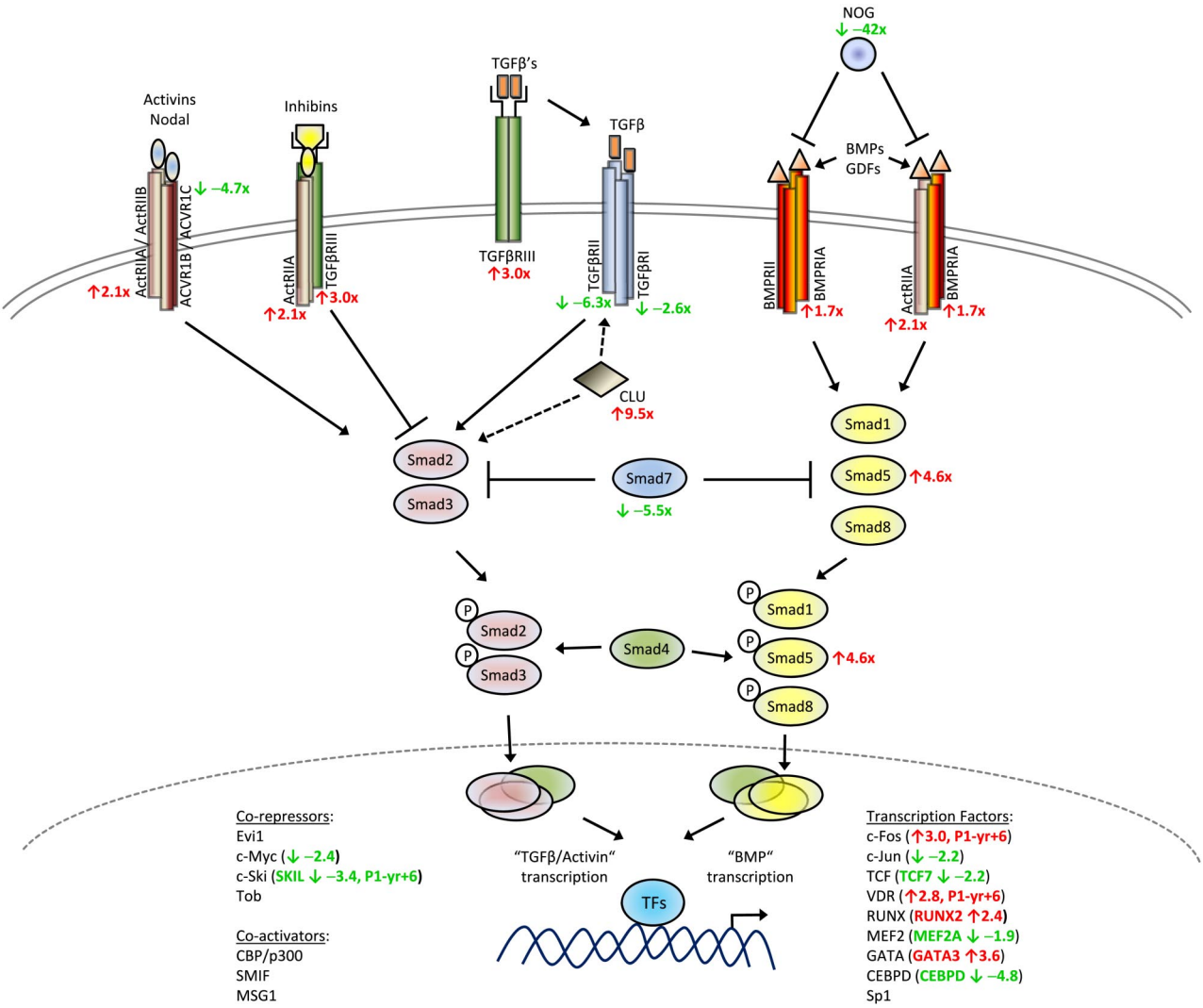

Figure 5. Schematic diagram of TGF-β signaling pathway. Altered expression in several TGF-β superfamily signaling genes was detected in L-HES patients CD3⁻CD4⁺ T cells. The mean fold changes detected in the microarrays for P1 to P3 relative to the controls are indicated in red (up-regulated genes) and green (down-regulated genes).

the noggin gene (*NOG*) was substantially decreased in the abnormal T cells and acts as an antagonist for the TGF-β superfamily members, BMP2 and BMP4, both of which play a role in early thymocyte differentiation.[39] Altogether, these alterations in gene expression reflect a shift in the balance of TGF-β superfamily–dependent intracellular pathways in the CD3⁻CD4⁺ T cells, with uncontrolled signaling via the BMP pathway possibly disrupting normal homeostasis and favoring abnormal cell survival and growth.

This hypothesis is further substantiated by altered expression of Smad proteins, which transmit signals downstream from the TGF-β superfamily receptors. We observed increased receptor regulated *SMAD5* gene expression together with decreases in the inhibitory *SMAD7* gene, both confirmed by quantitative RT-PCR. Although their function in hematopoietic cells is not as well defined as Smad2, Smad3, and Smad4, Smad5 is involved in regulating BMP signaling whereas Smad7 negatively regulates receptor regulated Smad signaling and has been implicated in mature hematopoietic cell development.[40] Receptor regulated Smad proteins specific for the BMP pathway, such as Smad5, interact with a variety of proteins, including Runx family transcription factors. The Runx genes have been shown to function as tumor suppressors in human cancer, although their overexpression in murine models revealed an oncogenic role in the development of hematopoietic tumors, including T lymphomas.[41] Runx2 mediates cellular responses to signaling pathways hyperactive in tumors, including TGF-β family pathways, by forming coregulatory complexes with Smads and other coactivator and corepressor proteins to regulate gene transcription. Runx2, better known for its role in bone development and maintenance, was up-regulated in all patients and then again during P1's evolution to T lymphoma (Table 1). *RUNX2* and RANKL (*TNFSF11*) are targets of transcriptional regulation by the vitamin D receptor (*VDR*; up-regulated in P1 yr +6) and were up-regulated in the CD3⁻CD4⁺ T cells from chronic disease. In addition, several target genes known to be induced by TGF-β were also decreased, including *JUN, MYB, FLT3LG*, and *CXCR4* (the latter confirmed by flow cytometry). The clusterin gene (*CLU*), which interacts with TGF-βRII to modulate Smad signaling,[42] was also significantly up-regulated in the abnormal T cells. Further investigation into the perturbations detected in the TGF-β superfamily signaling pathways and the role they play in the persistence of the CD3⁻CD4⁺ T-cell clone in L-HES are ongoing.

Gene expression changes in the CD3$^-$CD4$^+$ T cells associated with the evolution from chronic L-HES to T lymphoma

Cryopreserved blood samples from P1 spanning her 6-year progression from chronic disease to T lymphoma provided a very rare opportunity to assess changes in gene expression associated with malignant transformation in vivo. Our previous studies found that over time the initial CD3$^-$CD4$^+$ T-cell clone spawned subclones containing 2 independent 6q deletions (6q11-6q23.1 and 6q13-6q22.1) with progressive outgrowth of the 6q13q22.1-deleted subclone detected in 91% of the malignant T cells.[2,8,15] Gene expression profiles of P1's CD3$^-$CD4$^+$ T cells at diagnosis (yr 0), during chronic disease (yr +4), and with T lymphoma (yr +6) revealed 349 genes (450 probe sets) that were differentially expressed in the malignant compared with the premalignant T cells (Table 3 and supplemental Table 3). Remarkably, approximately one-third of the probes (126 of 450), corresponding to 87 of 349 genes, were also initially altered in patients with chronic L-HES (Table 1), with the majority of these genes displaying stepwise alterations in expression (decreases or increases), first in all patients with chronic disease and then with P1's T lymphoma development. Progressive decreases were observed in several apoptosis genes, growth factors, and transcription factors along with progressive increases in surface receptors, signaling proteins, as well as additional growth factors and transcription factors. Among the genes whose expression changed progressively from chronic to malignant L-HES, only 6 genes (*BCAT1, HLA-DQA1/2, HLA-DQB1, HLA-DRA, IL17RB*, and *SOS1*) also increased and only the *FAIM3* gene decreased after in vitro activation (Table 2). These data suggest that the genes whose expression is altered in the abnormal T cells from chronic and malignant L-HES reflect genuine changes in CD3$^-$CD4$^+$ T-cell physiology and not a transient response to external stimuli, such as signaling molecules, surface receptors, and cytokine production.

Genes with potential relevance to malignant transformation include progressive increases in surface receptors, growth factors, transcription factors, and signaling proteins, as shown in Table 3. The limited number of genes whose expression decreases in the T lymphoma cells is distinguished by signaling proteins and transcription factors. One potentially relevant gene is the IL-9 receptor (*IL9R*), which was up-regulated on the CD3$^-$CD4$^+$ T cells from chronic L-HES; however, in contrast to its increased expression in mice overexpressing IL-9 that develop thymomas[43] and some Hodgkin lymphomas, we did not observe further up-regulation on P1's T lymphoma cells. Overall, these alterations reflect progressive activation, altered signaling, and/or homing of the CD3$^-$CD4$^+$ T cells to specific sites and/or their adaptation to a specific microenvironment. The stepwise-modulated genes as well as those newly deregulated in the malignant T cells are of particular interest and relevance as potential therapeutic targets and new diagnostic markers.

T-cell trafficking and migration. Leukocyte migration is mediated by a network of trafficking receptors expressed both on lymphoid and nonlymphoid tissues such that specific combinations of these adhesion and chemoattractant molecules act as traffic signals for directing extravasion and migration.[44] Trafficking genes play distinct roles as leukocytes migrate through blood vessels. The initial step is mediated by selectins; and although we did not detect statistically significant changes in *SELL* (L-selectin; CD62L) gene expression, flow cytometry revealed surface receptor up-regulation on the CD3$^-$CD4$^+$ T cells, which continued to increase as P1 progressed to T lymphoma (Figure 4C). Rolling over endothelial cells exposes leukocytes to chemokines, which in turn provoke conformational changes in integrins that increase their affinity. The $\alpha4\beta1$ integrin (VLA-4) is composed of 2 subunits, CD49D ($\alpha4$; *ITGA4*) and CD29 ($\beta1$; *ITGB1*), both required for VLA-4 surface expression. Down-regulation of CD49D in association with a slight increase in CD29 was observed in the CD3$^-$CD4$^+$ T cells during chronic disease (Figures 2,4C and supplemental Tables 2,4). CD49D was reexpressed in concert with increased VLA-4 surface expression as P1 developed enlarged lymph nodes and progressed to T lymphoma (Table 1, Figure 4C).

Changes in other trafficking receptor genes were detected both in chronic disease and during the evolution to T lymphoma. Down-regulation of *CXCR4, CXCR6, CCR6*, and *CCR7* was detected in patients during chronic disease with some *CCR7* expression returning as P1 progressed to T lymphoma (Tables 1, 3). Increases in *CCR3, ICAM3, LFA-3, CD82*, and *CD99* were observed in all patients with up-regulation of *CCR2* in P1-yr +4 and *CCR5, CCR10, CD96*, and *PECAM1* in P1-yr +6. *CCR8* expression levels increased stepwise, 10-fold in chronic patients and a further 1.5-fold in P1-yr +6. CCR8 binds CCL1, which like CCL17 can be induced in bronchial epithelial cells by the Th2 cytokines IL-4 and IL-13.[45] Although we have previously shown that serum CCL17 levels are extremely high in patients with L-HES,[16] serum CCL1 levels and the functional role of CCR8 on CD3$^-$CD4$^+$ T cells remain unknown. The altered expression of trafficking receptors and ligands observed on the CD3$^-$CD4$^+$ T cells probably directs their movement to specific sites during premalignant and malignant L-HES disease,[46] exposing the cells to external activation signals and/or costimulatory cells present locally.

Differential microRNA expression in the CD3$^-$CD4$^+$ T cells

MicroRNAs are endogenously expressed noncoding RNAs that regulate gene expression via mRNA degradation, mRNA destabilization, or translation inhibition. There is growing evidence that deregulated microRNA expression contributes to oncogenesis with an increasing number of identified microRNAs targeting genes involved in immune development, proliferation, and apoptosis.[47] We extended the molecular profile of L-HES using quantitative RT-PCR to quantify changes in mature microRNA expression. Initially, we compared the expression of 156 microRNAs in CD3$^-$CD4$^+$ T cells from P1-yr.6 with control CD3$^+$CD4$^+$ T cells (supplemental Table 6). Thirty-eight microRNAs that decreased or increased greater than 2-fold in 2 independent experiments were selected for further analysis in CD3$^-$CD4$^+$ T cells from 6 chronic L-HES patients (P1-P5, P7) and CD3$^+$CD4$^+$ T cells from the same 4 controls. Using the nonpaired Student *t* test, we identified 23 microRNAs that were differentially expressed in the abnormal T cells (Table 4). The majority (19 of 23) of the selected microRNAs were down-regulated with increases found for only 4 microRNAs.

One effect of the interaction between a microRNA and its target mRNA can be transcript cleavage and degradation. We searched for a correlation between global changes in mRNA and microRNA expression in the CD3$^-$CD4$^+$ T cells but did not observe a statistically significant correlation. We then used Ingenuity Pathways Analysis to assess the potential biologic importance of the predicted target genes as a group and determined that the best scored functional networks included the cell cycle, cell death, and hematologic system development and function. Individual microRNAs and their putative gene targets were generated using MirBase and included some of notable interest and potential relevance. The expression of 3 Th2 genes in the CD3$^-$CD4$^+$ T cells inversely paralleled several microRNAs predicted to target them, including increases in

Table 3. Selected gene expression changes in the CD3⁻CD4⁺ T cells during P1's evolution from chronic L-HES to lymphoma

Probe set	Symbol	Name	P1 yr0 vs C[d]	P1 yr+4 vs yr0[c]	P1 yr+6 vs yr+4[b]	P1 yr+6 vs yr0[a,e]
236796_at	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	-4.31			-1.78
203685_at	BCL2	B-cell CLL/lymphoma 2				-1.64
232210_at			-3.65			
201849_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-3.10			-2.27
204655_at	CCL5	chemokine (C-C motif) ligand 5 (RANTES)	-33.99	-2.09	3.65	
206978_at	CCR2	chemokine (C-C motif) receptor 2 (CD192)		2.69		2.15
208304_at	CCR3	chemokine (C-C motif) receptor 3 (CD193)	3.70	3.09		
206991_s_at	CCR5	chemokine (C-C motif) receptor 5 (CD195)		2.67		1.80
206337_at	CCR7	chemokine (C-C motif) receptor 7 (CD197)	-13.03		1.91	3.13
208059_at	CCR8	chemokine (C-C motif) receptor 8 (CDw198)	9.75			1.50
220565_at	CCR10	chemokine (C-C motif) receptor 10				2.81
1555120_at	CD96	CD96 molecule				1.62
1556209_at	CLEC2B	C-type lectin domain family 2, member B		-1.49		-1.59
209732_at			-1.59			
207630_s_at	CREM	cAMP responsive element modulator		1.50		1.99
210140_at	CST7	cystatin F (leukocystatin)	-4.44		2.99	3.61
244413_at	DCAL1	CLECL1, dendritic cell-associated lectin-1	10.34			1.56
221601_s_at	FAIM3	Fas apoptotic inhibitory molecule 3	-3.68			-1.62
224840_at	FKBP5	FK506 binding protein 5				1.60
224856_at				1.33		
225262_at	FOSL2	FOS-like antigen 2			2.52	2.95
229055_at	GPR68	G protein-coupled receptor 68	2.88			1.50
205488_at	GZMA	granzyme A (granzyme 1, CTL-associated serine esterase 3)	-6.65		2.20	2.40
226878_at	HLA-DOA	major histocompatibility complex, class II, DO alpha				1.86
203290_at	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	5.73	1.84	1.81	3.33
212998_x_at	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	6.02		1.56	2.49
208894_at	HLA-DRA	major histocompatibility complex, class II, DR alpha	2.41	2.08		3.01
215193_x_at	HLA-DRB1	major histocompatibility complex, class II, DR beta 1	9.74	1.35		1.64
221491_x_at	HLA-DRB1,3,4,5	major histocompatibility complex, class II, DR beta	9.47			1.77
222396_at	HN1	hematological and neurological expressed 1	1.62			1.36
1554618_at	HRBL	HIV-1 Rev binding protein-like	3.16			-2.23
201601_x_at	IFITM1	interferon induced transmembrane protein 1 (9-27)	-1.88		1.32	1.57
201315_x_at	IFITM2	interferon induced transmembrane protein 2 (1-8D)				1.59
210095_s_at	IGFBP3	insulin-like growth factor binding protein 3				-2.44
201508_at	IGFBP4	insulin-like growth factor binding protein 4			2.92	3.45
219255_x_at	IL17RB	interleukin 17 receptor B	30.68	1.42		1.55
203828_s_at	IL32	interleukin 32	-2.02		2.19	2.50
213416_at	ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-7.38		1.85	2.08
1554306_at	ITPKB	inositol 1,4,5-trisphosphate 3-kinase B				-2.15
203542_s_at	KLF9	Kruppel-like factor 9			-2.12	-2.66
203543_s_at			-2.72			-2.12
201105_at	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)			1.64	2.40
208949_s_at	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	-19.96		2.93	
35974_at	LRMP	lymphoid-restricted membrane protein	-3.23		1.62	1.81
206584_at	LY96	lymphocyte antigen 96	1.70			1.52
206363_at	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	-2.04	1.68	2.06	3.45
218918_at	MAN1C1	mannosidase, alpha, class 1C, member 1	-2.09		-2.16	-1.66
216765_at	MAP2K5	Mitogen-activated protein kinase kinase 5	2.06			-1.75
227357_at	MAP3K7IP3	mitogen-activated protein kinase kinase kinase 7 interacting protein 3				1.45
235421_at	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	3.49	1.42		1.93
212022_s_at	MKI67	antigen identified by monoclonal antibody Ki-67		1.99		2.37
209928_s_at	MSC	musculin (activated B-cell factor-1)	2.60			1.95
202364_at	MXI1	MAX interactor 1				-1.60
203413_at	NELL2	NEL-like 2 (chicken)	-4.22	-2.40	-2.54	-6.10
224975_at	NFIA	nuclear factor I/A				1.88
203574_at	NFIL3	nuclear factor, interleukin 3 regulated			2.19	2.73
213028_at	NFRKB	nuclear factor related to kappaB binding protein	-1.34			1.34
204088_at	P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	1.56		1.43	1.64
217738_at	PBEF1	pre-B-cell colony enhancing factor 1	1.82	1.53		1.88
212390_at	PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin)				-2.59
236704_at			-2.62			
208983_s_at	PECAM1	platelet/endothelial cell adhesion molecule (CD31)				2.00
202014_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A		-1.33	1.61	
37028_at			1.64			
1562467_at	PPP3CA	Protein phosphatase 3, catalytic subunit, alpha isoform (calcineurin A alpha)	2.94			-3.53
228964_at	PRDM1	PR domain containing 1, with ZNF domain	1.92		1.49	2.07
204061_at	PRKX	protein kinase, X-linked				1.61
233314_at	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)				4.61
244050_at	PTPLAD2	protein tyrosine phosphatase-like A domain containing 2	1.97			1.34
204201_s_at	PTPN13	protein tyrosine phosphatase, non-receptor type 13 (CD95 (Fas)-associated)	-3.64		1.57	
203029_s_at	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	7.50	1.51	1.45	2.20
206039_at	RAB33A	RAB33A, member RAS oncogene family				1.62
228113_at	RAB37	RAB37, member RAS oncogene family				1.54
215070_x_at	RABGAP1	RAB GTPase activating protein 1				-1.71
203344_s_at	RBBP8	retinoblastoma binding protein 8	2.41			1.73
202988_s_at	RGS1	regulator of G-protein signalling 1	7.13		-1.49	
204319_s_at	RGS10	regulator of G-protein signalling 10	-2.31			1.40
232231_at	RUNX2	runt-related transcription factor 2				1.86
204351_at	S100P	S100 calcium binding protein P			1.73	1.91
217591_at	SKIL	SKI-like			-2.88	-3.37
212780_at	SOS1	son of sevenless homolog 1 (Drosophila)	2.38		1.42	1.45
235526_at	SOX6	SRY (sex determining region Y)-box 6				1.59
213994_s_at	SPON1	spondin 1, extracellular matrix protein	-3.59		-2.07	-2.57
241860_at	STK17B	Serine/threonine kinase 17b (apoptosis-inducing)				-1.64
234050_at	TAGAP	T-cell activation GTPase activating protein				-1.46
208944_at	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	-3.08		-1.59	
210166_at	TLR5	toll-like receptor 5		1.65	1.94	3.21
214228_x_at	TNFRSF4	tumor necrosis factor receptor superfamily, member 4 (CD134, OX40)				1.65
207426_s_at	TNFSF4	tumor necrosis factor ligand superfamily, member 4 (CD252, OX40L)			2.79	2.77
204529_s_at	TOX	thymus high mobility group box protein TOX				2.20
224412_s_at	TRPM6	transient receptor potential cation channel, subfamily M, member 6		3.53	7.09	25.05
204254_s_at	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor			2.71	2.74

[a]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr 0 compared with 4 controls; data are from supplemental Table 3. Progressively up-regulated genes are highlighted in dark gray and progressively down-regulated genes are highlighted in light gray.

[b]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +4 compared with yr 0; data are from supplemental Table 3.

[c]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +6 compared with yr +4; data are from supplemental Table 3.

[d]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +6 compared with yr 0; data are from supplemental Table 3.

Table 4. microRNAs that are differentially expressed in the CD3⁻CD4⁺ T cells from L-HES patients compared with CD3⁺CD4⁺ T cells from controls

	Patients vs controls		
miRNA name	*P**	Fold change†	Chromosomal location‡
hsa-let-7b	.032	3.2	22q13.31
hsa-miR-26a	.019	−2.3	3p22.3
hsa-miR-31	.004	−111.4	9p21.3
hsa-miR-95	.025	−2.6	4p16.1
hsa-miR-99a	.011	−60.9	21q21.1
hsa-miR-100	.010	−57.6	11q24.1
hsa-miR-126	.030	−9.1	9q34.3
hsa-miR-130a	.034	−6.2	11q12.1
hsa-miR-135b	.011	−11.8	1q32.1
hsa-miR-135a	.008	−10.9	3p21.1
hsa-miR-151	.019	−12.1	8q24.3
hsa-miR-181a	.010	−34.6	1q31.3
hsa-miR-181b	.010	−19.3	1q31.3
hsa-miR-193a	.017	−4.6	17q11.2
hsa-miR-213	.011	−78.8	1q31.3
hsa-miR-215	.019	−3.1	1q41
hsa-miR-221	.010	3.4	Xp11.3
hsa-miR-222	.010	3.7	Xp11.3
hsa-miR-335	.010	−8.0	7q32.2
hsa-miR-340	.019	−4.9	5q35.3

**P* values were corrected using the false discovery rate calculation.

†Fold change in the L-HES patients' CD3⁻CD4⁺ T cells (P1-P5 + P7) relative to controls (4).

‡Chromosomal locations were obtained from Ensembl.

GATA3 with decreases in miR-10a, miR-95, and miR-130a, *IL4R* increases in concert with decreased miR-126 and miR-340, and increased *CCR3* in parallel with decreased miR-181a, miR-181b, and miR-335. Genes whose mRNA expression changed in the abnormal T cells that were also predicted targets of 2 or 3 altered microRNAs included: *IL18RAP* (let7b, miR-221), *CD99* (miR-31, miR-95, miR-135a), *TRADD* (miR-31, miR-125a), *CD58* (miR-95, miR-135b), *PPP3CA* (miR-99a, miR-100), RANKL (*TNFSF11;* miR-126, miR-335), *DMN3* (miR-126, miR-151), *RGS1* (miR-130a, miR-335), and *PRMT2* (miR-221, miR222).

Perhaps of greatest potential biologic significance were 3 genes whose expression increased in patients with chronic disease (*RBBP8, CLU*, and *MAP3K8*) with further increases associated with P1's evolution to T lymphoma (*RBBP8* and *MAP3K8*) that were also predicted targets of 4 different down-regulated microRNAs. The retinoblastoma binding protein 8 gene (*RBBP8*), a predicted target of miR-31, miR-126, miR-130a, and miR-335, is thought to function as a tumor suppressor in conjunction with the transcriptional corepressor CTBP and BRCA1. Clusterin (*CLU*) is a calcium regulated protein whose expression has been associated with tumorigenesis and malignant progression, perhaps in part by modulating TGF-βRII signaling (Figure 5). The nuclear form is proapoptotic and the secretory form is antiapoptotic,[48] with both forms involved in DNA repair and cell cycle regulation. Clusterin expression was significantly up-regulated in the CD3⁻CD4⁺ T cells in concert with the down-regulation of miR-99a, miR-100, miR-126, and miR-335. Thus, miR-126 and miR-335 potentially target both *RBBP8* and *CLU*. A recent study of breast cancer found that miR-126 expression reduced tumor growth, whereas miR-335 suppressed lung and bone metastasis,[49] with miR-335 loss leading to *SOX4* and tenascin C (*TNC*) activation, which are both implicated in the acquisition of metastatic properties. We detected a 9-fold decrease in *SOX4* (no change in *TNC*), which paralleled a 9-fold decrease in miR-335, suggesting that this microRNA targets other critical genes in T cells.

Many of the gene changes detected in patients relative to controls and then during P1's evolution to T lymphoma are involved in cell signaling. The *MAP3K8* (Tpl2/Cot) oncogene expression increased stepwise first in patients during the chronic disease phase and again during P1's evolution to T lymphoma. The *MAP3K8* gene is a predicted target of miR-135a, miR-135b, miR-181a, and miR-181b, which were all decreased in the CD3⁻CD4⁺ T cells. Studies have shown that decreased miR-181b expression in B chronic lymphocytic leukemia patients is associated with up-regulation of the TCL1 oncogene.[50] The *MAP3K8* gene is of particular interest because studies have shown that it is differentially regulated in hematopoietic cells and plays a role in tumor development. Overexpression and truncation of MAP3K8 lead to the activation of several T-cell signaling pathways and have been associated with large granular lymphocyte proliferative disorders.[51] miR-181a also positively modulates TCR/CD3 sensitivity and affinity by suppressing phosphatases involved in negatively regulating TCR/CD3 signaling.[52] The miR-181 family is involved in controlling hematopoietic cell differentiation and maturation with miR-181a levels fluctuating during thymopoiesis and its repression shown to diminish T-cell sensitivity in both primed and stimulated naive T cells.[53] MiR-181a has also been shown to inhibit *CD69*, *BCL2*, and TCRα gene transcription.[52] Intriguingly, our data revealed a substantial decrease in miR-181a and miR-181b associated with low CD69, *BCL2*, and TCR/CD3 expression levels in the CD3⁻CD4⁺ T cells, suggesting that the complex interactions between the TCR/CD3 signaling genes and the miR-181 family require further analysis. Experiments designed to approach the functional relevance of decreased expression of miR-135 family members, about which little is known, were accomplished by transfecting miR-135a and miR-135b mimics together in the CD4⁺ Jurkat T-cell line. These preliminary data indicate that the miR-135 mimics decrease *MAP3K8* (−2.6-fold) and *SMAD5* (−2.3-fold) expression compared with irrelevant sequence controls (data not shown).

Changes in microRNA expression during P1's clinical evolution

We also quantified expression of the same 38 microRNAs in association with P1's evolution to T lymphoma and found that only miR-125a changed significantly. miR-125a levels were 5.7-fold lower ($P = .059$) in the L-HES patient cohort relative to controls; and as P1 evolved to T lymphoma, miR-125a expression progressively decreased with an additional 2.8-fold drop ($P = .003$) detected at yr +6. Predicted gene targets of miR-125a were generated using MirBase and compared with the mRNA expression profiles of P1's CD3⁻CD4⁺ T cells (supplemental Table 6). The up-regulated target genes included another gene involved in signaling, *PTPRN2*, which is a member of the receptor-type protein tyrosine phosphatase family. *PTPRN2* expression increased in parallel with the progressive decrease of miR-125a expression in the CD3⁻CD4⁺ T cells from chronic and malignant disease. *PTPRN2* (IA-2β) is a pancreatic β-cell autoantigen for type 1 diabetes; and although its function is largely unknown, our studies suggest its role in L-HES warrants further investigation. A second miR-125a target gene, the Abelson helper integration site 1 (*AHI1*), was significantly up-regulated in the latter stages of P1's evolution to T lymphoma. AHI1 has been implicated in the development of T- and B-cell malignancies with increased expression detected in CD4⁺CD7⁻ T cells from Sezary syndrome patients.[54] Although the function of miR-125a remains unknown, its homolog miR-125b

has been shown to posttrancriptionally target TNF-α and decrease cell proliferation.[55] The stepwise down-regulation of miR-125a detected in L-HES disease suggests a potential role for this microRNA in the persistence and progressive transformation of the CD3⁻CD4⁺ T cells.

Discussion

L-HES associated with a clonal population of CD3⁻CD4⁺ Th2 cells is a rare benign lymphoproliferative disorder that can progress to malignant T lymphoma after a prolonged period of chronic disease. During the chronic phase, patients generally seek medical attention because of cutaneous symptoms, including severe eczema, angioedema, and urticaria. The Th2 nature of the underlying T-cell disorder is responsible for the marked hypereosinophilia, which also leads to frequent patient follow-up by physicians and treatment well before the development of malignancy. However, the mechanisms underlying clonal T-cell persistence and transformation remain unknown, precluding the development of a targeted therapy capable of eradicating the abnormal T cells during the chronic disease phase and thereby short-circuiting malignant transformation.[1,56] The abnormal T cells persist for many years in vivo during which their levels are frequently stable in conjunction with corticosteroid treatment. The CD3⁻CD4⁺ T cells may eventually become refractory to treatment in parallel with malignant progression, which currently leaves only allogeneic stem cell transplantation as possibly curative.[57]

Our earlier work identified a CD3⁻CD4⁺ T-cell population as the source of Th2 cytokines in a symptomatic L-HES patient.[17] We further demonstrated that the CD3⁻CD4⁺ T-cell population in patients was monoclonal based on its T-cell receptor rearrangement[2] and that loss of surface TCR/CD3 expression was the result of a defect in *CD3*γ gene transcripts.[15] We detected multiple chromosomal aberrations within the clonal T-cell population and found that evolution to malignancy in vivo was associated with the emergence of a subclone characterized by a specific 6q deletion in one patient.[8] The present global gene expression study was undertaken to identify, in an unbiased manner, the specific genes and cellular pathways involved in the complex interplay between persistence and control of the CD3⁻CD4⁺ T cells in chronic L-HES and during their progression to full-blown malignancy.

The microarray analysis of patients with chronic disease provides a detailed immunophenotype/genotype, confirming the Th2 nature of the abnormal T-cell clone and offering insight on activation pathways and their homing state. Comparison of gene expression profiles from patients CD3⁻CD4⁺ T cells during chronic L-HES versus CD3⁺CD4⁺ T cells from healthy controls, activated or not by CD2/CD28 costimulation, demonstrated that altered gene expression in the abnormal T-cell clone does not simply reflect an activated memory T-cell phenotype. In addition, these data confirm that other previously reported functional characteristics of the CD3⁻CD4⁺ T cells, such as Th2 cytokine production and altered surface receptor expression, occur on engagement of membrane costimulatory receptors. We further assessed the importance of increased IL-25 receptor (IL-17RB) expression on the CD3⁻CD4⁺ T cells given the expected significant in vivo exposure to eosinophil-derived IL-25 in L-HES patients. These data demonstrate that the CD3⁻CD4⁺ T-cell response to IL-25 is characterized by Th2 cytokine production and increased proliferation in vitro. Given the premalignant nature of the CD3⁻CD4⁺ T cells during chronic disease, our findings indicate that controlling eosinophil levels should be a therapeutic endpoint for these patients, even though their frequently isolated cutaneous manifestations may not appear to warrant systemic therapy. Taken together, our data suggest that the blood-derived CD3⁻CD4⁺ T cells are in a transient state of ingress and egress with tissue microenvironments where they receive the signals for aberrant cytokine production and expansion.

We also identified genes whose expression deviated from the normal pattern of checks and balances controlling T-cell signaling and survival and thereby maintaining homeostasis. One of the more intriguing findings is the apparent switch in TGF-β superfamily signaling from TGF-β/Activin-directed to BMP-directed gene expression (Figure 5). TGF-β has been extensively characterized for its immune suppressive functions and is known to play critical roles in controlling thymocyte development and limiting effector/memory T-cell responses. Activin A is produced by activated Th2 cells and plays a role in Th2-mediated responses of B cells and macrophages.[58] BMPs were initially identified for their growth factor effects on bone formation but have since been shown to regulate neurogenesis and hematopoiesis during embryonic development; and although little is known about BMP-mediated control of mature T-cell responses, BMPs have been shown to play a role in T-cell differentiation in the thymus.[39] Several studies have described aberrations in BMP signaling pathways in solid tumors, suggesting that survival and expansion of the CD3⁻CD4⁺ T-cell clone in L-HES could be in part the result of a switch from negative TGF-β regulation to positive BMP signaling, and the processes involved are currently under investigation.

The sequential analysis of P1's clinical evolution revealed that almost one-third of the genes whose expression changed in association with the development of T lymphoma were already abnormally expressed in L-HES patients during chronic disease. The majority of these genes were not altered in response to in vitro activation, further suggesting that they reflect inherent changes in CD3⁻CD4⁺ T-cell biology associated with transformation and deregulated growth. These genes include progressively deregulated oncogenes, transcription factors, and signaling genes. Together with the genes that were newly altered in P1's T lymphoma cells, this relatively small number of genes identifies critical players in chronic and malignant L-HES. Good examples are the 3 genes, *RBBP8, MAP3K8*, and *PTPRN2*, whose expression increased stepwise CD3⁻CD4⁺ T cells, first in chronic disease and then in association with T lymphoma. Furthermore, their increased expression paralleled decreases in microRNAs predicted to target them, and our preliminary miRNA transfection experiments lend credence to the functional consequences. For example, the MAP3K8 oncogene is a member of the serine/threonine protein kinase family that was identified by its transforming activity. Activated MAP3K8 induces the ERK1/2, JNK, NF-κB, and p38MAPK pathways, and a study has shown that it is constitutively activated in HTLV-I-transformed human CD4+ T-cell lines.[59] The *MAP3K8* gene therefore illustrates a gene deregulation (increased expression) detected in our patient cohort during chronic disease, which was further augmented in L-HES–associated T lymphoma and identified as a potential target of microRNAs shown to be downmodulated in the patients' cells.

Our objective in this study was to provide a global assessment of gene expression changes characteristic of the CD3⁻CD4⁺ T cells during chronic and malignant L-HES as a means of

identifying the deregulated pathways that underlie their abnormal persistence and expansion in vivo. These data reveal important gene expression changes in receptors whose altered expression may contribute to the $CD3^{-}CD4^{+}$ T cell–modified responses to environmental stimuli as well as deviations in homeostatic growth control pathways whose perturbations may favor outgrowth of the abnormal T-cell clone. Preliminary functional experiments confirmed that the aberrant pathways identified in the $CD3^{-}CD4^{+}$ T cells warrant further in-depth exploration, and several specifically deregulated genes point to potential new drug targets and diagnostic markers.

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Authorship

Contribution: M.R. and K.W.-G. designed the research; M.R. and M.L. performed the research; M.R., M.L., C. Sibille, F.R., M.G., and K.W.-G. analyzed and interpreted the data; C. Sotiriou contributed vital analytical tools; C.G. and B.H.-K. performed the statistical analysis of microarray data; and M.R., F.R., and K.W.-G. wrote the manuscript.

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Part 3: Identification of *BACH2* as a tumour suppressor gene in CD4+ T-cells

The two first parts of this thesis aim at providing the most complete overview of the immune and genetics characteristic of the CD3-CD4+ T-cells from the L-HES patient cohort. In this section, with the information collected through the author's collaborative work, the functional studies of altered genes potentially implicated in the survival of the L-HES T-cell clones have been privileged, with the ultimate goal to identify one or several tumour suppressor gene(s) in the 6q region. This section is divided into two reports: first, a summary of the main results extracted from Article 3 demonstrating the deregulated pro- and anti-apoptotic pathways in the L-HES T-cell clones (Annex 3) and second, the Article 4 containing the functional data that has led to the identification of BACH2 as a tumour suppressor gene in CD4+ T-cells.

Annex 3

Pro-Apoptotic and Anti-Apoptotic Deregulated Pathways in CD3-CD4+ T-cells from L-HES Patients.

Introduction

Cancer cell accumulates mutations in different genes that collectively cause unrestrained growth. The loss of control of cell proliferation consequently results in the to failure to regulate cell cycle division or apoptosis. In order to ultimately understand the cause of the abnormal persistency of the CD3-CD4+ T-cell clones, it was thus important to evaluate the apoptotic pathway in these cells. Apoptosis or programmed cell death is activated through at least one of the two major pathways, the intrinsic (mitochondria-mediated) and extrinsic apoptotic (receptor-mediated) signalling pathways. It is generally admitted that the ratio of pro- to anti-apoptotic proteins determines cell faith. In previous reports, L-HES CD3-CD4+ T-cells were shown to express a high level of FAS (CD95/TNFRSF6) on their cell surface and to undergo apoptosis *in vitro* under soluble FAS-L (TNFSF6) induction at a higher rate than controls [142]. However, although these cells are characterized by high spontaneous apoptosis when cultured *in vitro*, they persist *in vivo* leading to a Th2 clonal expansion causing hypereosinophilia, thereby suggesting deregulation of the apoptotic pathway. Both microarrays and the flow cytometry data described in Article 3 during the chronic phase of the disease for the three L-HES patients and during the lymphoma development of patient 3 confirmed this contradiction and are detailed below [145].

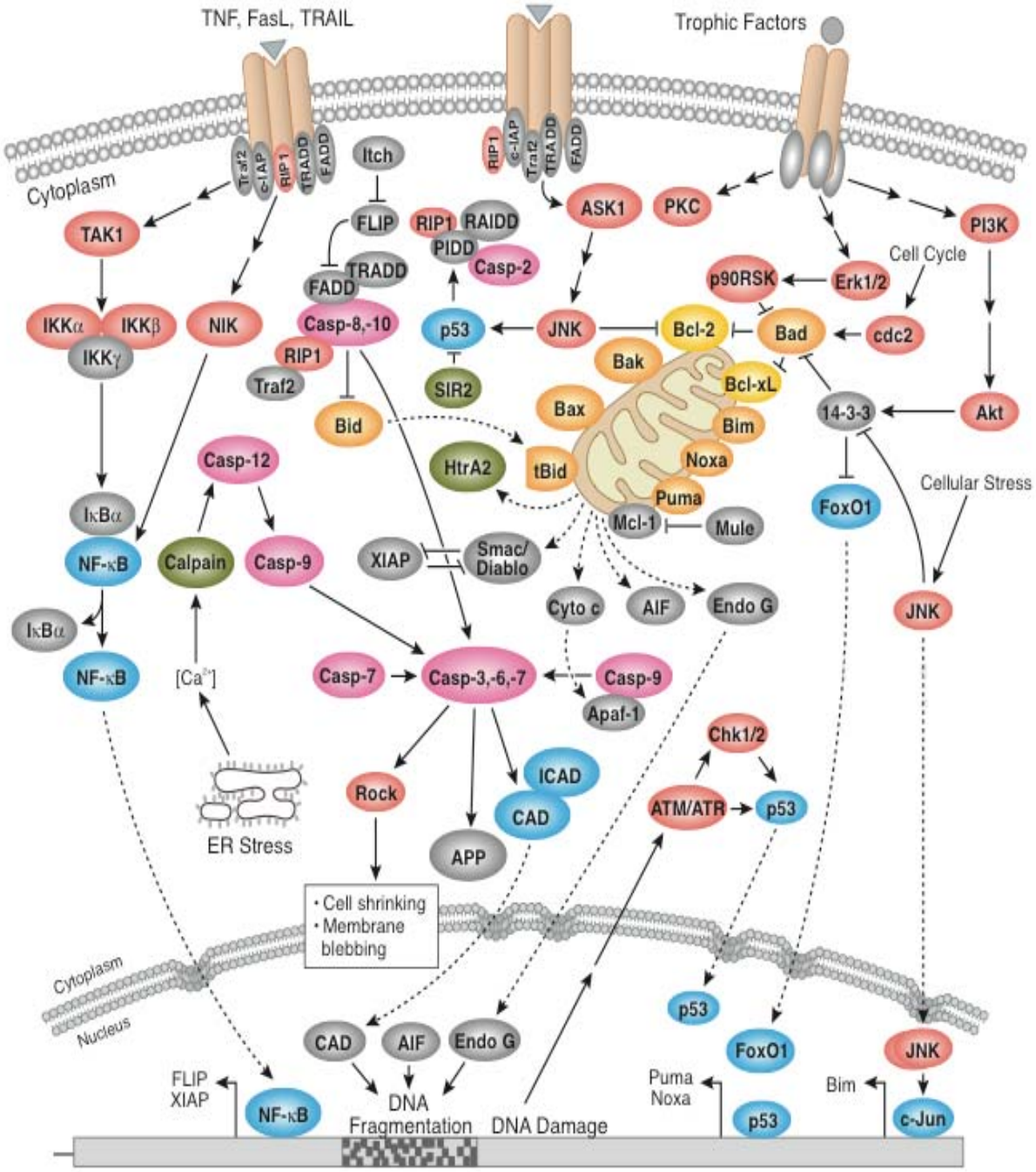

Figure 8.Detailled pathways of extrinsic and intrinsic apoptosis (Source: *www.cellsignal.com*)

3P L-HES CD3-CD4+ VS CTRL CD3+CD4+		**P1 EVOLUTION year0 versus year+6**	
GENES	**FOLD CHANGES**	**GENES**	**FOLD CHANGES**
Upregulated Pro-apoptotic		Upregulated Pro-apoptotic	
RBBP8	3	RBBP8	1,7
TNFSF10	2,9	TNFSF10	1,6
FAS	2,5		
TRADD	1,8		
DIABLO	1,8	DIABLO	2,5
Downregulated Pro-apoptotic		Downregulated Pro-apoptotic	
RIPK2	-5,9		
BACH2	-4,3	BACH2	-1,8
SESN1	-4,3		
BNIP3	-4,2		
TNFRSF10D	-3,4		
BCL2L11	-2,6		
CASP10	-2,3		
ATM	-2,2		
CD47	-2		
RBL2	-1,9		
TNFRSF10B	-1,7		
Upregulated Anti-apoptotic		Upregulated Anti-apoptotic	
TNFSF11	10,4		
TNFRSF11A	7,5		
TNFSF14	3,7		
MAP3K8	2,9	MAP3K8	1,9
RUNX2	2,4	RUNX2	1,9
BIRC4	2,1		
		PRDM1	2
		TNFS4	2,7
		TNFRSF4	1,6
Downregulated Anti-apoptotic			
CD27	-42,4		
BCL2	-5,23		
FAIM3	-4,6	FAIM3	-1,6
MYB	-2,8		
MYC	-2,4		

Results

Major expression changes of genes with pro-apoptotic or anti-apoptotic functions are summarized in the above table (supra). Firstly, the up-regulation of pro-apoptotic genes such as RB-1 binding protein (RBBP8), TNF death receptor ligand (TNFSF10/APO2/TRAIL), FAS, TNF receptor 1-associated death domain (TRADD) and Diablo homolog (DIABLO/SMAC) genes, combined with the down-regulation of pro-survival genes such as BCL2, FAIM3, MYC and MYB, may explain the surprising sensitivity of these cells to the apoptosis observed by the adjunct of soluble FAS-L *in vitro* [142]. In particular, two major anti-apoptotic genes are shown repressed in CD3-CD4+ T-cells including FAIM3 which is a known inhibitor of the FAS-mediated cell death during IL2-mediated AICD [92] and BCL2 which encodes the pro-survival mitochondrial protein BCL2, protecting cells from initiated apoptosis. In addition, DIABLO that activates the formation of the apoptosome by counteracting the X-linked inhibitor of apoptosis (XIAP/BIRC4) is also seen up-regulated [278]. Together, the deregulations of these eight genes should promote the elimination of the CD3-CD4+ T-cells, which, even so, continue to persist *in vivo*. However, it should be mentioned that CD2/CD28 costimulation of CD3-CD4+T-cells from the three LV-HES with IL-2 did not significantly increase the endogenous level of FAS-L as normally expected [145] (supplementary data microarray).

However, the long list of inversely down-regulated pro-apoptotic and antioncogenic genes associated with several up-regulated anti-apoptotic or oncogenic genes may help researchers to understand the paradox of CD3-CD4+ cell survival. Indeed, an increase of TNFS10 which is the ligand for the TNF death receptors 1 and 2 (TNFRSF10D/TRAILR1 and TNFRSF10B/TRAILR2), is shown here to be associated with the up-regulation of the TRADD gene that promotes apoptosis when recruited to the cytoplasmic domain of TNFRSF10D. However, it is also important to note that no parallel increase of the two corresponding receptors TNFRSF10D and TNFRSF10B is observed in CD3-CD4+ cells, which are both downregulated. Therefore, these results indicate that L-HES T cells might not be sensitized to the TNFSF10/TNFRSF10–induced apoptosis (or TRAIL-induced apoptosis) during chronic and acute phases, despite an overexpression of the TNFS10 ligand also observed during P1's disease progression. Another member of the TNF receptor superfamily, CD27 (TNFRSF7), is also not expressed by CD3-CD4+ T-cells, as reported here. This confirms the previous flow cytometric data [142]. Contrasting the constitutive expression of this receptor on the surface of most conventional T-cells and its normal upregulation

following T-cell activation, no subsequent CD27 increase was observed following CD3-CD4+ T-cells stimulation [73, 145].

Furthermore, the down-regulation of the other indicated pro-apoptotic genes is not coincidental. For example, receptor-interacting serine-threonine kinase (RIPK2) is a member of the receptor-interacting protein (RIP) family that contains a C-terminal caspase activation and recruitment domain (CARD). It promotes extrinsic FAS-mediated programmed cell death by enhancing caspase 8 activity [279]. Caspase 10, apoptosis-related cysteine peptidase (CASP 10), is a member of the cysteine-dependent aspartate-directed proteases that mediate apoptosis and whose mutation has been associated with autoimmune lymphoproliferative syndrome (ALPS) [280]. It is processed by caspase 8 and cleaves downstream caspases 3 and 7 for activation. It is also a crucial component during TNFR10/TRAIL-mediated apoptosis [281]. BCL2-like 11 (BCL2L11/BIM) interacts with other members of the BCL-2 protein family, including BCL2 and BCL2L1 and acts as an apoptotic activator, notably during thymocyte-negative selection in mice [282]. In addition its expression is increased during the transforming growth factor-beta1 (TGFβ1)-mediated apoptosis of osteoclasts [283]. RBL2 belongs to the Rb family of tumour suppressors and plays a pivotal role as negative regulator of cell cycle progression [284]. Another critical tumour suppressor gene shown downregulated is the ataxia telangectasia mutated gene (*ATM*) belonging to the PI3/PI4-kinase family [285]. It is a master controller of cell cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability. Biallelic mutations of this gene are associated with Ataxia Telangectasia (A-T) rare disease. A-T patients show an increased rate of lymphomas and leukaemias, preferentially of T lineage [286]. In addition, ATM deficiency causes resistance to Fas-induced apoptosis by upregulating CASP8 and FADD-like apoptosis regulator protein (FLIP) levels, a well-known inhibitor of FAS-induced apoptosis [287]. CD47 is a membrane protein that can either trigger T-cell apoptosis in the presence of high amounts of thrombospondin (TSP) or mediate T-cell activation. CD47-/- deficient mice sustain prolonged inflammation accompanied by a deficiency of T-cell apoptosis [288]. Upon activation, normal T-cells increases expression of the Bcl2/adenovirus E1B 19-kDa interacting protein (BNIP3) and undergo CD47-mediated apoptosis. BNIP3 is a potent pro-apoptotic protein at the mitochondrial site by selectively interacting with BCL2 and BCL2L1 pro-survival proteins [289]. The CD47/BNIP3 pathway sensitizes activated T-cells to apoptosis, thereby limiting inflammation by controlling the number of T-cells. Given that CD3-CD4+ T-cells are deficient for CD47 and CD3/TCR expression, it can be predicted that they may be resistant to CD47/BNIP3-mediated apoptosis,

despite the potential TSP secretion *in vivo* from inflamed skin that characterizes L-HES disease. Repression of the SESN1 and BACH2 genes with upregulation of PRDM1 are discussed in Article 4 and in the general discussion of this thesis (section 4). Notably, upregulation of the tumor necrosis factor receptor superfamily, member 11a (TNFRSF11A/RANK) and of its corresponding ligand TNFSF11 (RANKL), might confer a positive autocrine loop for the growth and survival of CD3-CD4+ T-cells since both are shown up-regulated. As described in the literature, this effect is generally mediated through positive induction of the NF-kappa B pathway and by activation of c-Jun NH2-terminal kinase (JNK) [290]. RANKL also augments the costimulatory effect of Langerhans dendritic cells, preventing their apoptosis and therefore possibly increasing the DC-mediated activation of CD3-CD4+ T-cells *in vivo* [291]. Interestingly, RANKL has also shown to be overexpressed in CD4+ T-cells from Sezary syndrome; its inhibition by specific antibody (denosumab) may represent a promising therapeutic tool, as shown in bone diseases [292-293]. The overexpression of the tumour necrosis factor (ligand) superfamily, member 14 (TNFS14/LIGHT), probably plays an additional role in L-HES pathogeny, as it regulates various cellular responses, including proliferation, differentiation, inflammation and apoptosis [294]. It binds three types of receptors such as the tumour necrosis factor receptor superfamily, member 14 (TNFRSF14/HVEM), lymphotoxin beta receptor (LTBR/TNFRSF3) and tumour necrosis factor receptor superfamily, member 6b, decoy (TNFRSF6B/DCR3). LIGHT binding to LTBR and TNFRS6B results in anti-apoptotic signals where binding to the HVEM of cancer cells mediates pro-apoptotic signals. The LIGHT/HVEM pathway is an important co-signalling pathway for T-cells [294], whereas LIGHT/LBTR modifies the function of stromal cells by creating microenvironments promoting immune responses [295]. For example, ectopic expression of LIGHT in pancreatic islets is necessary and sufficient to promote the formation of lymphoid-like structures and to induce pancreatic islet destruction [296]. Furthermore, secreted LIGHT has been shown to contribute to inflammation and pathogenesis in diverse mucosal, hepatic, joint, vascular and endocrine tissues; thus, it represents an interesting therapeutic target [297]. RUNX2 is a member of the RUNX family of transcription factors, which have been shown to function as tumour suppressors in human cancer. RUNX2 plays a primary role in osteogenesis and is involved in cellular responses to signalling pathways including TGF-β. In transgenic mice, the overexpression of RUNX2 interferes with early T-cell development and predisposes it to the development of T-lymphomas [298]. It is noteworthy that RUNX2 was shown overexpressed in the three L-HES patients and continuously upregulated during P1's evolution, thereby suggesting a

possible RUNX2 oncogenic effect. Since this increase could be mediated by the isochromosome 6p observed in P3's CD3-CD4+ T-cells, further experiments are being conducted to verify whether such 6p triplication is also present in the L-HES T-cell clones from the other patients. Mitogen-activated protein kinase kinase kinase 8 (MAP3K8/Tpl-2/Cot) possess oncogenic properties [299] It is a serine/threonine kinase that activates IkappaB kinase and thus induces the nuclear production of NF-KappaB. It also promotes IL2 and TNFα during T-cell activation and induces the ERK1/2 and JNK pathways [300]. Compatible with the view of a growth advantage mediated by the activation of these pathways is the observed overexpression of MAP3K8 transcripts correlating with the disease evolution of P1 in T-lymphoma. Finally, repression of the X-linked inhibitor of apoptosis XIAP (BIRC4) clearly favour an inhibitory effect on the apoptosis of CD3-CD4+ T-cells, since XIAP not only inhibits caspase 9, 3 and 7, but also reduces apoptosis through binding to other proteins such as DIABLO, and tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 [301-303]. Moreover, disequilibrium of XIAP and DIABLO expression has also been shown to be associated with germline cancer development, and the mutation of *BIRC4* causes X-linked lymphoproliferative disease (XLP) [304-305].

Conclusion

Consistent with the observed persistence and expansion of CD3-CD4+ T-cells, the results of this study have shown that at least two major TNF superfamily death receptor/ligand systems are deregulated in the CD3-CD4+ T-cells of L-HES patients, namely, TNFRSF10/TNFSF10(TRAIL) and FAS/FAS-L(TNFSF6), which could protect CD3-CD4+T-cells from programmed cell death. Furthermore, tumour suppressor genes including *RB-1* and *ATM* with antioncogenic properties in T-cell lineage were seen repressed in CD3-CD4+ T cell-clones. In addition, the downregulation of crucial mediators of the extrinsic and intrinsic apoptotic pathways such as *RIPK2*, *BNIP3, CASP10, DIABLO* and *BIM* (BCL2L11) associated with the co-stimulation signalling provided by the RANK/RANKL loop or LIGHT overexpression may lead overall to L-HES T-cell clone survival and proliferation *in vivo*. Interestingly, both RANK and LIGHT have been studied as recognized targets for immunotherapy in inflammatory conditions, autoimmune diseases and cancer. Therefore, it would be very interesting to explore whether blocking these pathways is an effective way to treat L-HES patients.

Article 4

BACH2 repression in CD4+ T-cells modulates their resistance to apoptosis demonstrating its function as a tumour suppressor gene.

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In our previous reports, 6q chromosomal deletion was observed in the CD3-CD4+ T-cells from two L-HES patients. In the present work, an additional 6q- was detected by FISH within the CD3-CD4+T-cell nuclei of a third L-HES patient, thereby indicating that 6q- was the most frequent chromosomal aberration characterizing this disease in this cohort patients. The established persistence of the 6q13-q22.1 deletion during P1's disease progression also suggests that this abnormality was contributes to the L-HES pathogenesis. With the final objective to identify a potential suppressor gene in the 6q13-22.1 region, the genes contained in the deletion were correlated with the transcriptomic profile of the CD3-CD4+ T-cell clones taken from three patients during their chronic phase and during the transformation to T-lymphoma of P1. The expression profiles confirmed by quantitative RT-PCR displayed 11 commonly repressed genes at 6q13-22.1 in the three L-HES T-cell clones relative to the CD3+CD4+ control T-cells. The microarray and RTPCR results indicating the continuous repression of solely the *BACH2* gene during the disease progression of P1 and coincident with data from the literature, identified *BACH2* as the most relevant candidate tumour suppressor gene located in the 6q deletion. This study's functional analysis with shRNAs, further demonstrates the tumour suppressive properties of the *BACH2* gene by revealing its ability to modulate the apoptosis level of CD4+ Jurkat T-cells during genotoxic stress. By using a specific apoptosis RT-PCR profiler and flow cytometric analysis, it was demonstrated that the BACH2 supressive effect on Jurkat cell's apoptosis was mediated through *FAS-L* gene transcriptional regulation, thereby opening a new way to the understanding of CD4+ T lymphomagenesis. As FAS-L is also a pivotal mediator of AICD, it can be postulated that the deletion of BACH2 may explain the survival of CD3-CD4+ T cells associated with the hypereosinophilia and the predisposition to T lymphoma transformation *in vivo*.

BACH2 REPRESSION IN CD4+ T CELLS MODULATES THEIR RESISTANCE TO APOPTOSIS DEMONSTRATING ITS FUNCTION AS A TUMOUR SUPPRESSOR GENE

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Abstract

Hypereosinophilic Syndromes (HES) cover a wide variety of disorders characterized by sustained hypereosinophilia inducing tissue damage and distinct from secondary causes such as allergic or parasitic conditions. Lymphocytic variant (L-HES) has been shown to be caused by the clonal expansion of abnormal T cells producing high levels of IL-5 that drives secondary proliferation of eosinophils. Contrasting with the myeloproliferative HES variant (M-HES) associated with the FIP1L1-PDGFR fusion gene, the genetic defect underlying the survival of the L-HES T cell clones is still unknown. Our previous observations of chromosome 6q deletions and more recent micro-array studies of the CD3-CD4+ T cells from L-HES patients have led us to investigate in depth the potential pathogenic role of repressed genes located on the deleted segment. In the present work, an additional 6q loss was detected in the CD3-CD4+T-cell nuclei of a third L-HES patient, thereby indicating that 6q- is the most frequent chromosomal aberration characterizing this disease in our cohort of patients. The established persistence of the 6q13-q22.1 deletion during one patient's disease progression towards lymphoma, suggested also that this abnormality could contribute to transformation. With the final objective of identifying a potential suppressor gene in the 6q13-22.1 region, a correlation was made between the genes contained in the deletion and their corresponding expression level in CD3-CD4+ T cell clones from 3 L-HES patients. The comparison displayed 11 commonly repressed genes at 6q13-22.1 in the 3 L-HES T cell clones. Furthermore, microarray and RT-PCR results indicated the continuous repression of only *BACH2* gene during T lymphoma development of one patient and identified *BACH2* as the most relevant candidate tumour suppressor gene located in the 6q deletion. Functional analysis with shRNAs, demonstrated further the tumour suppressive properties of the *BACH2* gene by revealing its ability to modulate the apoptosis level of CD4+ Jurkat T cells during genotoxic stress. By using a specific apoptosis RT-PCR profiler and flow cytometric analysis, we demonstrated that the suppressive effect of BACH2 on apoptosis of Jurkat cells was mediated through transcriptional regulation of the *FAS-L* gene thereby leading to the proposal of a new model for CD4+ T lymphomagenesis. As FAS-L is also an important player in the AICD pathway, we postulated that the deletion of BACH2 could explain the survival of CD3-CD4+ T cells and the predisposition to T lymphoma transformation in L-HES.

Introduction

Hypereosinophilic Syndromes (HES) are a group of orphan diseases that encompass a wide range of clinical manifestations secondary to persistently elevated eosinophilia [1]. Based on recent advances in clinical and molecular diagnosis, HES is generally divided into three major types: myeloproliferative (M-HES), lymphocytic (L-HES) and idiopathic (I-HES) variants. M-HES characterized by the FIP1L1-PDGFR fusion gene results in clonal proliferation of malignant eosinophils and responds usually to imatinib treatment [2]. In contrast, hypereosinophilia observed in L-HES is secondary to the clonal expansion of an aberrant T cell population, generally CD3-CD4+, overproducing IL5 and other cytokines [3]. L-HES patients benefit from mixed therapies including interferon alpha and corticosteroids with variable results [4-6]. While the clinical course of L-HES appears benign, the abnormal T cell clones persist for years and sometimes evolve in T lymphomas despite long term therapy [5, 7]. Therefore, CD3-CD4+ clones should be regarded as premalignant cells and L-HES patients considered at risk for T lymphomagenesis requiring close survey. In a previous study, molecular profiling of aberrant CD3-CD4+ T cell clones from 3 patients with L-HES revealed deregulation in TGFβ and growth pathways while the immunological profile defined these cells as clonal memory Th2 effectors [8]. However, contrasting with the understanding of the molecular underpinnings of the M-HES, the genetic events leading to the malignant transformation of the CD4+T cell clones of L-HES patients remain largely unknown.

Chromosomal imbalances are often observed in haematological malignancies and somatic deletions are believed to indicate the location of tumour suppressor genes whose alteration produces loss-of-function in a recessive (knudson's model) or haploinsufficient context [9-11]. Tumour suppressor genes function by restraining cell cycle progression, maintaining genomic integrity or promoting apoptosis [12]. Our group has previously described the occurrence and persistence of hemizygous 6q13-q22 deletions in abnormal CD3-CD4+ T cell clones from two L-HES patients over a 6-year study period during which one patient developed a T-cell lymphoma [7, 13]. Recurrent finding of 6q deletions in various solid tumours and lymphoid diseases has strongly suggested the presence of several tumour suppressor genes residing on the long arm of chromosome 6. The first evidence that 6q loci are involved in tumour suppression came from somatic cell hybrid experiments inducing senescence in fibroblasts and from microcell-mediated chromosome 6 transfer within breast and ovarian cancer cell lines [14-18]. Despite heterogeneity, several studies refining the regions of minimal deletion (RMD) in large patient cohorts with lymphoid malignancies highlighted preferential loss of 4 distinct regions at bands 6q14-q15, 6q16-q21, 6q23 and 6q25-27, respectively [19-21] [20-28] [22, 29-31] [18, 22, 29, 32]. Notably, deletions of chromosomal bands 6q16-21 are preferentially associated with ALL of T-lineage and high-grade prostate cancers [21, 28, 33]. With recent advances in high resolution array-based comparative genomic hybridization (aCGH), a growing number of 6q tumour suppressor genes have been identified at different loci as potential candidates for specific diseases. However, with the exception of the *TNFAIP3* (*A20*) gene, located at band 6q23 and recently recognized as a tumour suppressor gene of B-lineage lymphoma subsets, no definitive evidence of suppressive activity has been obtained for other 6q gene-locus candidates in separate entities [34-35].

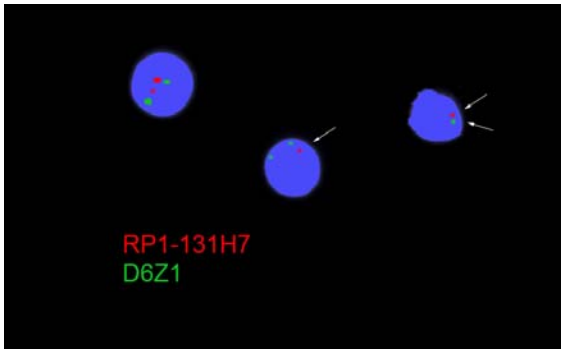
In the current study, the transcriptomic profile of 3 L-HES T-cell clones has been correlated with the minimal deleted region (MDR) at 6q13-22.1 allowing identification of 13 candidate tumour suppressor genes. In order to increase statistical significance, an expression study of these 13 repressed genes was extended to a cohort of 5 L-HES patients. By comparing observations made during disease progression of one of our patients and published data about the 11 selected genes, BTB and CNC homology 1, basic leucine zipper transcription factor 2 (*BACH2*) gene located at 6q15 emerged as a strong candidate tumour suppressor gene [8]. With the objective of demonstrating *BACH2* suppressive activity, this gene was further analyzed molecularly for mutations and functionally for apoptosis in the CD4+ Jurkat T cell line by using small hairpin RNA inactivation technology. Moreover, in order to decipher cellular pathways targeted by *BACH2* gene silencing, an informative

profiling of the apoptosis related genes was also obtained.

Results

Gene expression profile focused on the 6q13-22.1 minimal deleted region in CD3-CD4+ T cells from L-HES patients

Figure 1.A 25 % loss of the 6q region is observed by interphase dual-color FISH on CD3-CD4+ purified T cells from Patient 3. The single arrow indicates the clonal 6q deletion detected in 16% of the nuclei with FITC- labelled RP1-131H7 BAC probe at 6q15. The two arrows show the complete loss of chromosome 6 identified in 9% of the nuclei with the 6q15 FITC labelled probe and the rhodamine-labeled D6Z1 centromeric probe.



A commonly deleted region located between bands q13 and q22.1 of the long arm of chromosome 6 was previously identified in CD3-CD4+ T cells from 2 L-HES patients (P1 and P2) (ref [13]MRHaematologica 2005). To complete the genomic characterization of the CD3-CD4+ T cell clone of the third patient (P3), FISH experiments were performed using a 6q15-specific BAC probe. A complete 6q loss was identified in 25% of nuclei of uncultured CD3-CD4+ T cells from patient P3 (Figure 1) and found associated with an isochromosome 6p (i(6)(p10;p10)) as confirmed by CGH (data not shown).

Figure 2.Heat map of the 13 repressed 6q13-q22.1 genes detected by comparing transcription profiles of 3 patients L-HES (P1-P3) versus 4 controls (C1-C4).
The genes shown are commonly altered in all 3 patients relative to controls zooming in the minimally deleted region shown in the ideogram of normal human chromosome 6 (R-Banding ~700 resolution). Each gene probe is shown representing the greatest absolute fold change in patients compared to healthy controls. The columns represent the expression level of an individual probe set obtained from the 4 controls and the three patients, respectively, in triplicates for P1-year 0 and duplicates for P2 and P3. The clustering and the heat map were produced using R2.5.1 and Tree View programs. The dendogram was derived from a group of selected genes using the hierarchical clustering method and shows the similitude of gene repression patterns in the 3 L-HES patients versus controls [36] (http://project.tcag.ca).

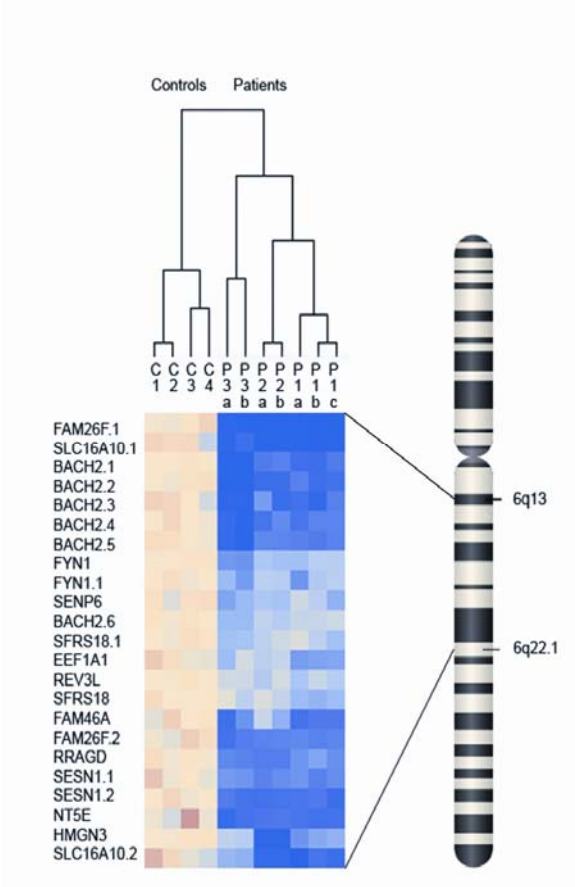
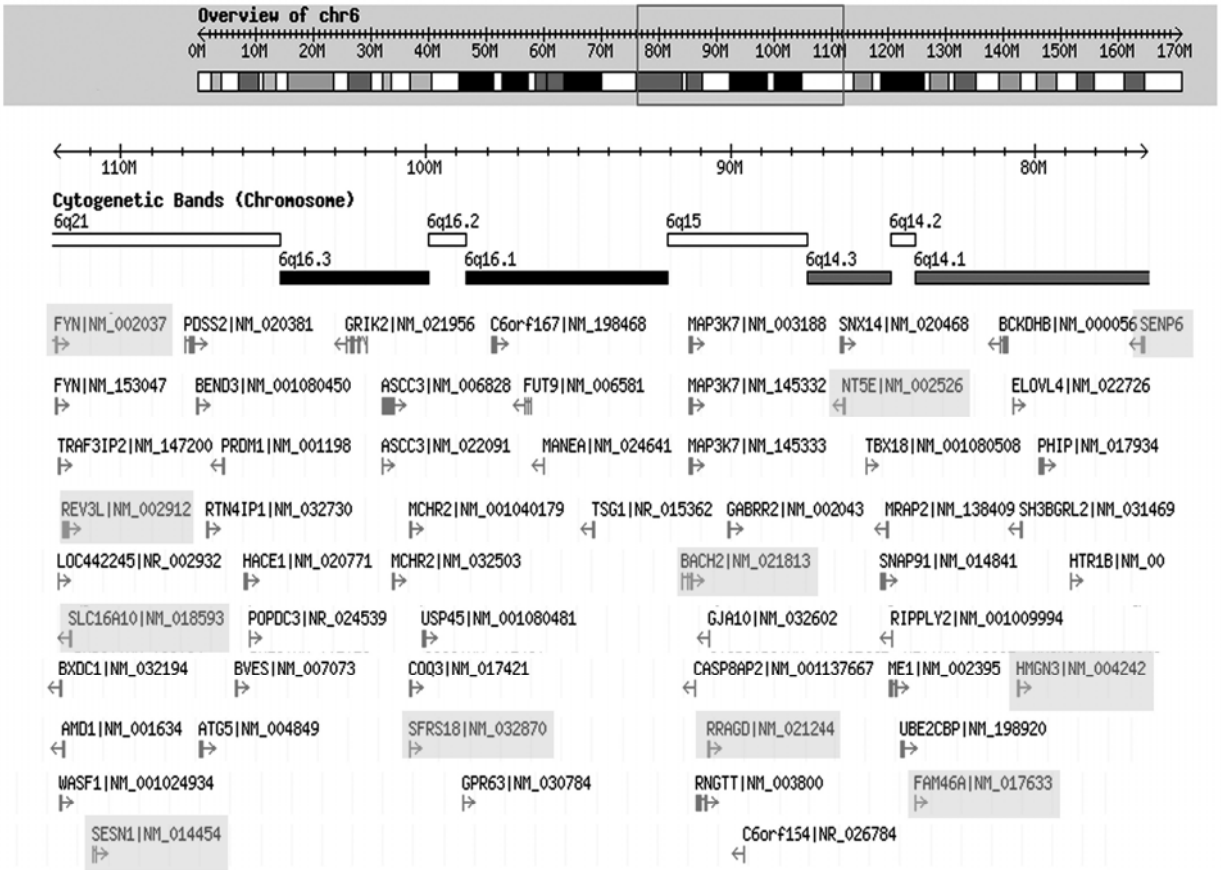


Table 1.Validation of reduced expression of 12 genes on the 6q13-22.1 region by real-time quantitative RT-PCR in 5 patients L-HES. Results are normalized with average levels in 5 healthy controls.

Table 1	Gene Symbol	Name	Cytoband	Average DCt 5 Patients	Rank 5 Patients	Average DCt 5 Controls	Rank 5 Controls	Average 2^(-DDCt) 5P vs 5	Fold Change 5P vs 5C	*p*-value 5P vs 5C
NM_001402.5	EEF1A1	Eucaryotic translational elongation factor 1 alpha	6q13-14	-6,64	[-9,73 : -5,55]	-9,06	[-9,31 : -8,87]	0,19	-5,38	0,0155
NM_00100409.1	SENP6	sentrin specific peptidase 6	6q14.1	1,73	[0,91 : 2,59]	-0,09	[-0,48 : 0,38]	0,28	-3,54	0,0007
NM_004242.2	HMGN3	High mobility group nucleosomal binding domain 3	6q14.1	1,92	[-0,54 : 4,08]	-0,50	[-0,72 : -0,3]	0,19	-5,34	0,0143
NM_017633.2	FAM46A	Family with sequence similarity 46, member A	6q14.1	5,63	[1,88 : 7,95]	2,67	[2,33 : 2,86]	0,13	-7,73	0,0192
NM_002526.2	NT5E	5'-nucleotidase,ecto (CD73)	6q14.3	9,38	[6,65 : 11,19]	4,94	[3,12 : 6,25]	0,05	-21,74	0,0012
NM_021244.4	RRAGD	Ras-related GTP binding D	6q15	5,98	[3,14 : 7,19]	2,53	[1,98 : 2,78]	0,09	-10,97	0,0021
NM_021813.2	BACH2	BTB and CNC homology 1, basic leucin zipper transcription factor	6q15	4,73	[2,41 : 6,83]	1,22	[0,64 : 1,66]	0,09	-11,42	0,0019
NM_01591.1	SFRS18	Splicing factor, arginine/serine-rich 18	6q16.3	-0,47	[-0,98 : 0,63]	-2,93	[-3,2 : -2,34]	0,18	-5,50	0,0001
NM_014454.1	SESN1	Sestrin 1	6q21	3,86	[1,88 : 4,83]	0,98	[0,41 : 1,4]	0,14	-7,36	0,0008
NM_018593.4	SLC16A10	Solute carrier family 16, member 10	6q21	7,66	[4,64 : 9,85]	2,38	[1,97 : 2,71]	0,03	-38,96	0,0025
NM_002912.3	REV3L	REV3-like,catalytic subunit of polymerase zeta	6q21	3,51	[2,49 : 4,5]	1,07	[0,38 : 1,72]	0,18	-5,43	0,0003
NM_002037.3	FYN	FYN oncogene related to SRC,FGR,YES	6q21	-1,18	[-2,39 : -0,06]	-2,52	[-2,69 : -2,35]	0,39	-2,54	0,0114
NM_001010919.1	FAM26F	Family with sequence similarity 26, member F	6q22.1	5,41	[0,79 : 8,65]	3,35	[2,34 : 4,09]	0,24	-4,16	0,3140

Figure 3.Mapping of the 11 repressed genes (grey colour) on the 6q14.1-22 region.



The identification of three L-HES patients carrying a 6q genomic defect in their CD3-CD4+ T cell clones was an additional argument to focus on the gene expression pattern from this region. By comparing the gene expression profiles of CD3-CD4+ T cell clones from 3 L-HES patients with CD3+CD4+ T cells from healthy controls, we have detected 13 repressed genes within the commonly deleted region 6q13-q22.1 (Figure 2). The heat map was produced by extracting a part of the original data published in [8], accessibleat http://www.ncbi.nlm.nih.gov/geo/) and by using the R2.5.1 and Tree View programs. Interestingly, no enhanced expression was found even in the totality of the long arm of chromosome 6, in contrast to several over-expressed transcripts of the 6p short arm such as *HLADR* and *RUNX2* genes (supplementary data of [8]). Thus, with stringent statistical analysis, the only altered pattern of gene expression relative to controls observed from cytoband 6q13 to 6q22.1 is a common repression or loss of namely: *EEF1A1*, *SENP6*, *HMGN3*, *FAM46A*, *NT5E*, *RRAGD*, *BACH2*, *SFRS18*, *SESN1*, *SLC16A10*, *REV3L*, *FYN* and *FAM26A* genes, respectively (Figure 2).

Validation of the microarray data was performed for the 13 mentioned genes by real-time quantitative RT-PCR on purified T cells from an enlarged patient cohort of 5 L-HES patients and 5 healthy controls (Table 1). Clinical description of the 5 L-HES patients has been reported previously [5, 7, 37]. With the exception of FAM26F (p= 0.314) a significant *p*-value was obtained for the 12 remaining genes, confirming repression or loss of their corresponding mRNA (Table 1). A consistent *p*-value (<0.0004) was obtained for FAM26 only when unpaired Student's T test was restricted to patients P1, P2 and P3 (with a detected genomic defect) versus 5 controls, corroborating the previous microarray data. The minimal deleted region previously defined in two L-HES patients extending from band 6q13 to q22.1 covers 301 common genes and ESTs (Entrez Map viewer human Genome browser built 37.1). Beside *EEF1A1* for which reported oncogenic propertieshave already been reported, 11 genes of interest are significantly down regulated between 6q14.1 and 6q21 bands in a 35,88 Mb region [38].

As shown in Figure 3 with genes indicated in grey, the newly defined region extends from the *SENP6* gene located proximally at 74,225,473 bp to the *FYN* gene distally positioned at 112,194,627 bp (http://project.tcag.ca).

Changes in gene expression within the 6q14.1-22.1 region during P1's evolution towards T cell lymphoma

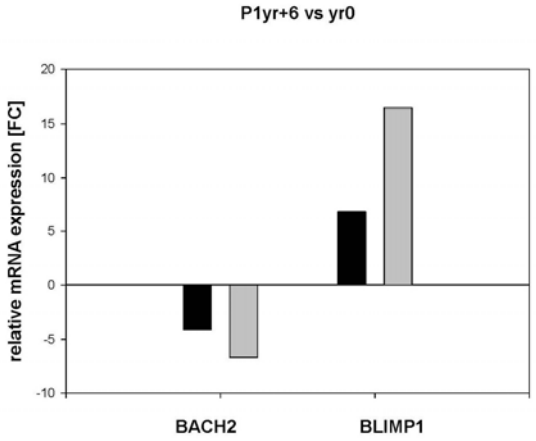


Figure 4.Real-time quantitative RT-PCR showing progressive repression of *BACH2* mRNA and over-expression of *BLIMP1* mRNA during clinical evolution of P1. After normalization with average expression levels in 8 healthy controls, fold changes of *BACH2* and *BLIMP1* genes are indicated at yr 0, in black, and at yr+6, in grey, respectively (data are representative of two separate experiments).

The chronological profiling of CD3-CD4+ T-cells from P1 was previously described starting with a chronic indolent clinical phase and continuing to the full blown T lymphoma stage [8]. It was therefore interesting to look over time at changes in expression of the genes contained within the 6q14.1-22.1 region during disease progression to understand the transforming steps. Of the 11 repressed genes, only *BACH2*'s transcript level was shown to be significantly and progressively under-expressed when tested by microarray (p = 0.00001) (in [8] Table S3-1) and by real-time quantitative RT-PCR (Fig.4). In particular, a ~1,7 fold additional decrease in *BACH2* mRNA level was confirmed between yr 0 and yr+6 corresponding, respectively, to samples taken during the chronic and lymphomatous phases of the

disease (Figure 4). Of interest, at that advanced stage, *BACH2* repression was associated with a significant 2,4 fold over-expression of *BLIMP1* mRNA (microarray, p = 0.0001) located precisely at band 6q21 of the same deleted region (Figure 4) [8]. However, contrasting with *BACH2* (p= 0.0019), the level of *BLIMP1* mRNA was not altered during the chronic phase in 5 L-HES patients relative to the 5 healthy controls (p=0,18) (supplementary data, Figure S1 and S2) suggesting that it represents a secondary or late event associated with the progression of disease in P1. Therefore, while *BACH2* repression occurs at early stages of the disease (Figure S1), these two genes appeared to be epigenetically and inversely regulated since they are both located on the same hemizygous deleted region.

Comparison of the expression level of BACH2 mRNA in normal and L-HES T lymphocytes

In humans, BACH2 is expressed mostly in the thymus, the spleen and leukocytes and at a lower level in the small intestine and the brain [39]. *BACH2* mRNA is notably present in pre-B lymphocytes [40] and, expressed up to fourfold more strongly in cord blood (UCB) and naïve CD4+CD45RA+ T cells [41] compared to adult CD4+ T cells [42]. The repression of *BACH2* gene in CD3-CD4+ TH2 cells of L-HES patients was detected by reference to polyclonal CD3+CD4+ T cells purified from healthy controls. Because of the heterogeneity of these CD3+CD4+ T cell populations and in order to exclude a clonal artefact, it was important to verify whether similar repression could be observed when comparing the abnormal CD3-CD4+ T clones with antigen-specific CD3+CD4+ T helper clones or various functional CD4+ sub-sets. The relative expression of *BACH2* was found significantly decreased in the group of 3 CD3-CD4+ T cell clones from L-HES patients versus the group of 3 CD3+CD4+ T helper antitumor clones (p=0.0023) [43] (Figure S3). Moreover, expression of *BACH2* mRNA in, respectively, one CD4+ Treg clone [43], one CD8+ T effector clone and purified memory CD4+ CD45RO+ T cells from a healthy control was found to be 5 to 10 fold higher relative to the 3 CD3-CD4+ T cell clones (Figure S3).

Molecular analysis of the *BACH2* gene in CD3-CD4+ T cell DNA from 2 L-HES patients

To identify possible mutations at the *BACH2* locus within the remaining allele, sequence analysis was performed on DNA extracts from CD3-CD4+T cells of P1 and P3 displaying heterozygous deletion in the 6q15 region. However, for both patients, only wild type sequences of the coding exons of *BACH2* gene were obtained (data not shown).

To exclude epigenetic events repressing expression of the *BACH2* wild type allele, we verified the methylation pattern of the *BACH2* promoter sequences. Following amplification, bisulfite treatment and subcloning of CD3-CD4+ DNA from patient P3, no specific promoter region was found to be hypermethylated compared with CD3+CD4+ T cell DNA from healthy controls.

Transduction of BACH2 shRNA BACH2 into the Jurkat E6.1 cell line

Primitive CD3-CD4+ T cells from L-HES patients are available in restricted amounts and rapidly undergo apoptosis *in vitro* in absence of growth factors hampering the efficient restauration of *BACH2* gene by means of transfection experiments using vectors encoding the complete *BACH2* cDNA sequence (BACH2-pCDNA3) [4]. Therefore, in order to demonstrate anti-oncogenic properties of BACH2, functional assays were performed on the CD3+CD4+ E6.1 Jurkat T cell line, in which BACH2 expression is similar to normal helper T cell clones and no 6q deletions are present. First, transfections with BACH2-pCDNA3 aiming at reinforced expression of BACH2 in Jurkat cells, resulted in the suppression of E6.1 clonogenicity as repetitively observed by the total absence of BACH2-pCDNA3 growing transfectants in contrast to control clones (with pCDNA3 vector alone).

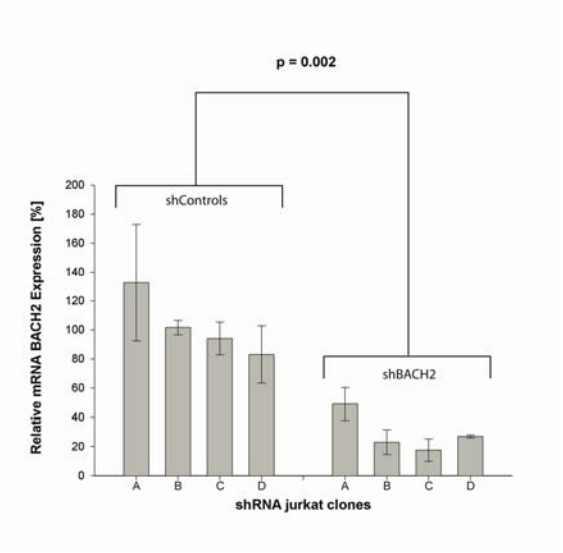


Figure 5.Relative mRNA *BACH2* expression level in the Jurkat T cell line transduced with control shRNA or BACH2 shRNA lentiviral vectors (4 clones shCTRL-A,B,C,D and 4 clones shBACH2-A,B,C,D respectively). Columns with standard deviations represent the mean percentage of 3 experiments.

Next, we turned to the use of small hairpin RNAs (shRNAs) for the targeted repression of gene expression, a technique that has been elegantly used at the cellular level for demonstrating loss-of-function tumour suppressor gene activity [44]. A BACH2 shRNA lentiviral vector was designed by inserting miR RNAi sequence spanning position 2405bp to 2425bp of *BACH2* gene in pLenti6.4/R4R2/V5-DEST (Invitrogen). Two successive cycles of lentiviral infection of E6.1 with, respectively, BACH2 shRNA or a negative-control shRNA preceded cloning of EGFP-positive cells by FACS sorting. Interestingly, clonogenicity was always superior in the shRNA BACH2 group as the number of shRNA BACH2 clones was at least twice the shRNA control clones (>50%). Figure 5, shows a representative panel of 4 transduced E6.1 clones with different levels of *BACH2* transcript silencing compared to the negative controls (p=0.002). In addition, western blotting with cell extracts from shRNA CTRL-A, -B clones compared to shRNA BACH2-B, -C were performed with specific anti-BACH2 monoclonal antibodies and confirmed the extinction of BACH2 protein in the 2 shRNA silenced clones (Figure 6).

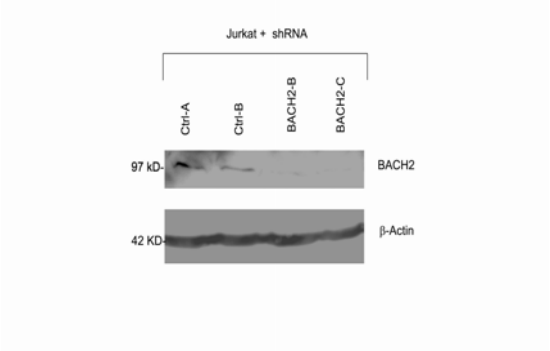


Figure 6.Extinction of BACH2 protein in shRNA BACH2-C,D Jurkat clones versus shRNA CTRL-A,B clones measured by western blotting. 90Kd BACH2 protein is detected in the 2 shRNA control lanes and absent in the shRNA BACH2 -B, and -C lanes in contrast to Beta-actin endogenous expression.

Apoptotic resistance of BACH2 shRNA Jurkat clones to topoisomerase inhibitor VP16

Etoposide (VP16), commonly used for treatment of non-Hodgkin lymphomas (NHL), induces intracellular oxidative stress by inhibiting topoisomerase II thereby generating DNA-strand breaks followed by cell apoptosis. Increased sensitivity to genotoxic drugs like etoposide has been shown in the RAJI (B-lymphoïd) cell line after forced *BACH2* overexpression [45]. We thus asked if, inversely, *BACH2* silencing could modify resistance to apoptosis of Jurkat T cell clones treated with etoposide.

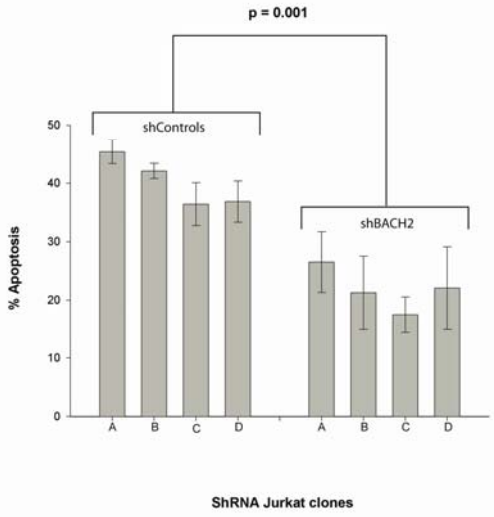


Fig.7. Silencing of *BACH2* in Jurkat clones treated with etoposide markedly reduces apoptosis. The 8 clones were incubated for 18 hours with 1.2 µg/ml of etoposide before staining with annexin V-FITC and PI. Apoptosis was assessed by flow cytometry. Columns with standard deviations represent the mean percentage of 3 experiments.

Data presented in Figure 7 indicate that the 4 BACH2 shRNA clones are on average twice as resistant to apoptosis as the 4 control clones (p= 0.001) following etoposide treatment. Moreover, a correlation could be drawn between the level of mRNA BACH2 repression and the level of apoptosis resistance observed for each clone (R^2=0.9251) (see Graph S1 in supplementary data). Comparison between clone shRNA BACH2-C and shRNA control-A presenting the same mean EGFP fluorescence intensity demonstrated a twofold reduction in the number of apoptotic cells (AnnexinV+) in the BACH2-repressed clone at 1µg/ml of etoposide (Figure S3).

Further, serial etoposide dilutions demonstrated a 30% to 70% gain in apoptosis resistance of clone shRNA BACH2-C relative to clone shRNA CTRL-A (Figure 8). In contrast, no significant difference in apoptosis resistance was found between the clones in presence of FAS ligand (FASLG/TNFSF6) or PMA (phorbol myristic acetate) (see Table 2) or following 18-hour stimulation with anti-CD2, -CD28 or anti-CD3 antibodies (data not shown).

Treatment	Apoptosis response % shRNABACH2	shRNACTRL	Difference %	p
PMA	16,17(+/-2,26)	16,7(+/-6,12)	-0,5	0.928
Fas-L	33,18(+/-2,32)	34,51(+/-3,75)	-1,3	0.719
Etoposide	41,2 (+/-4,94)	18,27(+/-2,71)	23	0.013

Table 2.

Table 2.Apoptosis resistance of shRNA BACH2 and shRNA CTRL Jurkat clones following 18 hour incubation with etoposide, PMA and, Fas-L. In contrast to etoposide treatment, no significant difference of surviving cells is observed in presence of Fas-L or PMA.

Comparative apoptosis gene-array profiling of Jurkat shRNA BACH2 clone C versus shRNA CTRL clone A at 4 h post-etoposide treatment

In order to define specific genes implicated in resistance to apoptotis of *BACH2*-silenced Jurkat cells relative to controls, comparative gene expression profiling was performed by using the apoptosis focused RT² profiler PCR array system (Sabiosciences). The genes in shRNA BACH2-C cells showing significantly modified expression compared with shRNA CTRL-A cells are indicated in Table 3 with specific functions related to apoptosis.

Gene number	Name	Function	Fold change
NC_000022.10	BIK	BCL2-interacting killer, apoptosis-inducing, shares a BH3 domain as BAX and BAK proteins	-3,3
NC_000005.9	CARD6	caspase recruitment domain family, member 6, positively modulates signalling converging on activation of NF-kB	-2,6
NC_000011.9	CASP1	apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) plays a central role in the execution-phase of cell apoptosis	-2,1
NC_000001.10	FAS-L	Fas ligand (TNF superfamily, member 6), is critical in triggering apoptosis	-4,6
NC_000008.10	RIPK2	receptor-interacting protein (RIP) family of serine/threonine protein kinases, potent activator of NF-kappaB and inducer of apoptosis	-2

Table 3

Table 3.Comparison of relative mRNA expression of apoptosis-related genes in Jurkat shRNA BACH2 clone C versus Jurkat shRNA CTRL clone A following 6 hour incubation with 1.2 µgr/ml etoposide or without treatment.

Notably, at 6 hours post-etoposide induction, *BACH2* silencing is associated with the repression of 4 pro-apoptotic genes such as *BIK*, *CASP1*, *FAS-L*, *RIPK2*, and of one mediator, *CARD6*, without upregulation of survival genes. In particular, BCL2 interacting killer protein (BIK) shares a BH3 domain with the other death-promoting proteins BAX and BAK and induces apoptosis by interacting with BCL2-related cellular survival-promoting proteins [46]. Caspase 1 (CASP1) is a member of the cysteine-dependent aspartate-directed proteases that mediate apoptosis and is activated by inflammosomes usually during micro-organism infections [47]. Receptor-interacting serine-threonine kinase (RIPK2) is a member of the receptor-interacting protein (RIP) family that contains a C-terminal caspase activation and recruitment domain (CARD). It promotes NF-kappaB activation and apoptosis in response to various stimuli [48]. Fas ligand or TNF superfamily,member 6 (FASLG/TNFSF6) is the ligand for Fas receptor or TNFRSF6 [49-50]. These transmembrane proteins are critical in triggering extrinsic apoptosis in lymphoid and various non lymphoid tissues. Caspase recruitment domain family, member 6 (CARD6) encodes a protein that transmits positive signals from receptor protein kinases

resulting in NF-kB activation [51]. Altogether, among these repressed pro-apoptotic genes, two belong to the receptor mediated pathway and one to the mitochondrial pathway. Interestingly, no upregulation of *CASPASE 3* or *8* genes could be detected at this early phase of apoptosis. Moreover, flow cytometric analysis specific for the active form of caspase 3 showed no involvement of caspase 3 in the early apoptotic process although it became evident 12 hours later (data not shown).

Comparative cytometric FAS/FAS-L profiles of Jurkat shRNA BACH2 clone C versus shRNA CTRL clone A at 4 h post-etoposide treatment

Since significant (p=0.0469) repression of the *FAS-L* gene was confirmed in silenced BACH2 clones under genotoxic stress (Figure S4), it was important to verify both constitutive and induced level of expression of the FAS/FAS-L proteins in these cells compared to controls.

As generally observed in the absence of activation via the antigen receptor or exogenous cytotoxic stimulus, no constitutive FAS-L was observed at the surface of untreated CD4+ Jurkat T cell clones (data not shown). However, at 6 hours post-etoposide treatment, flow cytometric analysis detected upregulated FAS-L on shRNA control cells (Figure 9B) but no upregulation on shRNA BACH2 clone C (Figure 9C) consistent with the transcriptional inhibition of the *FASL* gene in the *BACH2*-silenced Jurkat cells shown by quantitative RT-PCR at 4 hours post-etoposide (Table 3 and Figure S4). Moreover, the constitutive Fas expression shown here and previous apoptosis experiments carried out following addition of FAS-L (Table 2), demonstrate that Fas receptor signalling is intact in the BACH2-silenced Jurkat clones, indicating that only the FAS-L pathway is deregulated in the absence of BACH2 upon genotoxic activation.

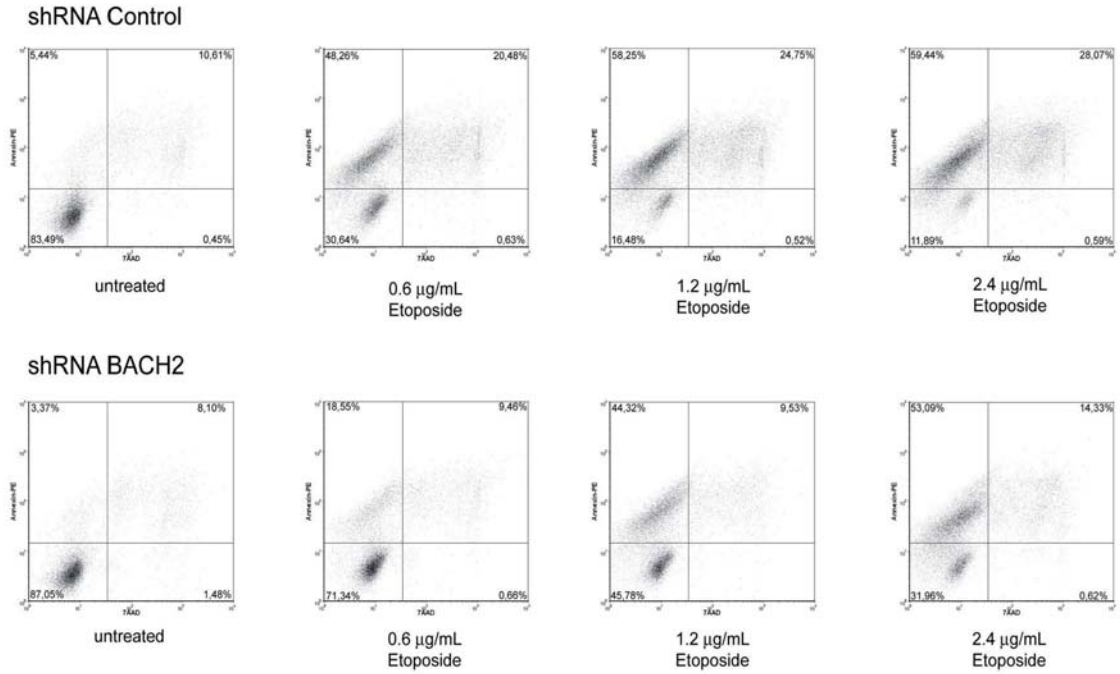
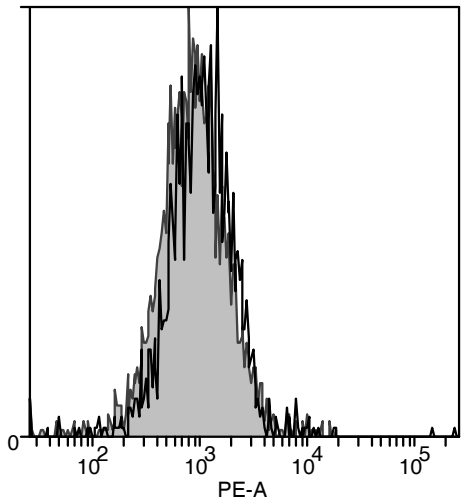


Figure 8.Apoptosis assay of shRNA BACH2 clone C and shRNA CTRL clone A untreated or treated for 18 hours with various concentrations of etoposide before staining with annexin V-FITC and PI. Percentages of apoptotic cells are shown. The experiment is representative of three individual experiments where the p value of the mean of the triplet samples comparison is <0.005 with 1.2 µgr/ml concentration of etoposide.

A Fas untreated



B sh CTRL + Fas-L + Etoposide C sh BACH2 + Fas-L + Etoposide

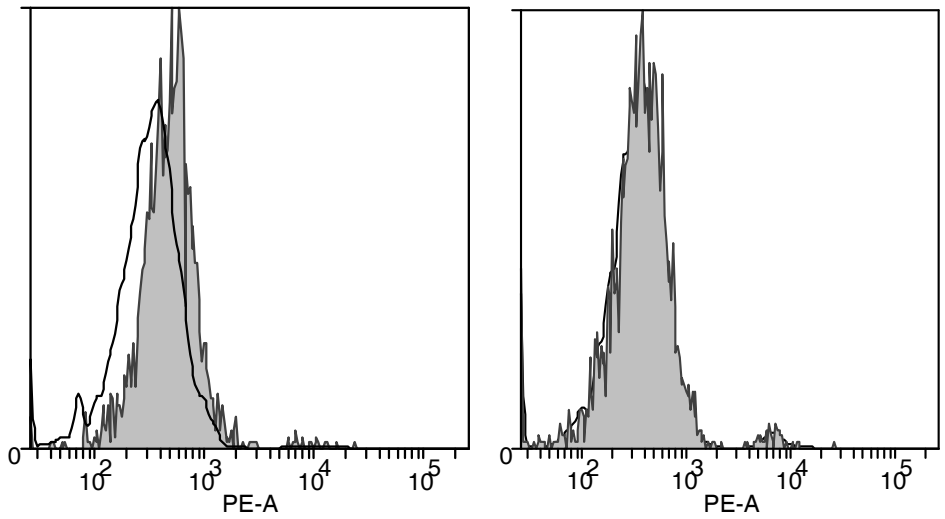


Figure 9.Flow cytometric profiles of shRNA BACH2 clone C and shRNA CTRL clone A. Overlay histograms of both clones labelled with anti-FAS antibody (grey histogram) are shown in A. Histograms of FAS-L labelled shRNA CTRL clone A and shRNA BACH2 clone C at 6 hours post etoposide are provided respectively in B and C (grey histogram) with isotype control (open histogram). As shown in C, there is no FAS-L expression induced by etoposide at the surface of shRNA BACH2 clone C in contrast to the upregulated FAS-L expression on the surface of shRNA CTRL A. In A, there is no difference in FAS receptor expression detected between the two clones.

Discussion

L-HES patients are characterized by the presence of an abnormal circulating CD4+ Th2 cell clone in their blood causing hypereosinophilia. Although the clinical course is generally indolent, the long term prognosis of this disease can be modified in some patients by the development of T lymphomas. For instance, in our L-HES patient cohort, one developed an anaplastic null cell lymphoma and two others a peripheral T cell lymphoma with variable latency [5, 7]. Therefore, this predisposition to T lymphomagenesis characterizing L-HES offers a rare opportunity to study the critical genetic changes leading to T cell transformation.

We previously described, a commonly deleted region located between bands q13 and q22.1 of the long arm of chromosome 6 was identified in CD3-CD4+ T cells from 2 L-HES patients (P1 and P2) [7, 13]. Further, our timeline studies of chromosomal aberrations of the CD3-CD4+ subclones from P1 have demonstrated the persistence and the emergence of one 6q- subclone in parallel with full-blown T cell lymphoma development [13]. Moreover, a new translocation t(6;11)(q21;q23) has been reported in a patient with hypereosinophilia and T cell lymphoma [52]. In the present report, genomic characterization of a CD3-CD4+ T cell clone from a third L-HES patient (P3), has shown a complete 6q loss associated with an isochromosome 6p (i(6)(p10;p10)) in 25% of CD3-CD4+ T cell nuclei (Figure 1). Therefore, isolated 6q aberrations, are over-represented in L-HES patients and might be specific for this condition. However, use of 6q- as a diagnostic marker or prognosis factor in L-HES requires further validation through prospective studies using accurate diagnostic techniques including CGH array and FISH on purified aberrant T cells.

These observations also suggest that 6q loss may contribute to the pathologic disease process ultimately leading to T cell lymphoma. In this study, we investigated the pathogenic role of this chromosomal defect in this rare entity with the objective of ultimately identifying potential tumour suppressor gene(s). Close examination of the transcriptomic profile of 3 CD3-CD4+ T cell clones, focusing on genes located on the commonly deleted region 6q13-22.1, previously described for two L-HES patients, has targeted 13 repressed genes (Figure 2). By comparing the expression pattern of CD3-CD4+ T cells from an extended cohort of 5 L-HES patients with normal CD3+CD4+ cells from 5 healthy controls, 11 gene loci of interest have been selected at 6q14.1-22.1 namely *SENP6*, *HMGN3*, *FAM46A*, *NT5E*, *RRAGD*, *BACH2*, *SFRS18*, *SESN1*, *SLC16A10*, *REV3L* and *FYN* genes (Table1 and Figure 3). Although *RRAGD*, *SESN1* and *REV3L* genes possess tumour-related functions ([53], [54]and [55]), we identified the *BACH2* gene as the most relevant candidate suppressor gene in the CD3-CD4+T cells. Indeed, among the 11 repressed genes, an early and progressive repression of only the *BACH2* transcript was demonstrated during disease evolution of patient 1 towards T cell lymphoma [13]. The *BACH2* gene is known to be well expressed in CD4+ T cell lines [39] and, in normal CD4+ T cells such as umbilical cord blood or adult CD4+ T cells, T helper or T reg subsets and naïve or memory CD4+ T cell populations [42]. Furthermore, observation of non random BACH2 gene inactivation either by Epstein-Barr virus (EBV) integration into 6q15 band of a Burkitt lymphoma cell line (Raji) or by recurrent insertions of human immunodeficiency virus (HIV) at the *BACH2* locus in resting CD4+ T cells from patients receiving antiretroviral therapy have suggested target-site preference contributing to lymphomagenesis or viral persistence [39], [56]. Finally, several reports pinpointed *BACH2* as a candidate tumour suppressor gene in the B cell compartment by showing frequent loss of heterozygosity in non Hodgkin lymphomas and a reduction of clonogenicity with increased sensitivity to apoptosis in *BACH2* over-expressing Raji cells [45]. Moreover, besides its critical role in class switch recombination (CSR) and somatic hypermutation of immunoglobulin genes [57], transcription factor *BACH2* has also been described as a useful prognostic marker in diffuse large B-cell lymphomas [58].

In contrast to B cell malignancies, the potential tumour suppressor activity of BACH2 in T cell disorders has never been reported. Therefore, to test directly the suppressive properties of BACH2 in a robust T cell lineage, experiments were performed using the Jurkat E6.1 CD3+ CD4+T cell line. We first observed that enforced BACH2 over-expression completely abrogated

clonogenicity. Since no homozygous 6q15 deletion or biallelic inactivation of the *BACH2* gene was found in the CD3-CD4+ T cell clones as expected when fulfilling Knudson's classic two-hit criteria, the first hypothesis was that *BACH2* could represent a haploinsufficient tumour suppressor gene. Therefore short hairpin RNA suppression technology mediated by lentiviral expression vectors to stably suppress *BACH2* gene expression was adopted in Jurkat cells similar to the strategy used successfully for identifying the *RPS14* tumour suppressor gene by loss-of-function in a haploinsufficient context [44]. The 50% gain of clonogenicity observed in the BACH2-silenced Jurkat clones was in itself consistent with a tumour suppressor phenotype. We then questioned whether BACH2 gene ablation had a survival-promoting effect on Jurkat T cells under genotoxic stress, as demonstration of the anti-oncogenic properties of other haploinsufficient genes such as CDKN2B, has required the presence of genotoxic agents [59]. As demonstrated with various etoposide concentrations, significant gain (30-70%) of apoptosis resistance was obtained in *BACH2* silenced Jurkat clones. Moreover, the extent of *BACH2* transcript extinction was correlated with the percentage of apoptosis protection, providing evidence for a "gene dosage" effect. Together, these data support tumour suppressor activity of BACH2 through modulation of apoptosis resistance of CD4+ T cells such as a rheostat.

Cytotoxic drugs including etoposide activate apoptosis through at least one of the two major pathways, the intrinsic (mitochondria-mediated) and extrinsic (receptor-mediated) apoptotic signalling pathways [60-61]. It is generally considered that the ratio of pro- to anti-apoptotic proteins determines the sensitivity of a cell to a given apoptotic trigger. *BACH2* silencing in the Jurkat cells was shown to be associated with early decreased expression of a limited set of 4 pro-apoptotic genes namely *BIK, CASP1, FAS-L* and *RIPK2* genes (Table 3). Notably, *BIK*, redundant with *BCL2-like 11* (*BCL2L11*), is a strong mitochondrial pro-apoptotic inducer in several tissues, which is transcriptionally activated by several anti-cancer drugs [46], while *RIPK2* has been reported to induce extrinsic FAS mediated-programmed cell death by enhancing caspase 8 activity [62]. Interestingly, *RIPK2* and *BCL2L11* transcripts were also found consistently repressed in CD3-CD4+ T cells from L-HES patients [8]. Therefore, these results demonstrate the repression of two important apoptotic mediators in BACH2-silenced Jurkat T cells and in BACH2 deleted CD3-CD4+ T cells. Furthermore, etoposide is a DNA-damaging drug that has been shown to promote extrinsic apoptosis by inducing transcription of FAS-L and TRAIL in responsive tumour cells [63-65]. Altogether, these data suggested that resistance to etoposide-induced apoptosis of *BACH2*-silenced clones may be secondary to a FAS/FAS-L system defect. Consistent with this hypothesis, no upregulation of the *FAS-L* gene transcript was detected under etoposide treatment in the shRNA BACH2 clones in contrast to the shRNA control clones (figure S4). In addition, FAS-L induction 6h post-etoposide was observed on control clone-A by flow cytometric analysis, but not on the *BACH2*-silenced clone-C (Figure 9: B, C). No differences in expression of FAS (Figure 9A) or in apoptosis in response to exogenous FAS-L (Table 2) were detected between shRNA BACH2 and shRNA control clones. We therefore conclude that the *BACH2* gene regulates transcription of *FAS-L* and consequently modulates the autocrine FAS/FAS-L apoptotic loop in CD4+ Jurkat T cells, a fundamental extrinsic apoptotic mechanism involved in the activation-induced cell death (AICD) [66]. In this line, the *FAS-L* gene promoter contains an AP1 site towards which BACH2 is known to display binding specificity [63, 67].

Normal effector memory T cells are intrinsically sensitive to apoptosis and have upregulated levels of *FAS-L* [68]. In contrast, L-HES memory CD3-CD4+FAS+ T cells under-expressing *BACH2* exhibit low levels of *FAS-L* and are unable to upregulate consistently *FAS-L* transcription upon CD2, CD28, IL2 and etoposide stimulation (Table S1 and [8]). Furthermore, contrasting with autologous CD3+CD4+ T cells increasing FAS-L expression, 6q deleted CD3-CD4+ T cells from L-HES P3 are shown to be resistant to etoposide-induced apoptosis (Figure S5). Moreover, lack of TCR/CD3 expression on the surface of these cells precludes *FAS-L* upregulation by antigenic or anti-CD3 stimulation. Thus, although CD3-CD4+T cells from L-HES patients have been shown to undergo apoptosis in response to exogenous

FAS-L *in vitro* [4], it can be hypothesized that they do not undergo autocrine FAS-L-mediated apoptosis *in vivo* due to the repression of *BACH2*. Therefore, it is suggested that abrogation of *FAS-L* transcription consequent to deletion of the *BACH2* locus plays an important role in L-HES pathogenesis by rendering CD3-CD4+ T cell clones resistant to apoptosis and can be considered as an early and novel transforming event in CD4+ T lymphomagenesis.

Supporting this view, autoimmune diseases have been often reported associated with a significant risk of cancer or lymphoma development [69-72]. Moreover, germinal mutations in *FAS-L* result in the autoimmune lymphoproliferative syndrome (ALPS) type IB (ref OMIM 601859), which is characterized by the uncontrolled proliferation of CD3+CD4-CD8- T cells progressing in some cases to T lymphoma [69, 73]. Furthermore, published data with ours indicate that memory Th2 cells are potent producers of eosinophilopoietic lymphokines modifying tissue environment [3, 74]. Therefore, our results give a reasonable explanation of why *BACH2*-deficient L-HES T cells, which are unable to undergo FAS-L-apoptosis are predisposed to T lymphoma development and, induce at the same time the hypereosinophilia secondary to their Th2 nature. This is believed to be the first description of a BACH2/FAS-L defect that may lead to the perpetuation of a Th2 disease. In addition, our observations suggest that BACH2 reflects the function of a landscaper tumour suppressor gene, which may modifie the cellular microenvironment in a variable mode depending on the lineage of the mutated CD4+ T-cell clone. Recent reports using genome-wide association studies in human subjects have pinpointed the *BACH2* gene as a risk locus for type I diabetes and celiac disease among genes with immune functions [75-77]. In addition, deregulation of the FAS-L/FAS system has been described in thymocytes from the non-obese diabetic mouse (NOD) studied as an animal model for type I diabetes [78]. Having demonstrated here the critical role of BACH2 in FAS-L-mediated CD4+ T cell apoptosis, it would be worthwhile to verify the functionality of the *BACH2*/*FAS-L* pathway in T lymphocytes from subjects with diabetes and celiac disease.

In conclusion, the identification of *BACH2* as a haploinsufficient suppressor gene in CD4+ T cells by our functional experiments has lead to the proposal of a new model of T lymphomagenesis in L-HES. Moreover, by providing evidence that the suppressive effect of BACH2 is mediated via expression of the *FAS-L* gene, the product of which is a major actor in AICD, our results have potentially unravelled an essential function of BACH2 in CD4+ T cell homeostasis. To our knowledge, this is the first report of a regulatory effect of BACH2 on the FAS-L extrinsic apoptotic pathway in CD4+ memory T cells. Further investigations on the integrity of the FAS-L pathway modulated by BACH2 in other persisting CD4+ autoimmune, infectious and lymphoproliferative diseases may prove to be rewarding. Finally, our data have demonstrated that *BACH2* is a tumour suppressor gene whose repression modulates apoptosis resistance of CD4+ T cells *in vitro*. To understand further the tumour suppressive role of BACH2 *in vivo*, it would be interesting to follow hemizygous +/- *BACH2* knockout mice for autoimmune diseases and neoplastic development in a genotoxic or infectious context.

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GENERAL DISCUSSION

1. Phenotypic Characterization of CD3-CD4+ Th2 Cells

As part of the collaborative team, the author was involved in the genetic diagnosis of the majority of the patients included in the L-HES cohort. The initial reports focused on the selection of L-HES patients based on the strict criteria proposed by Chiusid et al. [98]. In the first two studies, the systematic investigation of the CD4+ T-cells isolated from the peripheral blood allowed the inclusion of five L-HES patients [117-118]. In particular, the molecular and cytogenetic analysis realized in the researcher's laboratory led to the identification of T-cell receptor clonality and specific chromosomal anomalies [117-118, 124]. Immunophenotyping of the purified CD4+ T-cells was included in the diagnosis process, and demonstrated the presence of an aberrant population of clonal CD2+CD5+CD3-CD4+CD7-CD45RO+CD69- T-cells [117-118, 306]. The abnormal CD4+ T-cells expressing intracellular TCR-α/β chains and a high level of FAS-R (CD95) in the absence of CD27 (TNFRSF7) were further characterized by their ability to produce high levels of Th2 cytokines such as IL-5 and IL-13 upon stimulation provided by dendritic cells [145]. Moreover, an autocrine IL-2/IL-2R loop was demonstrated to be initiated by dendritic cells through the B7/CD28 and LFA-3/CD2 interactions. The gene profiling in this study established the type-2 signature of these aberrant memory T-cells by detecting high expression levels of Th2 cytokines receptors and transcription factors genes, contrasting with the downregulation of genes that determine TH1 differentiation [145]. For example, the upregulation of *IL-4R*, *IL-9R*, *GATA3*, *CCR3* and *CCR8* type-2 genes was shown to be associated with the downregulation of *BTLA*, *IL18R*, *CCL5*, *NOTCH2*, *JUN* and *SLAM7* type-1 genes.

As reported earlier, a lack of surface TCR/CD3 expression was caused by a defect in CD3γ chain expression, thus preventing the correct assembly and surface exportation of the TCR/CD3 complex, and thereby hampering full activation of the CD3-CD4+ T-cells of P1 [306]. It was also shown that binding of the inhibitory factor NFATc2 to the NFATγ2 site in the *CD3G* promoter was greatly increased in these abnormal CD3-CD4+ T-cells [307-308]. Molecular profiling confirmed the previous characterization of surface receptor expression by revealing corresponding changes in the transcription levels of several genes, including the loss of *CD3G* gene's mRNA with an additional decrease of *CD3Z* gene transcripts in the CD3-CD4+ T-cells of three L-HES patients [145]. There have been an increasing number of

human conditions exhibiting reduced expression of TCR/CD3 surface expression, since its initial description associated with T-cell malignancies [309]. A loss of TCR/CD3 was also observed in HTLV-1 or HIV-infected T-cell lines and associated with a growth advantage [310-311]. The underlying mechanisms maintaining the survival of aberrant CD4+ T-cells through ablation of surface TCR/CD3 complexes are still largely unknown. However, it can be speculated that reduced TCR/CD3 triggering might be advantageous to these cells in order to escape AICD.

Additional changes for new receptor genes were unravelled with this study's gene expression profiling array of abnormal CD3-CD4+ T-cell populations. Notably, high expression levels for *IL17RB* (IL-25 receptor) and for *CRTH2* (Prostaglandin D2 receptor) genes were detected and further correlated by flow cytometry with their relevant proteins detection. *In vitro* proliferation and Th2 cytokines production of CD3-CD4+T-cells from P3 was demonstrated when cultured with rhIL-25. With low CCR7, the immunophenotype of the abnormal L-HES T-cell clone is consistent with a T effector memory (TEM) subtype. However, TEM cells normally do not express CD62-L, contrasting with L-HES T-cell clones *in vitro*. It has also been reported that upon IL-17 RB engagement, TCM CTRH2+ cells repress surface CCR7 and CD27 while retaining CD62L and CTRH2, thereby suggesting that CCR7-CD27-CD62L+CRTH2+ expression characterizes an acquired effector-memory phenotype [76]. The fact that the T-cell clones share a similar phenotype suggests that freshly isolated CD3-CD4+ T-cells from the blood of this study's patients have acquired a TEM phenotype, which they maintain after stimulation in vivo. The same study also demonstrates that IL-25 promotes strong Th2 cells expansion and Th2 cytokines production when stimulated with thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine expressed by skin and bronchial cells [76]. Keratinocytes, mast cells eosinophils and basophils express IL-25 transcripts, while IL-5-activated eosinophils and basophils secrete IL-25 protein, which augments the activity of Th2 memory cells. Therefore, with the results, a model of a cell-mediated activation loop in inflammatory environments can be presented where the CD3-CD4+ cells are stimulated by activated DCs which enhance their IL-5 production and increased surface expression of IL17RB. IL-5-mediated activation of eosinophils promotes increased IL-25 secretion, which in turn further enhances the activation level of the IL-17RB-expressing CD3-CD4+ T-cells. Consistent with the presented model is the elevated expression of IL-25 and IL17-RB transcripts found in asthmatic lung tissues and atopic dermatitis skin lesions [76]. Furthermore, in allergic sites, mast cells and basophils

secrete PGD2, the CRTH2-ligand in order to locally attract Th2 cells and other CRTH2+ effectors including eosinophils and basophils [312]. As previously described, the engagement of CTRH2 strongly enhances IL-5 and IL-13 secretion in the absence of TCR/CD3 stimulation, and TLSP-activated DCs promote the proliferation of CRTH2+CD4+ Th2 cells [312-313]. However, such PDG2/CTRH2+ stimulation of CD3-CD4+ T-cells has not yet been tested *in vitro*. In conclusion, these two activation models are consistent with the general view of the persistence of CD3-CD4+ T-cells in inflamed skin environment, as observed in L-HES *in vivo*.

2. Molecular Characterization of the TGF-β Deregulated pathway

The altered expression of numerous genes involved in the TGF-β signalling pathway was observed in the molecular study of CD3-CD4+ T-cells from L-HES patients. The TGF-β superfamily includes approximatively 30 growth and differentiation factors divided into TGF-β, activin, inhibin, growth/differentiation factor (GDF) and bone morphogenetic protein (BMP) subgroups that regulate multiple cellular processes such as proliferation, differentiation, apoptosis, embryonic development, angiogenesis and other functions in a wide variety of cells [314-315]. They are three types of TGF-β receptors, respectively, I, II and III. In contrast to TGFβR1 and TGFβR2 receptors, TGFβR3 has no intrinsic signalling capacity but binds TGF-β with a high affinity increasing the local concentration of ligands and then transferring the cytokine to the other receptors TGFβR1, TGFβR2, activins and inhibins. When TGF-β ligand binds to activated TGFβRII, it induces the recruitment of TGFβR1 and activation by autophosphorylation of the TGFβR1/TGFβR2 heteromeric complex. This complex transphosphorylates the receptor-regulated Smad (R-Smad) proteins, Smad2 and Smad 3, that are associated with Smad4 as they are translocated to the nucleus. In contrast to TGFβs, the activins/nodal and inhibins that mediate activation through Smad2 and Smad3, BMPs and GDFs transactivate Smad1, Smad5 and Smad8. Consequently, they form two distinct nuclear transcription complexes with Smad4, the “TGFb/activin” and the “BMP” complexes, targeting different genes, respectively. In addition, this process is controlled by two inhibitory Smads (I-Smads): Smad6 and Smad7 [314-315]. Importantly, the TGF-β cytokines can also mediate their activity through the MAPK signalling pathway [316].

A decreased expression of type 1 TGF-β (*TGFBR1*) and activin (*ACVRIC*) genes with the type II receptor gene (*TGFBR2*) was found in CD3-CD4+ T-cells suggesting a negative regulation of the TGF-β signalling pathway. Interestingly, the downregulation of *TGFBR2* was reported twice in CD4+ T-lymphoma cells and related to reduced responsiveness to TGFβ1-mediated growth inhibition [292, 317]. In addition, it was speculated that the enhancement of TGFβRIII receptor's association with ActRIIA receptors- binding inhibins resulted in the inhibition of the TGFβ/activin pathway, to the benefit of the antagonist BMP pathway in the CD3-CD4+ cells. This hypothesis was further supported by the confirmation of the upregulation of *TGFBR3*, the type II activin receptors (*ACVR2A* and *ACVR2B*), BMP type I receptor *BMPRIA* and the *SMAD5* genes. Moreover, observation of the repression of *SMAD7* and *NOG* inhibitory genes associated with the upregulation of Smad5 target genes was consistent with an increased transcriptional activity mediated by Smad5/BMPR1 activation. It is worth noting that such Smad5 targets, which included *RUNX2* and oncogenic *MAPK8* genes, were both shown as upregulated in all three L-HES patients and overexpressed during P1's disease progression to T-lymphoma. Altogether, the signalling switches from the TGF-β to the BMP pathways seen in CD3-CD4+ T-cells possibly reflect the disruption of a normal homeostatic pathway, to the advantage of an abnormal unregulated growth process.

TGF-β induces the immune suppression of CD4+ T-cell proliferation without increasing apoptosis or modifying IL-4, IL-10 or INF-γ production, but by down-regulating IL-2 production upon TCR/CD3 triggering [318]. It has been shown that deprived serum CD4+ T-cells lose their TGF-β responsiveness and can proliferate at suboptimal antigen doses [319]. TGF- β also represses Th2 development by inhibiting GATA3 [320]. In addition, eosinophils activated by Th2-mediated inflammation secrete TGF-β1 that suppresses CD4+ proliferation in response to antigen activation *in vitro* and *in vivo* [321]. Together, these data are consistent with the hypothesis that the CD3-CD4+ T-cells from L-HES patients have persisted in an inflamed microenvironment secondary to their resistance to TGF-β and possibly helped by the combined absence of TCR expression.

3. Pro-Apoptotic and Anti-Apoptotic Deregulated Pathways in CD3-CD4+ T-Cells

Cancer results from the stepwise accumulation of genetic alterations in the essential genes that control normal pathways of cell proliferation, reparation and death. One of these fundamental pathways is programmed cell death or apoptosis whose deregulation promotes uncontrolled cell expansion. In the previous report, persistent L-HES CD3-CD4+ T-cell clones were shown to express a high level of FAS (CD95) and to be highly sensitive to spontaneous apoptosis in IL-2-deprived medium compared to control cells [142]. Moreover, supporting this paradox, an adjunct of soluble FAS-L *in vitro* induced a higher rate of apoptosis in the CD3-CD4+ T-cells relative to normal CD3+CD4+ T cells [142]. Furthermore, the microarray's results showing upregulated *FAS* transcription in CD3-CD4+ T-cells and the down-regulation of major anti-apoptotic genes such as *BCL2* and *FAIM3* confirmed the natural predisposition of L-HES T-cell clones to apoptosis *in vitro* contrasting with their expansion *in vivo*.

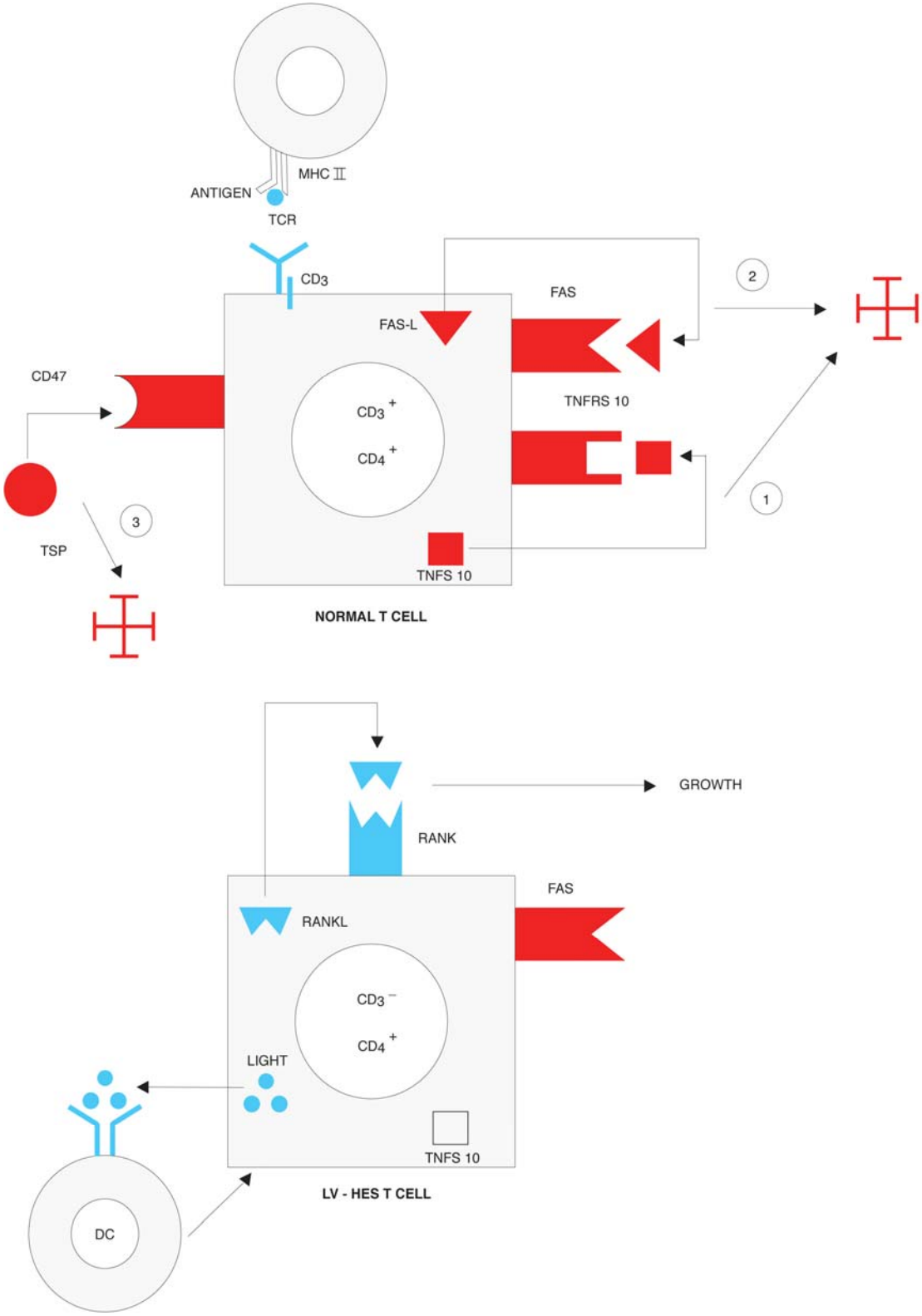
However, by exploring in details the expression profile of CD3-CD4+ L-HES clones under stimulated conditions and during disease progression, additional apoptotic and proliferative disrupted pathways (summarized in Annex 3 and Figure 9) were identified that might explain the survival of these cells *in vivo*. In particular, contrasting with normal T-cells, the data of this study have shown that CD2/CD28 and IL-2 co-stimulation of FAS+CD3-CD4+T-cells from three L-HES patients did not increase FAS-ligand gene transcription (*FAS-L/TNFSF6*) [145]. Since conventional CD4+ T-cells have been shown to increase FAS-L upon stimulation, these results demonstrate the disruption of the FAS/FAS-L activation pathway in CD3-CD4+ T-cells [91]. In addition, the results reveal an uncoupling of three other pro-apoptotic systems in CD3-CD4+ T-cells, including TNFRSF10/TNFSF10(TRAIL), CD27(TNFRSF7)/CD70 and TSP/CD47 caused by the absence of constitutive TNFRSF10(B–D), CD27 and CD47 receptors, respectively. Although the T_{EM} phenotype of CD3-CD4+ T-cells could have resulted in the down-regulation of CD27 and CD47 genes, the finding concerning the upregulation of the *TNFSF10* gene dissociated from the corresponding transcription of *TNFRSF10B/D* receptor genes is consistent with the alteration of the TNFRSF10/TNFSF10 pathway in L-HES T-cell clones during both chronic and acute phases of the disease [73, 145].

In addition, repression of critical genes coding for mediators of the extrinsic and intrinsic apoptotic programmes, or major tumour suppressors in T lymphoid neoplasm such as ATM (listed in Annex 3) was further detected in L-HES clones. [286].

Furthermore, three TNF-superfamily genes involved in T-cell mediated inflammation and proliferation were found overexpressed in CD3-CD4+ T-cells, namely, TNFRSF11A/RANK, its corresponding ligand TNFSF11/RANKL, and TNFS14/LIGHT. As the co-expression of RANK/RANKL might lead to an autocrine loop, and since RANK-L with LIGHT amplify the co-stimulatory signals provided by DC and stromal cells, these results suggest that the upregulation of RANK/RANKL and LIGHT could activate the growth of CD3-CD4+ T cells *in vivo* [291, 295]. Moreover, both RANK-L and LIGHT are under study for immunotherapy in cancer, inflammatory and autoimmune diseases [292-293]. Therefore, antibody directed against these proteins could represent promising therapeutic targets for L-HES in the future.

In conclusion, consistent with the observed persistence of CD3-CD4+ T-cells *in vivo*, the results of this study have demonstrated the transcriptional deregulation of important genes related to apoptosis/survival cellular mechanisms. In particular, the expression of major effector genes belonging to the TNF receptor/ligand superfamily was found altered. A proposed model summarizing such deregulation is provided in Figure 9.

Figure 9.Proposed model of the deregulated TNF super-family apoptotic pathways in CD3- CD4+ T-cells versus CD3+CD4+ T-cell controls



4. *BACH2* as a Haploinsufficient Tumour Suppressor Gene.

To date, 70 percent of cancer genes with acquired mutations are associated with leukemias and lymphomas, whereas chromosomal translocations represent the most common somatic mutation [322]. Although the vast majority of these translocations generate chimerical fusion genes with oncogenic properties, tumour suppressor genes have rarely been recognized in lymphoid neoplasm [259-260]. Furthermore, with the exception of the ATM gene, no tumour suppressor gene has been identified with T lymphoid lineage preference [286]. Expansion of abnormal circulating CD4+ Th2 cell clones causing secondary hypereosinophilia distinguishes L-HES from the other variants [112]. Moreover, although the clinical short-term evolution of L-HES seems benign several patients in this study have developed T-cell lymphomas with variable latency [113]. Therefore, the predisposition to T lymphomagenesis of L-HES has presented an invaluable model for studying the underlying genetic events leading to T-cell transformation, some of which will be discussed in the final part of this thesis.

4.1 Persistence and Recurrence of 6q- in CD3-CD4+ T-Cells

With the final objective to find new cancer genes flagged by chromosomal imbalances, the major cytogenetic changes occurring in the L-HES T-cell clones were first analyzed. The first cytogenetics analysis revealed a commonly deleted region located between bands q13 and q22.1 of the long arm of chromosome 6 in CD3-CD4+ T-cells from two L-HES patients [118, 124]. Next, the timeline studies of chromosomal aberrations objectivised the persistency and emergence of only one 6q- subclone associated with T-cell lymphoma development for one L-HES patient [124]. Furthermore, the last report described a complete 6q loss in CD3-CD4+ T-cell nuclei from a third L-HES patient and a translocation t(6;11) (q21;q23) was reported in a new patient with hypereosinophilia and T-cell lymphoma [123]. Finally, with the exception of primary central nervous system lymphomas where the deletion 6q22-23 reaches 66 percent, the frequency of 6q- observed in lymphoid neoplasms ranges between 20 and 40 percent in NHL and 10 percent in T-ALL ([185, 215-216, 218, 323-328]. Together, the cytogenetic data demonstrate for the first time that the 6q aberration is the most frequent and persistent anomaly present in the L-HES T-cell clones, reaching 60 percent of the cases cohort. However, with the experience gained in this work, 6q abnormalities can be

easily missed if they are not carefully analyzed on purified cells. Consequently, to accurately evaluate the incidence and prognosis significance of the 6q aberration in the next prospective studies, it is recommended to investigate the 6q13-22.1 region on DNA isolated from abnormal T-cells by CGH and to apply 6q13-22.1 FISH-probe analysis only on purified nuclei for practical purposes.

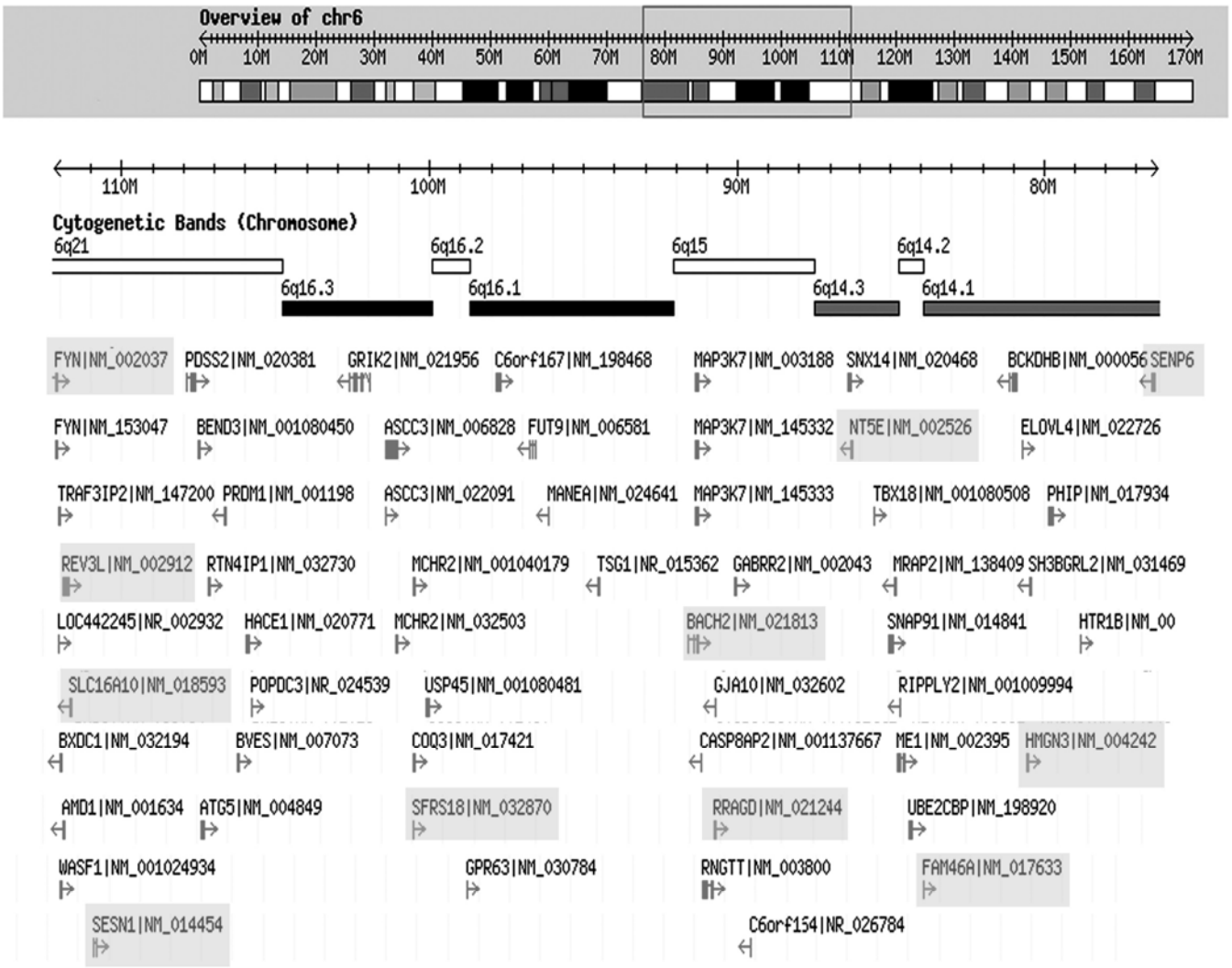
Although cytogenetic analysis has been rarely included in the diagnostic algorithm of L-HES, the results suggest that 6q aberration as a sole anomaly might be a specific disease characteristic that could be used as an early diagnostic marker for discriminating reactive from pre-/malignant lymphoproliferative T-cell conditions. It is also proposed that 6q- could potentially serve as an important prognosis indicator for the survey of the L-HES patients, although this will require careful analysis in the long term.

4.2 Identification of Candidate Suppressor Gene(s) of the 6q13-q22.1 Region

The finding that three out of five of the L-HES patients in this study were carrying a 6q genomic defect in their CD3-CD4+ T-cell clones provided a strong argument to focus on the gene expression pattern from this chromosomal region in order to pinpoint potent suppressor gene(s). From previous studies, 5 candidate tumour suppressor genes such as *BACH2* at band 6q15, *GRIK2* at 6q16.3, and *HACE1*, *PRDM1* and *REV3L* at 6q21 have been located in the 6q13-q22.1 commonly deleted region of chromosome 6 (Figure 10)[215, 220, 234, 247, 252].

In the last report, transcriptomic profile of three L-HES T-cell clones correlated with the minimal deleted region (MDR) at 6q13-22.1, which enabled the identification of 13 repressed target genes. By increasing statistical significance with a study of five L-HES patients, eleven potent anti-oncogenes were selected, including *SENP6*, *HMGN3*, *FAM46A*, *NT5E*, *RRAGD*, *BACH2*, *SFRS18*, *SESN1*, *SLC16A10*, *REV3L* and *FYN* genes (grey colour, Figure 10). By analyzing data from the literature, only *RRAGD*, *BACH2*, *SESN1* and *REV3L* genes appeared to possess tumour-related functions and/or evidence of constitutive expression in CD4+ T-cells [224, 250, 253, 329], therefore representing an interesting candidate for suppressor genes. However, the timeline of the transcriptomic analysis, which revealed the early and continuous repression of solely *BACH2* during T-lymphoma transformation, suggests that among these four genes, only *BACH2* is the most relevant candidate suppressor gene that plays an early role in L-HES pathogenesis.

Figure 10.Mapping of the candidate tumour suppressor genes of the 6q14.1-21 region



In contrast, although GRIK2, *HACE1*, *MAP3K7* and *CASP8AP2* genes located within the 6q13-22.1 deleted region (Figure 10) were previously described either as a candidate tumour suppressor gene in T-ALL and NK/T-cell lymphomas [235] or prognosis markers in childhood T-ALL [185] and high grade prostate cancer [330], respectively, none of these genes were retrieved through the comparative expression array or quantitative RT-PCR analysis. Interestingly, similar to its neighbour *CASP8AP2* gene, *BACH2* gene transcription was shown to be significantly decreased in the transcriptomic profiles obtained from the childhood T-ALL cohort with 6q deletion (supplementary data from [185]). Therefore, these published data suggest an unexpected role for *BACH2* in this condition, which requires further investigation.

In parallel, the results of this study showed an increased expression of *PRDM1* tumour suppressor gene during P1's T lymphomagenesis. Hence, as overexpression of PRDM1β isoforms has been reported in both T and B lymphomas, the results provide a preliminary basis for further experiments to verify the nature and potential oncogenic role of the transcripts produced in P1's T-cell clone [244, 247]. Moreover, since *PRDM1* locus resides within the hemizygous deleted region at 6q21, and its expression is unchanged during the chronic disease of the L-HES patients, the results suggest that *PRDM1* upregulation could represent a late transforming and epigenetic event during P1's T lymphomagenesis.

4.3 *BACH2* as a Haploinsufficient Tumour Suppressor Gene

To directly test the suppressive properties of *BACH2* in a T-cell lineage, functional experiments were realized by using the Jurkat E6.1 CD3+CD4+ T-cell line. The first evidence of the anti-oncogenic effect of BACH2 was obtained by showing the abrogation of clonogenicity following enforced *BACH2* expression in Jurkat cells. Since no homozygous 6q15 deletion or biallelic inactivation of *BACH2* gene could be found in the CD3-CD4+ T-cell clones of this study's patients, as expected when fulfilling Knudson's two-hit paradigm, the first hypothesis was that *BACH2* could represent a haploinsufficient tumour suppressor gene. Therefore, an RNA-mediated interference (RNAi)-based approach to stably suppress *BACH2* gene expression was adopted in Jurkat cells as similar strategy was successfully used for identifying haploinsufficient *RPS14* tumour suppressor gene in the 5q- myelodysplastic syndrome [331]. An increase of clonogenicity observed for shRNA BACH2-silenced Jurkat clones was already consistent with the "loss of function" phenotype of a tumour suppressor gene. However, in order to further demonstrate the role of *BACH2* during cell apoptosis, the survival effect of *BACH2* gene ablation in Jurkat T-cells was examined under genotoxic stress. As demonstrated with various etoposide concentrations, the data showed a significant increase (30-70 %) of apoptosis resistance mediated by *BACH2* silencing in Jurkat clones. Moreover, as described for other suppressor genes such as *NKX3*, the level of *BACH2* transcript's extinction was correlated with the percentage of apoptosis protection, thus providing evidence for a "gene dosage" effect [169]. Together, the data demonstrate that the BACH2 transcription factor is a tumour suppressor gene whose haplo-insufficiency modulates the apoptosis resistance of CD4+ T-cells such as a rheostat.

4.4 BACH2 Mechanism of Suppressive Action

Cytotoxic drugs, including etoposide, activate apoptosis through at least one of the two major pathways: the intrinsic (mitochondria-mediated) and extrinsic apoptotic (receptor-mediated) signalling pathways [82, 332-334]. However, it remains unknown by which exact mechanism *BACH2* inhibition provides protection against the etoposide-mediated apoptosis of Jurkat T-cells. Following genotoxic stress, the apoptosis RT-profiler experiments showed early decreased expression of a limited set of four pro-apoptotic genes, namely *BIK*, *CASP1*, *FAS-L* and *RIPK2* genes, in BACH2-silenced clones. Notably, *BIK*, redundant with *BCL2-like 11* (*BCL2L11/BIM*), is a strong mitochondrial pro-apoptotic inducer in several tissues, and is transcriptionally activated by several anti-cancer drugs [335]. Furthermore, thymocytes from BIM-deficient mice are more resistant to many apoptotic stimuli such as IL-2 withdrawal [336]. However, *RIPK2* has been reported to induce extrinsic FAS-mediated-programmed cell death by enhancing caspase 8 activity [279]. Interestingly, among the differentially pro-apoptotic genes altered in CD3-CD4+ T cells from L-HES patients, *RIPK2* and *BCL2L11* transcripts were also found to be consistently repressed [145]. Hence, the results demonstrate the repression of two important mediators of both extrinsic and intrinsic apoptotic pathways in BACH2 silenced Jurkat T cells and in BACH2-deleted CD3-CD4+ T-cells. This suggests a key regulatory role of BACH2 in these two convergent intracellular death routes.

Furthermore, etoposide is a DNA damaging drug that has been shown to promote apoptosis by inducing the transcription of *FAS-L* and *TRAIL* in responsive tumour cells [337-339]. Since silenced BACH2 clones were shown to downregulate *FAS-L* transcription under etoposide induction, these data suggest a FAS/FAS-L apoptotic defect in these cells. Consistent with this hypothesis, the flow cytometric analysis following etoposide treatment confirmed the absence of FAS-L on *BACH2*-silenced Jurkat clones contrasting with the FAS-L positive controls. Importantly, no difference of CD95/FAS level or of apoptosis resistance to exogenous FAS-L addition was detected between the control and BACH2-silenced clones, excluding alteration of the FAS receptor pathway. Therefore, it can be concluded that the *BACH2* gene directly or indirectly regulates the transcription of solely *FAS-L* and consequently the autocrine FAS/FAS-L apoptotic loop in CD4+ Jurkat T-cells, a mechanism similar to the activation-induced cell death (AICD) used to shut down T-cell mediated

immune responses [340]. Cumulatively, the results suggest for the first time a potential role of BACH2 as a major switch control in CD4+ T-cell homeostasis.

4.5 Suggestion of a T lymphomagenesis Model in L-HES

Normal effector memory T-cells are intrinsically sensitive to apoptosis and have upregulated levels of *FAS-L* and *BIM* [336, 340]. In contrast, as discussed in section 3 of this work, memory CD3-CD4+FAS+ T-cells under-expressing *BACH2* exhibit low levels of constitutive *FAS-L* and are unable to upregulate consistently *FAS-L* transcription upon CD2, CD28 and IL2 stimulation [145]. Moreover, despite increased apoptotic response to adjunct of FAS-L, it has been shown in the present work, that similarly to BACH2-silenced Jurkat clones, the etoposide-treated CD3-CD4+T-cells from one L-HES patient do not undergo FAS-L-mediated apoptosis caused by the hemizygous deletion of *BACH2* [142] . This suggest that deletion of the 6q *BACH2*-locus plays an important role in L-HES malignant process by conferring a clear survival advantage to CD3-CD4+ T-cell clones, and as such, can be considered as an early transforming event that may lead to T lymphomagenesis in L-HES (Table 4.).

Th2 polarization	**L-HES**	**persistent L-HES**	**T Lymphoma**
	step 1	step 2	step 3
CD3+CD4+ T cell →	TCR clonality + CD loss →	BACH2↘ / 6q- (Fas-L resistance) →	further mutations (RUNX2/PRDM1,..)

Table 4. Model of progression to T lymphoma

Supporting this view, autoimmune diseases have been often reported to be associated with a significant risk of cancer or lymphomas development [273, 275-277]. Moreover, germinal mutations in *FAS-L* gene result in the autoimmune lymphoproliferative syndrome (ALPS) type IB syndrome which is characterized by the uncontrolled proliferation of CD3+CD4-CD8- T-cells progressing in some cases to T-cell lymphoma [273, 341]. In addition, published data and the finding of this study (discussed in section 2) indicate that memory Th2 cells are potent producers of lymphokines and are able to regulate both adaptive and innate immunity by modifying the tissue microenvironment [27, 108]. Therefore, the

deregulated L-FAS pathway mediated by *BACH2* hemizygous deletion in L-HES effector memory T-cells provides a relevant explanation why these premalignant cells persist in L-HES, at the same time inducing hypereosinophila with undesirable inflammatory effects linked to their specific Th2 nature. This is believed to be the first report of a BACH2/FAS-L defect that may lead to the perpetuation of a Th2 disease. In this manner, *BACH2* reflects the function of a landscaper tumour suppressor gene which modifies the cellular microenvironment, although in a variable mode, depending on the lineage of the mutated CD4+ clone. Recent reports using genome-wide association studies in human subjects have pinpointed the *BACH2* gene as a risk locus for type I diabetes and celiac disease among genes with immune functions [342-344]. In addition, deregulation of the FAS/FAS-L system has been described in thymocytes from the non-obese diabetic mouse (NOD) studied as an animal model for type I diabetes [345]. However, the relationship between these two observations remains unclear. Having demonstrated the functional role of BACH2 in CD4+ T-cell apoptosis mediated by FAS-L, it is relevant to verify the BACH2 pathway integrity in CD4+ T-cells of predisposed diabetic and celiac patients, since this mechanism may be implicated in the peripheral and/or central deletion of autoreactive T-cells. Moreover, further work is needed in order to decipher the positive and negative factors controlling BACH2 gene expression, and thereby fine tuning FAS-L-mediated apoptosis for new therapeutic targeting. Finally, by showing the tumour suppressive properties of *BACH2* in CD4+ T-cells, the results of this study simultaneously pave the way for new research perspectives for understanding of T-cell homeostasis.

CONCLUSION AND PERSPECTIVES

The phenotypic and genetic data presented in this thesis have shed light on new defects underlying L-HES pathogenesis, which potentially could lead to targeted therapeutic approaches. The data have identified immunophenotypic traits that more fully characterize the CD3-CD4+ T-cells, including increased expression of IL17RB and CTRH2, which is consistent with the clonal Th2 cell's inflammatory tropism, persistence and crosstalk with eosinophils. Furthermore, changes in TGFβ superfamily-directed and apoptotic pathways were detected that may play a role in the prolonged survival of the CD3-CD4+ T-cells *in vivo*.

The recurrence and persistence of the 6q chromosomal deletion observed in the CD3-CD4+ T-cells from three L-HES patients suggests that this chromosomal aberration could be a specific disease characteristic representing an early diagnostic marker that discriminates reactive and malignant lymphoproliferative T-cell diseases.

The identification of the 6q-located *BACH2* as a haploinsufficient tumour suppressor gene in CD4+ T cells may be the foundation for a new model of T lymphomagenesis. Simultaneously, it provides evidence that the suppressive effect of BACH2 is mediated via *FAS-L,* a major player in the activation-induced cell death (AICD) pathway. This suggests a critical role for BACH2 in CD4+ T-cell homeostasis. These data are the first experimental evidence that BACH2 exerts a regulatory effect on the FAS-L extrinsic apoptotic pathway in CD4+ effector memory T-cells.

Taken altogether, these data provide an explanation for why the *BACH2*-deficient L-HES CD4+ T-cells, which are unable to undergo FAS-L mediated apoptosis, can persist and expand to produce Th2 cytokines upon stimulation, thereby provoking secondary hypereosinophilia, and in some patients, accumulating additional abnormalities that eventually lead to full-blow T lymphoma.

The results of this study warrant further investigation to verify the integrity of the FAS-L pathway and its modulation by BACH2 in other persistent CD4+ T-cell based autoimmune, infectious and lymphoproliferative diseases such as lymphomas, asthma, diabetes, atopic dermatitis, arthritis, cancer, as well as celiac and parasitic diseases.

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