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Molecular characterization of unresponsiveness to BiTE CD19-CD3 therapy in adult acute lymphoblastic leukemia

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B-cell acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease at clinical and genetic levels, characterized by the sequential acquisition of genetic aberrations which drive leukemic clone initiation and maintenance. Furthermore, several evidences suggest that the pre-leukemic clone can seem dormant prior to activation by primary abnormalities, which are often chromosomal translocations, resulting in chimeric fusion genes, or the acquisition/deletion of genetic material, hyperdiploidy or hypodiploidy, respectively; whereas secondary abnormalities are commonly DNA copy number alterations (CNA) and sequence mutations (Fig.1)[2;4].

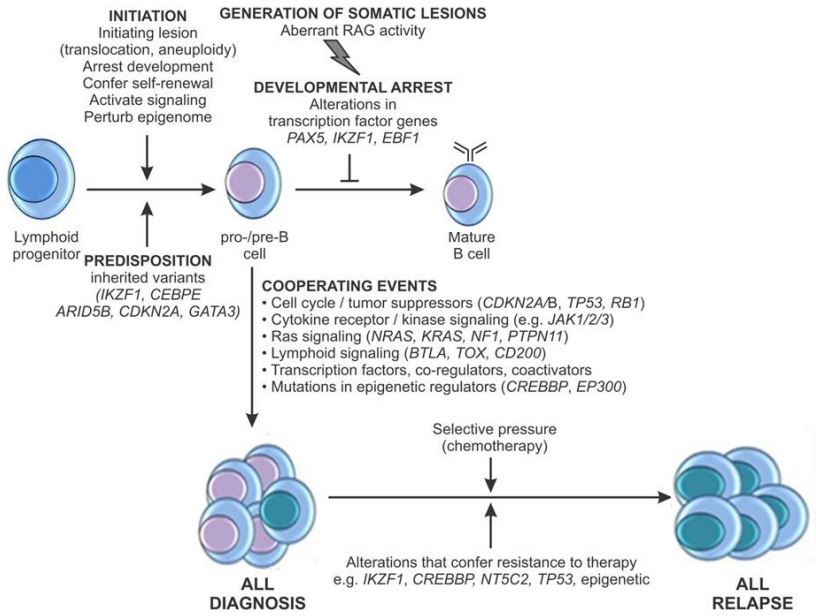

Fig.1[(2)] Schema for genomic alterations and their correlation with ALL development and resistance to therapy

The role of inherited predisposition to ALL development, in association with initiation alterations, remains still undefined; the Down syndrome is associated with an increased risk of developing both AML and ALL, but otherwise ALL has not been considered to have a strong heritable component. However, genome-wide association studies, using also ethnically matched controls, have identified an increasing number of common inherited variants that modulate risk of susceptibility to ALL and inherited mutations in familial ALL. *ARID5B* polymorphisms are associated with ALL risk and outcome in specific ethnic groups, *GATA3* variants are associated with Ph-like ALL and ALL in adolescents and young adults. Deleterious germline mutations have been identified in familial and sporadic ALL; in addition to *TP53* germline mutations in low-hypodiploid ALL, hypodiploid cases exhibit germline mutations known to activate RAS signaling (involving *NRAS* and *PTPN11*) and DNA repair that are likely to be pathogenic.

In addition to initiation alterations and inherited predisposition, the gene expression modulation could contribute to define distinct pathological entities because specific epigenetic signatures and mutations in genes encoding modifiers of the epigenome recur in different ALL subtypes and are associated with treatment failure. On the whole, hypermethylation is associated with gene silencing, while hypomethylation results in transcriptional activation. Regarding the cytosine methylation, leukemic cells, compared to their normal counterparts, have global hypomethylation with selective hypermethylation of CpG-rich regions in gene promoters. However, different ALL subtypes share a common set of aberrantly methylated genes in comparison to normal lymphoid progenitors; thus, perturbations in methylation might be a consequence of the founding genetic alterations and might act together with structural genetic changes to promote leukemogenesis. In ALL, also, recur mutations in genes encoding epigenetic regulators and chromatin-modifying proteins. In particular, *WHSC1* in *ETV6–RUNX1*-positive ALL, *CREBBP* (a H3K18 and H3K27 acetylase) in relapsed and hypodiploid ALL, mutations of *SETD2* (a H3K36 trimethylase), *KDM6A* (lysine [K]-specific demethylase 6A) and *MLL2* in relapsed ALL[3].

Overall, ALL is characterized by an arrest in lymphoid development and alterations of genes regulating early stages of this process involve most commonly *PAX5*, *IKZF1*. *PAX5* is required for B lymphoid lineage commitment and maturation; the related genetic alterations include deletions, sequence mutations and chromosomal rearrangements which encode *PAX5* chimeric gene fusions, resulting in impaired PAX5 function and contributing to arrest B-cell development. In addition, recently two subtypes are identified as *PAX5*-altered (PAX5alt) and PAX5 p.Pro80Arg in B-ALL; PAX5alt subgroup include several *PAX5* alterations (rearrangements, focal intragenic amplifications and sequence mutations), exhibiting an enriched frequency of *PAX5* rearrangements and nonsilent sequence mutations compared to other B-ALL cases. Instead, PAX5 p.Pro80Arg mutation affects the PAX5 DNA-binding domain, impairing ability to bind DNA and transcriptionally activate target genes[17].

The DNA-binding transcription factor IKAROS (IKZF1) is required for development of all lymphoid lineages. The *IKZF1* gene lesions involve frequently the whole gene (DEL1-8), resulting in loss of wild-type IKZF1 expression, as well as deletions that alter the IKZF1 function, such as the dominant-negative isoform IK6 (DEL4-7). Other common variants include deletions affecting exons 2-3, 2-7 and 4-8. In addition, IKZF1 function is compromised by insertions, frameshift and missense mutations, which represent ~7% of *IKZF1* alterations in B-ALL. Furthermore, have been identified rare in-frame fusions involving *IKZF1*, such as *IKZF1-NUTM1*, *IKZF1-SETD5* and the reciprocal *SETD5-IKZF1*; however, is still not established whether these fusion genes are pathogenic and contribute to leukemogenesis. The *IKZF1* gene alterations result in impaired

lymphoid maturation, but have additional roles in conferring hematopoietic stem cell-like properties such as enhanced self-renewal, aberrant adhesion to bone marrow stroma, resistance to chemotherapy and tyrosine-kinase inhibitors (TKIs) and disease relapse in both *BCR–ABL1*-positive and *BCR–ABL1*-negative ALL. Additional commonly mutated genes in ALL affect pathways involved in tumor suppression and cell-cycle regulation (*TP53, RB1* and *CDKN2A*), cytokine receptor, tyrosine-kinase and RAS signaling (*CRLF2, EPOR, IL7R, ABL1, ABL2, CSF1R, PDGFRB, JAK2, NRAS, KRAS* and *NF1*), lymphoid signaling (B and T lymphocyte associated protein [*BTLA*] and *CD200*) and epigenetic modification (*EZH2, CREBBP, SETD2, MLL2*, and *WHSC1*).

ALL involves B-cell precursor lineage (B-ALL) or, less commonly, T-cell precursor lineage (T-ALL). The genes altered in each pathway and types of alteration (chromosomal rearrangements, deletion/amplification or sequence mutations) vary according to ALL subtype and the frequency of each B-ALL subgroup varies with age (Fig.2-3). Indeed, the prevalence of hyperdiploidy and *ETV6–RUNX1*-positive ALL declines from 25%–30% in children to less than 3% in young adults (age 21–39). Conversely, *BCR–ABL1*-positive ALL is uncommon in children (found in only 2%–5% of patients), but accounts for one-quarter of adult patients with ALL[1;3;17;18].

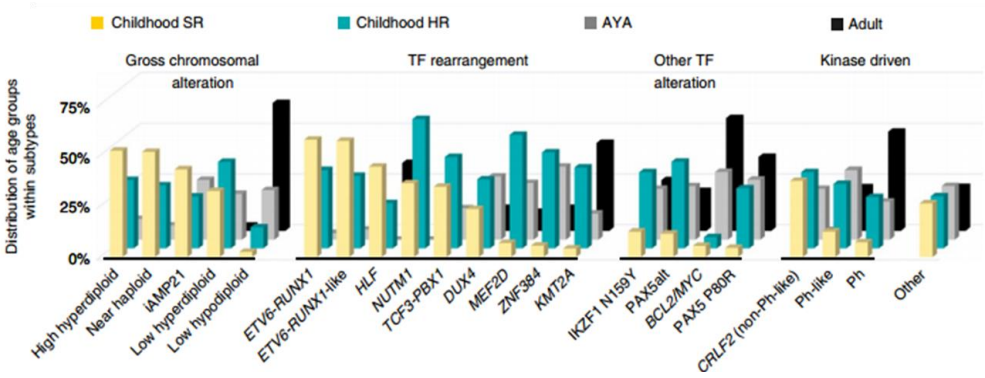

Fig.2[17] The distribution of B-ALL subtypes within each age group

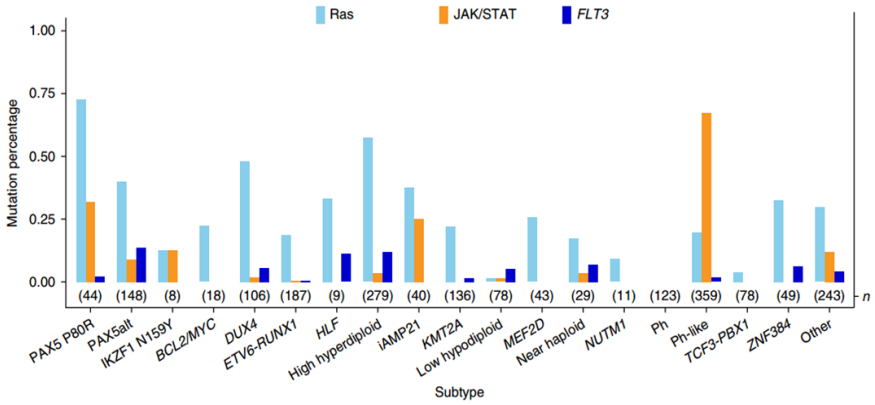

Fig.3[17] Distribution of mutations in three key signaling pathways in different B-ALL subtypes

The identification of alterations is important for diagnosis, risk classification, and, for some lesions, targeted therapy.

MLL-AF4

The *MLL* (mixed-lineage leukemia or myeloid-lymphoid leukemia) gene consists of at least 37 exons and its translated protein includes: three AT hooks motifs binding to the minor groove of DNA and influencing the chromatin structure, a transcriptional repression domain including a cysteine-rich region of homology with DNA methyltransferase, which is involved in the epigenetic regulation of transcription by methylation, 2 zinc-finger domain (PHD - plant homology domain) involved in protein-protein interaction, a serine/threonine rich region acting as a trans-activator and a SET domain, in the C-terminal region, through which MLL catalyses histone H3 Lys4 methylation. The MLL protein directly binds to *HOXA9* and *HOXC8* promoters, regulating *HOX* target genes expression, and interacts with the nuclear protein Menin (MEN1), which is essential for *MLL*-mediated transcription and for *MLL-AF4*-mediated transformation. MLL is, also, a member of a complex composed by at least 27 proteins involved in epigenetic regulation, including nucleosome remodeling, histone deacetylation and methylation processes; moreover, is involved in HSCs self-renewal and is essential to maintain the hematopoietic potential during both embryonic and adult development.

AF4 is a member of the AF4/FMR2 family, involved in RNA polymerase II releasing, and is part of the AEP complex, which includes other members of the AF4/FMR2 family (AF5Q31), the ENL family (ENL and AF9) and the p-TEFB elongation factor. AF4 is ubiquitously expressed, but its expression level is higher in the lymphoid compartment and placenta; can also promote CD133 expression, a cell surface marker of hematopoietic and cancer stem cells important for selectivity factor 1 (SL1) recruiting, which ensures the loading of TBP to the TATA box. Through N-terminal portion, AF4 can bind to the p-TEFB kinase, that phosphorylates the largest subunit of RNA polymerase II, DSIF, the NELF complex, UBE2A, but also recruits TFIIH and MEN1. AF4 is the docking platform for AF9 or ENL, which both interact via AF10 with DOT1L, enabling methylation of lysine-79 residues of histone H3 proteins, a prerequisite for the maintenance of RNA transcription[13;14].

The *MLL* gene (11q23) rearrangements are common genetic events in hematological malignancies and recur in around 10% of acute lymphoblastic leukemia and 5% of acute myeloid leukemia (AML). Genetic alterations involving the *MLL* gene are mainly the result of reciprocal recombination which leads to fusion of the 5'*MLL* gene portion with several translocation partner genes (TPGs); there are more than 80 genes that can be involved in chromosomal translocations

with *MLL* in leukemia, with *AF4*, *AF9* and *ENL* among the most common. The breakpoints can be various and the most of them localize in the region between exon 9 and intron 11, which represents the *MLL* gene breakpoints cluster region. Another alteration is *MLL* recombination involving only chromosome 11, based on two independent DNA strand breaks associated either with inversions or deletions on 11p or 11q (Inv, Del). The *MLL* fusion genes usually occur in tumors of specific hematological lineages (e.g. *MLL-AFX1* in T-ALL, *MLL-AF4* in B lineage ALL, *MLL-EEN* in AML, *MLL-ENL* in ALL/AML), suggesting that the *MLL* partner plays a critical role in disease phenotype definition. Thus, the fusion protein can affect the hematopoietic pluripotent stem cells or the lymphoid/myeloid committed stem cells differentiation. Indeed, the *MLL* fusion gene could immortalize an early progenitor cell having both lymphoid and myeloid potential or could, also, induce commitment to lymphoid or myeloid lineage followed by differentiation arrest. The epigenetic abnormalities result in upregulation of genes normally expressed in HSCs such as *HOXA9*, *MEIS1*, *HMGA2* and *RUNX1*, interfering with cell survival and apoptosis regulation and stem cells commitment. *MLL-AF4* can also increase the expression of BCL-2, resulting in resistance to apoptosis, MCL-1 and repress the transcription of *BIM* through promoters binding.

The frequency of $t(4;11)^{+}$/*MLL-AF4*$^{+}$ ALL is between 50%-85% in infant subgroup, decreases in older infants (10%–20%) and drops to 2% in children; in adult B-ALL accounts for ~10% of cases. The *MLL-AF4* fusion gene is recognized as prognostic biomarker of high-risk leukemia at all ages. Furthermore, the prognosis of *MLL-AF4*$^{+}$ leukemia can be predicted by FLT3 expression: high FLT3 expression is associated with a bad prognosis, a shorter overall survival and a shorter disease-free survival, while low expression is associated with a better prognosis. $T(4;11)^{+}$ leukemia blasts often express hematopoietic progenitor markers (CD34), B lymphoid and myeloid markers (CD19, CD22, CD79a, Tdt, CD15, CD65) and components of the leukocyte antigen complex (HLA-DR); are negative for CD24, IgM, CD13 and CD33.

The reciprocal fusion gene *AF4-MLL* is present in approximately 80% of patients diagnosed with $t(4;11)^{+}$/*MLL-AF4*$^{+}$ leukemia and, similar to *MLL-AF4*, contains cleavage sites for threonine-Aspartase 1 (Taspase 1), which are important for its activation and its resistance to proteasomal degradation. *AF4-MLL* can associate with proteins that modulate the activity of RNA polymerase II promoters (e.g. P-TEFB kinase) and alter the histone methylation signature by recruiting H3 arginine and lysine methyltransferases, such as CARM1 and DOT1L, leading to an increased H3 methylation signature. Therefore, both *MLL-AF4* and *AF4-MLL* can increase H3K79, H3R2, H3R17 and H3R26 methylation marks. Additionally, *AF4-MLL* can increase the H3K4 methylation status and extend the activation of gene expression.

However, MLL-AF4 expression in the stromal compartment may increase niche support for the cell-of-origin and leukemia blasts. In addition, *MLL-AF4* and *AF4-MLL* can modulate *ALOX5* gene expression through the activation of transcription and elongation; *ALOX5* gene encodes the 5-lipoxygenase enzyme, which is essential for proinflammatory leukotrienes production. These arachidonic acid metabolites can activate the immune response and help to establish a tumor microenvironment by acting both on cancer cells and stromal cells; are, also, associated with cell proliferation (Fig.4)[4;5;14].

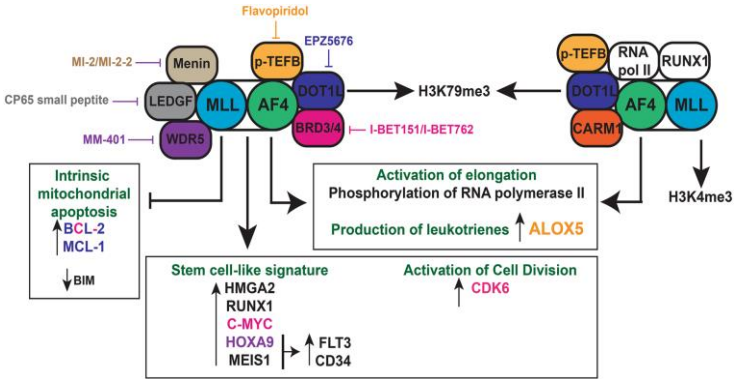

Fig.4[(5)] The MLL-AF4 fusion gene and AF4-MLL reciprocal fusion gene associated with their main interaction partners. The DOT1L inhibitor (EPZ5676), I-BET inhibitor (I-BET151), p-TEFB inhibitor (flavopiridol), WDR5 inhibitor (MM-401) and LEDGF inhibitor (CP65 small peptide) are potential therapeutic agents that target various members of the MLL-AF4 complex and its regulated genes

E2A-PBX1

E2A-PBX1 fusion gene, resulting by 1;19 chromosomal translocation, recurs in ~3% of children/adolescents and 6% of adults affected by ALL; *E2A-PBX1*$^{+}$ leukemia blasts express CD10, CD19, CD34 surface antigens. The t(1;19) results in chimeric transcription factor containing the N-terminal transactivation domain of E2A fused to the C-terminal DNA-binding homeodomain of PBX1. *E2A-PBX1* is associated with intermediate risk disease; however, some clinical study groups classify *E2A-PBX1*$^{+}$ adult patients as high risk class and treat this subgroup more aggressively[4;15].

The *E2A* locus, also known as *TCF3*, expresses two proteins: E12 and E47. Once these helix-loop-helix proteins dimerize, bind E-box sites on promoters and enhancers to regulate gene expression. *PBX1* is a gene coding for a TALE-class homeodomain transcription factor that interacts with other homeodomain-containing nuclear proteins such as HOX and MEIS to form heterodimeric transcription complexes. PBX1 is highly expressed in hematopoietic progenitors and, also, plays a pivotal role in maintaining long-term HSCs quiescence and in lymphopoiesis.

The *E2A-PBX1* fusion gene coding for a chimeric transcription factor that retains ability to interact with *HOX* genes, but not with MEIS; its activity can be regulated by interaction with GCN5 due to

increase its stability or with HDM2 to initiate its ubiquitination-mediated degradation. Under physiological conditions PBX1 transcriptional activity is low, while E2A-PBX1 can constitutively activate the transcription of PBX1 targets, including PBX, POU and STAT transcription factors families. E2A-PBX1 functionally affected pathways, involving in leukemogenesis, apoptosis, hematopoietic lineages regulation and cell cycle, include the WNT signaling. Among its related proteins, WNT16, a protein that can activate the expression of NOTCH ligands required for definitive hematopoiesis, is detected as a target of E2A-PBX1; in addition, E2A-PBX1 and GCN5 interaction facilitates WNT16 expression.

Furthermore, it's investigated HOXA9 and HOXB4 role in t(1;19)$^{+}$/*E2A-PBX1*$^{+}$ B-ALL mouse models. The overexpression of HOXA9 promotes B-cell proliferation followed by faster disease progression, in association with reduced PAX5 and EBF1 expression levels and FLT3 activation; while HOXB4 expression in vitro increases pro-B cells production and is concurrent with higher number of leukemia-initiating cells detection. In addition, in *E2A-PBX1*$^{+}$ mouse leukemia blasts occurs the hyperphosphorylation of PLCγ2, a key enzyme in B-cell receptor signaling. The hyperphosphorylation is associated with an increased proliferation and is mediated by ZAP70 (zeta chain associated protein kinase), LCK (lymphocyte-specific tyrosine kinase from the SRC family) and SYK (spleen tyrosine kinase), which are downstream targets of *E2A-PBX1* fusion gene. SYK can upregulate also BCL-6, involved in polyclonal B-cells formation and pre-B-cells self-renewal.

Moreover, the inhibition of PI3K/AKT/mTOR pathway through mTOR activity inhibitor, rapamycin, eradicates leukemia blasts in t(1;19)$^{+}$ leukemia cell lines, suggesting the PI3K/AKT/mTOR pathway involvement in *E2A-PBX1*$^{+}$ ALL. Furthermore, Aurora kinase B (AurB) expression is even higher in t(1;19)$^{+}$/*E2A-PBX1*$^{+}$ patients than many types of pediatric pre-B-ALL, in which is also overexpressed (Fig.5)[5;15].

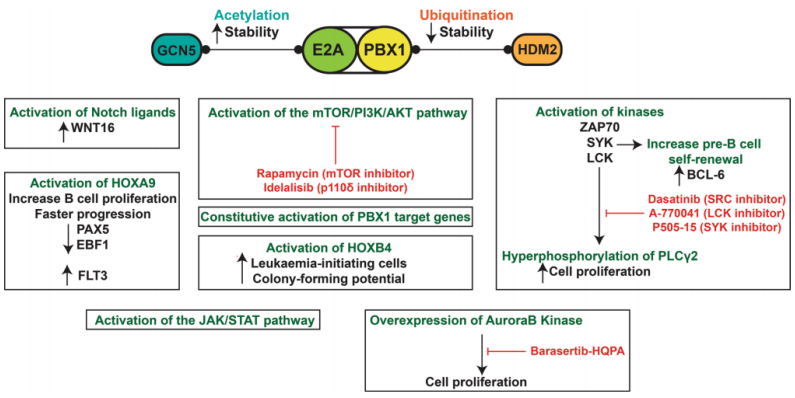

Fig.5[(5)] E2A-PBX1 can interact with GCN5 to increase its stability through acetylation or with HDM2 to initiate its degradation through ubiquitination. Potential therapeutic agents that target pathways affected by E2A-PBX1 gene fusion are shown in red

The Philadelphia chromosome in ALL

The Philadelphia (Ph) chromosome recurs in about 1%–3% of pediatric pre-B-ALL and around 25% of ALL adult patients. The chromosomal translocation leads to fusion the *BCR* gene 5' region with the sequences upstream of the second exon of the *ABL1* gene *(BCR-ABL1* or *BCR-ABL*); if the translocation involves *BCR* exon 1 resulting in the p190 fusion protein while, if the breakpoint affects *BCR* exon 13 or 14, the fusion gene coding for a 210 kDa protein (p210). Additional genetic features are a *GATA3* susceptibility to ALL locus (rs3824662), overrepresented in all patients, but even more in Ph^+ ALL adolescents and young adults and relapsed patients, and *IKAROS* deletion, pivotal for the leukemia maintenance.

BCR has an intrinsic serine/threonine kinase activity that is responsible of BCR serine and threonine residues phosphorylation; in addition, the BCR protein can also be phosphorylated by BCR-ABL1. The phosphorylation of BCR tyrosine residue 177 leads to the binding of GRB2, an activator of RAS, while the phosphorylated tyrosine 360 is important for the BCR-mediated trans-phosphorylation of casein and histone H1, but also for serine/threonine kinase activity inhibition. ABL is a member of the ABL cytoplasmic tyrosine kinases c-ABL family and can be found in the cytoplasm as well as in the nucleus, where exhibits DNA-binding activity; is involved in hematopoiesis and B lymphoid development so that the ablation of the ABL C-terminal portion in mice leads to a reduction in the B-cell compartment, especially at pro-B and pre-B stages. In addition, in $CD34^+$ human bone marrow cells, the inhibition of c-ABL through a specific antisense oligonucleotide leads to an accumulation of cells in the cell cycle G0/G1 phase and a decrease in proliferation, causing a significant loss in the formation of granulocyte-macrophage colonies. Moreover, ABL1 activates JAK2 phosphorylation in hematopoietic cells, whose pathway is involved and altered in many hematological malignancies[5].

Both BCR-ABL1 fusion proteins, p190 and p210, have constitutive kinase activity that can affect several pathways including RAS, RAC, PI3K/AKT/mTOR, NF-κB and JAK/STAT. Indeed, p190 and p210 can interact with the p85α regulatory subunit of PI3K through their SH2 domain and resultant activation of the PI3K/AKT pathway effects an upregulation of BCL-2 and C-MYC. Furthermore, interactors of both BCR-ABL1 fusion proteins are many cytosolic signaling components from tyrosine kinase and RAS–MAPK signaling pathways such as prototypic signaling adaptors (GRB2, SHC1, DOK1, GAB2), E3 ubiquitin ligases (Cbl, Cblb), phosphatases (Inppl1/Ship2 and Ptpn11/Shp2), kinases (Map4k1, Lrrk1), GTPase effector proteins (including the Ras GEFs Sos1/2) and cytoskeleton remodeling proteins (the Abi1–Wasf2–Cyfip2 complex), the tetrameric adaptor protein complex 2 (AP2), the kinase cochaperone Cdc37.

Overall, B-ALL displays a differentiation arrest at early B-cell development stages associated with constitutive RAG activity, terminal deoxy-transferase (TdT) expression and ongoing immunoglobulin gene rearrangements. RAG-mediated recombination can lead to *IKAROS* deletion in BCR-ABL1^{+} pre-B-ALL, facilitating leukemogenesis. Furthermore, the IL7 receptor (IL7R) is crucially involved in lymphoid development and its loss leads to reduced lymphoid cellularity in the peripheral blood. IL7R, once activated, can interact with JAK1 and JAK3 to phosphorylate and activate STAT5; the JAK/STAT pathway is often activated in leukemia blasts of Ph^{+} pre-B-ALL. P190 protein can mediate the tyrosine residues phosphorylation of STAT members, and JAK1 and JAK2 can be constitutively activated by mutations in the kinase and pseudo-kinase domains, which explains the sensitivity of Ph^{+} leukemia cells to JAK2 inhibitors (Fig.6)[5;19].

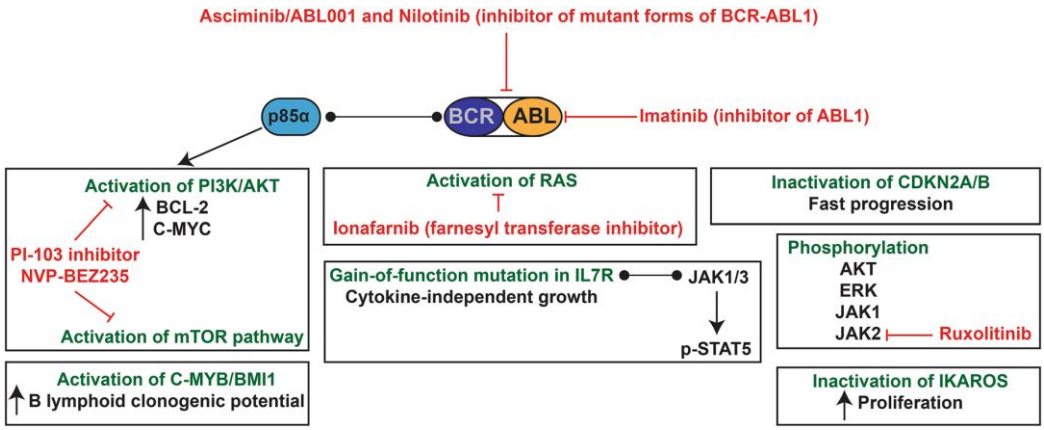

Fig.6[(5)] BCR-ABL1 pre-B acute lymphoblastic leukaemia

Chromosome number alterations

Chromosome number alterations characterize around 20%–30% of pediatric and around 3% of adult B-ALL cases. Additional mutations recur in *FLT3* (~25%), *NRAS/KRAS* (~15%) or *PTNP11* (~10%) genes, which are also detected in others cytogenetic classes of pro-B and pre-B-ALL; other frequent abnormalities involve deletions in loci of *ETV6*, *CKDN2A*, *PAX5* and *PAN3*. High hyperdiploidy (≥51 chromosomes) is associated with a good outcome and related overall survival rates at five years is over 90% in pediatric ALL and 55% in adult ALL, while the chromosome number less than 44 is associated with a poor outcome[4;5].

Hypodiploidy refers to whole chromosomes loss and can be further divided into low-hypodiploidy (LH) with 31–39 chromosomes or high-hypodiploidy (40–43 chromosomes). In addition, near-haploid (NH) ALL exhibits 24–30 chromosomes, while near-diploid cases are associated with 44–45 chromosomes. Furthermore, NH-ALL and LH-ALL are characterized by additional genetic alterations. In around 71% of near-haploid patients recur alterations that lead to tyrosine kinase receptors and RAS signaling activation, including focal deletions of *NF1*; 13% of near-haploid

cases exhibit deletions and one frameshift mutation in *IKZF3* gene. Instead, in LH-ALL patients recur *TP53* biallelic alteration (loss of one chromosome and a sequence mutation on second allele), deletions of *CDKN2A*/*CDKN2B* and/or *RB1* genes and *IKZF2* inactivation. Moreover, hypodiploid ALL blasts exhibit activation of PI3K/mTOR and MEK–ERK pathways.

Trisomy of chromosomes 4 and 10 accounts for 20%–25% of pediatric pre-B-ALL, but is very rare in adults; is associated with an extremely favorable 4-year event-free survival rate (~96%) compared to ~70% for patients with none or just one trisomy. Other evidences suggest, also, the involvement of chromosome 21 in leukemia; children with Down syndrome exhibit a 10–12 fold higher chance of developing an acute leukemia compared to children without Down syndrome. In addition, intrachromosomal amplification of chromosome 21 (iAMP21), defined as gain of at least three copies of large regions on chromosome 21 incorporating *RUNX1*, is observed in approximately 2% of B-ALL patients; iAMP21 can lead to an amplification of *RUNX1* locus, associated with a poor outcome and needy of more intensive chemotherapy. Overall, iAMP21 causes sequential amplification and deregulation of genes in the gain region[5;3].

***BCR-ABL*-like class**

Since 2016, the WHO revision of acute leukemia classification recognizes *BCR-ABL*–like or Ph-like ALL as a new leukemia entity associated with clinical relevance due to TKIs responsiveness, but an adverse prognosis. Patients affected by Ph-like ALL exhibit a gene expression profile similar to *BCR-ABL* positive ALL cases but *BCR-ABL* fusion gene lack, a poor outcome and frequently harbor alterations of *IKZF1* or other genes involved in B lineage lymphoid development. The Ph-like ALL prevalence rises with age, from 10%–15% of childhood B-ALL to over 25% of ALL in young adults; in addition, the genomic features frequency can vary according to ethnicity, as *CRLF2* alterations which recur in ~50% of Ph-like ALL patients and are associated with hispanic ethnicity.

Differently from many other ALL subtypes associated with a single founding genomic occurrence, Ph-like ALL is characterized by a range of genetic alterations that dysregulate several classes of cytokine receptors and tyrosine kinases, including rearrangements of *ABL*-class genes, *JAK2*, *EPOR* and *CRLF2*, mutations activating JAK–STAT signaling, *RAS*, and other less common kinase alterations (Fig.7)[3;8;9].

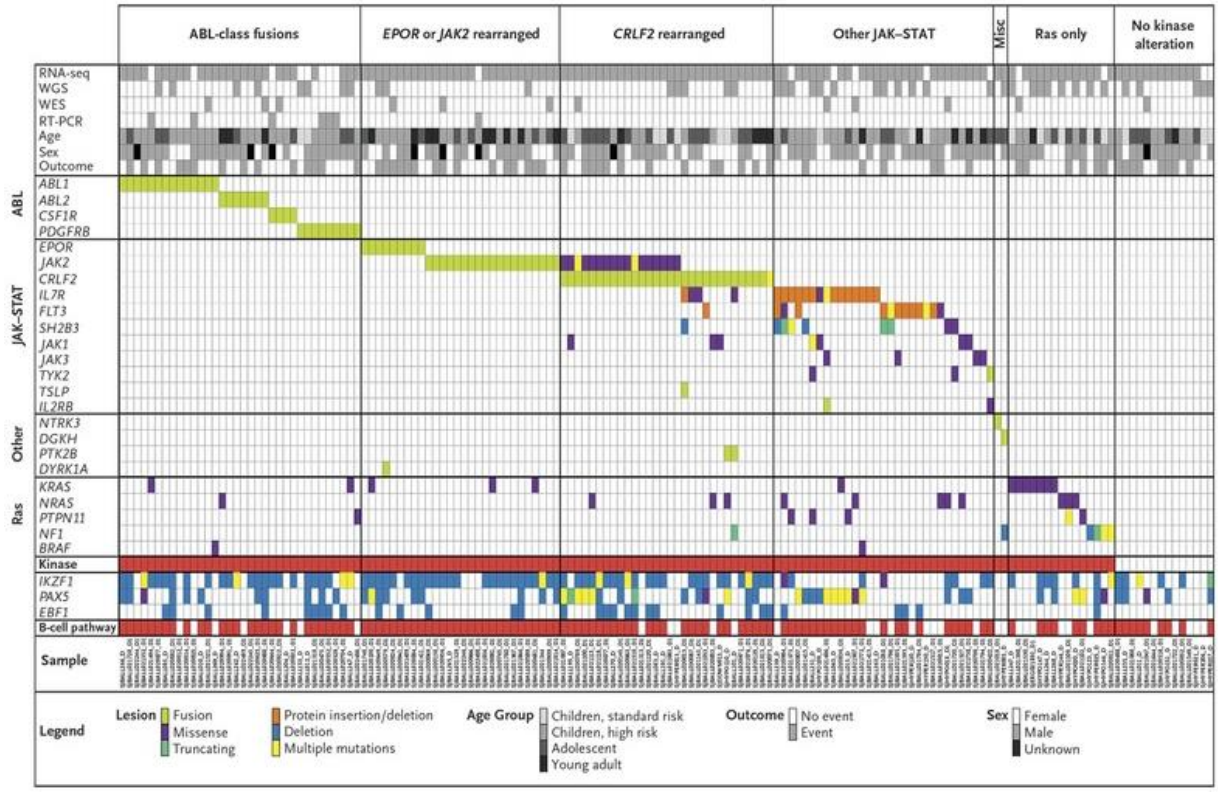

Fig.7[9] *ABL*-class fusions, *EPOR* or *JAK2* rearrangements, *CRLF2* rearrangements, other JAK–STAT–activating mutations, other kinase fusions, alterations in the RAS pathway, and no kinase alteration are recurrent class of alterations in Ph-like ALL

ABL-class rearrangements lead to expression of fusion genes that involve ABL1 or ABL2 (also known as ARG), CSF1R (encoding the macrophage colony stimulating factor receptor) or platelet-derived growth factor receptor-β (PDGFR-β). Despite multiple partner genes identified, all fusions retain the kinase domain.

JAK2/EPOR rearrangements include at least 10 different fusions of *JAK2* that preserve the JAK2 kinase domain and *EPOR* (erythropoietin receptor gene) rearrangements with immunoglobulin heavy (IgH) or kappa (IgK) loci resulting in overexpression and activation of receptor signaling.

CRLF2 at Xp22.3/Yp11.3 encodes for cytokine receptor-like factor 2, also known as the thymic stromal-derived lymphopoietin receptor, which in combination with interleukin 7 receptor α chain (IL7R) represents the receptor for thymic stromal lymphopoietin. *CRLF2* rearrangements are observed in approximately 50% of Ph-like ALL cases, but also in non Ph-like B-ALL, particularly in patients affected by Down-syndrome-related ALL. *CRLF2* is deregulated by rearrangement with the immunoglobulin heavy chain locus (*IGH–CRLF2*) or by an interstitial deletion in the PAR1 region of chromosome X and Y that gives rise to juxtaposition of the *CRLF2* locus to the *P2RY8* promoter; both alterations effect overexpression of *CRLF2* transcripts and CRLF2 protein on

leukemic blasts cell surface. Also, *CRLF2* sequence mutations recur in B-ALL and activate receptor signaling.

Additional *JAK* mutations arrange most frequently at or near the Arg683 residue in the *JAK2* pseudokinase domain, but less frequently are also observed in the pseudokinase and kinase domains of *JAK1*, particularly at Val658 residue, homologous of *JAK2* Val617 mutated in myeloproliferative neoplasms. In *CRLF2*-rearranged B-ALL patients lacking *JAK* mutations, recur additional alterations activating the JAK–STAT signaling such as *IL7R* sequence mutations, *SH2B3* deletions and *TSLP* rearrangements, *RAS* mutations. Thus, *CRLF2* genetic alterations result in overexpression of the cytokine receptor and additional activating mutations affect JAK–STAT, PI3K, mTOR, BCL2 and RAS pathways. In most studies, *CRLF2* rearrangements are associated with poor prognosis, particularly if recur along with *IKZF1* alteration.

The JAK–STAT signaling is an important mediator of stimuli from hematopoietic cytokine receptors. Approximately 12% of Ph-like ALL patients exhibits mutations that constitutively activate cytokine receptors, including *IL7R*, *FLT3* and *IL2RB* (encoding interleukin-2 receptor subunit β), activating mutations in the Janus kinases themselves (*JAK1* or *JAK3*), or mutations that impair function of JAK–STAT signaling negative regulators (SH2B adaptor protein 3, *SH2B3*). On the other hand, in some cases lacking a kinase rearrangement or JAK–STAT activating alterations recur mutations that activate RAS signaling; these include mutations in *NRAS*, *KRAS*, *PTPN11* and *NF1*. Moreover, few Ph-like ALL patients exhibit several uncommon kinase alterations such as rearrangements of *NTRK3* and *TYK2*.

Several studies highlight that Ph-like ALL hallmarks are associated with high risk clinical features, suboptimal response to remission induction therapy and low event free and overall survival rates. Ph-like ALL cell lines and xenografts harbor often kinase fusions, activation of oncogenic signaling pathways and cellular proliferation treatable with ABL inhibitors or JAK2 inhibitors (e.g. rearrangements and mutations activating the JAK–STAT signaling). These evidences suggest the requirement of Ph-like ALL identification flow, determining the underlying kinase-activating alterations, in order to identify suitable targeted therapy[9;3].

ERG alteration in B-other ALL group

Approximately 5%–10% of B-ALL cases exhibit a specific gene expression signature, few frequent genetic alterations and also lack recurring chromosomal rearrangements. Around 75% of these patients harbors a focal deletion of *ERG* gene, encoding an ETS-domain-containing transcription factor, that results in aberrant ERG protein expression. In addition, *IKZF1* deletions are relatively

common (~40%) in *ERG*-altered ALL but, differently from *BCR–ABL1*$^{+}$ and Ph-like ALL, are not associated with worse outcome[3;4].

New ALL subsets

New ALL subsets, such as *DUX4*-rearranged ALL subtype, are often not detected by conventional cytogenetic methods or involve diverse chromosomal rearrangements that converge on a single gene (e.g. *MEF2D*- and *ZNF384*-rearranged ALL). By an integrated genomic analysis performed at St. Jude Children's research Hospital some predominantly adult cases harbor rearrangements of *BCL2*, *MYC* and/or *BCL6* associated with B-cell-precursor immunophenotype, exhibiting alterations identical to those observed in double- or triple-hit lymphoma, but rare in ALL. In addition, 11/1.988 cases (0.6%) are characterized by rearrangements of *NUTM1* to six different partner genes (three with *ACIN1*, three with *CUX1*, two with *BRD9* and one for each of *IKZF1*, *SLC12A6* and *ZNF618*), and nine cases (0.5%) by rearrangement of *HLF* to *TCF3* (n.8) or *TCF4* (n.1). Moreover, three groups include patients with similar gene expression profiles to established subtypes but lacking the founding rearrangements: *ETV6-RUNX1*-like (n.42), *KMT2A*-like (n.5) and *ZNF384*-like (n.4).

Thus, genomic analyses disclose B-ALL complexity consisting of multiple distinct genetic alterations and several molecular subsets (Fig.8)[17].

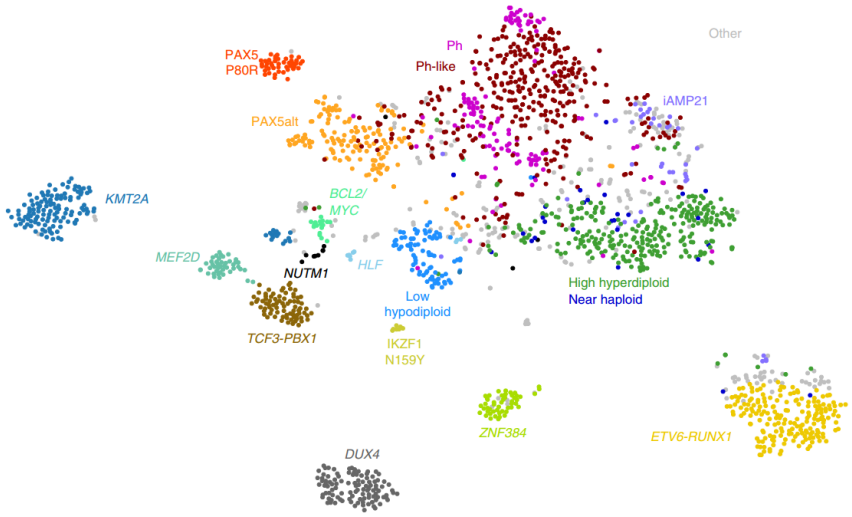

Fig.8[(17)] B-ALL genomic classification includes both major B-ALL subtypes (e.g. ETV6-RUNX1, TCF3-PBX1, *DUX4*-rearranged, *ZNF384*-rearranged, *MEF2D*-rearranged, Ph-like, High hyperdiploid, Low hypodiploid, Near haploid and cases with intrachromosomal amplification of chromosome 21 (iAMP21) and uncommon subtypes (e.g. *BCL2/MYC*-rearranged, *TCF3/TCF4-HLF* and *NUTM1*-rearranged)

Clonal heterogeneity, evolution and relapse in ALL

Despite long-term survival rates gain due to therapeutic improvements, approximately 15% to 20% of children and ~50% of adult ALL patients still relapse. Relapses are generally classified and stratified according to three prognostic factors: time, site and immunophenotype (B or T). The time of relapse is the most important prognostic factor: earlier the relapse occurs, worse is the prognosis. The most important factor after timing is probably the relapse anatomical sites, which are commonly divided into bone marrow alone, extramedullary alone, and combined relapses. Furthermore, many mutations associated with predominant clone at relapse might be detected in early therapeutic phase, determining implications for molecular monitoring and prediction of relapse risk.

Cumulative evidences suggest a clonal origin of ALL and clones evolve over time. In the linear succession model, one clone acquires stepwise novel mutations; subclones with different mutational features can exist in parallel but are related according to a linear genealogy. In the branching evolution model, on the contrary, subclones divide based on a branching, nonlinear manner; at different evolution phases subclones, emerged from a common ancestral clone, can exist in parallel but are not directly related with each other.

The clonal evolution is involved in relapse, with the possible emergence of subclones derived either from the major clone found at initial diagnosis, minor subclones, or a common ancestral pre-leukemic clone. The analysis of matched ALL samples obtained at diagnosis and relapse shows that the majority of patients exhibits substantial genomic changes from diagnosis to relapse, with acquisition of new deletions and mutations, and loss of diagnosis-specific lesions. On the other hand, most paired diagnosis-relapse samples share some DNA copy number alterations and antigen receptor rearrangements, the founding chromosomal translocation, and relapse-acquired deletions and/or mutations can often be detected at low levels at diagnosis. All these findings strongly hint for a branching Darwinian selection model associated with ALL propagation, evolution and probably relapse[16;3;11].

Relapses may originate from a chemoresistant clone, either already present as a minor clone at diagnosis or resulting from acquisition of secondary mutations during treatment (Fig.9 B-C-D); several studies describe chemoresistance-related mutations in BM samples of relapsed patients, such as those that affect the glucocorticoid response pathway (mutations of glucocorticoid receptor gene *NR3C1* or other genes interfering with its function, such as *BTG1*) or nucleotide metabolism (e.g. *NT5C2* or *PRSP1* genes). In addition, cells may be therapy resistant by residing in niches where the microenvironment confers protection from chemotherapy and drug concentration may be lower.

Aside from chemoresistance mechanisms, relapse can also be caused by dormant cells within the bulk population. These rare, non-dividing, quiescent cells are less sensitive to cytotoxic agents, can therefore resist to treatment and give rise to a "new" population after reentering the cell cycle, leading to relapse (Fig.9 A-E)[16;3].

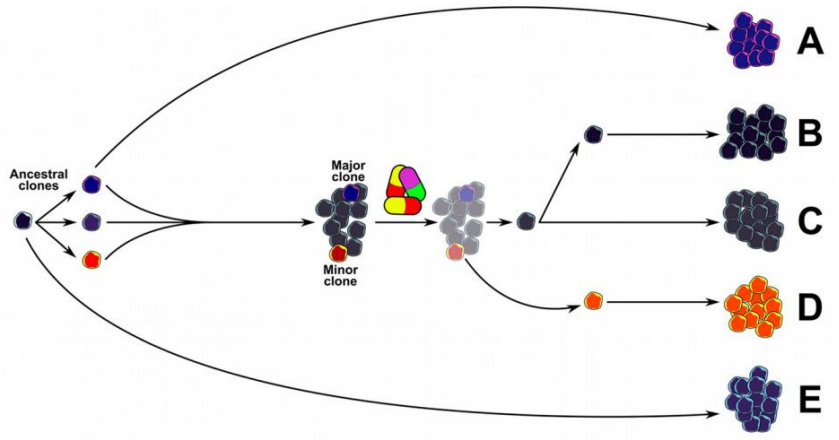

Fig.9[(16)] Clonal evolution of leukemic cells from preleukemic stage to relapse

Both intrinsic (leukemic cell features) and extrinsic factors, such as microenvironmental conditions or chemotherapeutic pressure, may favor a clone from which the relapse can develop, but mechanisms by which cells reach and invade distant sites are also yet to be clarified. Metastatic niches may share characteristics that attract leukemic cells offering a favorable microenvironment for maintenance and growth. CXCL12, also known as stromal derived factor 1 alpha (SDF1α), is one of the most important chemokines in the BM niche; through binding to its receptor, CXCR4, CXCL12 favors HSC homing in BM and participates in maintenance of these cells in the niche because is highly expressed by specialized cells close to sinusoid vessels within the BM. Thus, the CXCL12-CXCR4 axis is involved in leukemia pathogenesis, favoring ALL cells homing, survival and proliferation in the bone marrow. In addition, other sites such as liver and spleen appear as favorable niche promoting ALL cells engraftment through the CXCL12-CXCR4 axis.

Low oxygen concentrations, improperly termed hypoxia, are physiologically found in specific areas in mammalian organisms. Indeed, the BM is likely to be one of tissues in which the oxygen concentration is the lowest; increasing evidence suggests that the niches in which HSCs reside are hypoxic and that low oxygen concentrations may be needed to maintain their stemness. Furthermore, hypoxia affects cancer cells and tumor microenvironment promoting aberrant angiogenesis, epithelial-mesenchymal transition, radio- and chemoresistance and metastatic process. On the other hand, leukemic cells can remodel surrounding microenvironment secreting for example osteopontin, physiologically produced in the bone marrow, into the invaded

microenvironment, which in turn also produced osteopontin. Moreover, the direct communication between leukemic blasts and surrounding supportive cells, probably as well as distant interaction from the original to the secondary niche, appears to be crucial. Indeed, large extracellular vesicles (EVs), released through plasma membrane budding, may be important for communication between ALL cells and microenvironment, in addition to interactions with mesenchymal stromal cells through tunneling nanotubes, to niche set up. In turn, also leukemic cells intrinsic properties can affect interaction with surrounding microenvironment; for example, *IKAROS* gene alterations alter leukemic blasts adhesive properties and may influence their settlement in the niche.

Forthcoming treatment challenge is "niche" and leukemia cell properties targeted therapy to try effectively treat ALL[16].

Immune system cellular subsets

The immune system detail is the ability to discriminate between self and non-self entities and protect from non-self; the immune response is classified as innate or adaptive. The innate immune response includes the skin barrier, various cytokines, complement proteins, lysozyme, bacterial flora, and phagocytes (neutrophils, basophils, eosinophils, monocytes, macrophages, dendritic cells), reticuloendothelial system, natural killer cells (NK cells), epithelial cells; the adaptive acquired immune response involves T and B lymphocytes, characterized by specificity, heterogeneity and memory, which release specific products to generate a response against non-self entities.

All hematopoietic lineages are derived from the long-term hematopoietic stem cells (LT-HSCs) in bone marrow (BM); LT-HSCs divisions can result in self-renewal or differentiation into multipotent and lineage-committed hematopoietic progenitor cells, such as CLP (common lymphoid progenitors), CMP (common myeloid progenitors), GMP (granulocyte-macrophage progenitors), all of which lack or retain limited self-renewal capacity (Fig.10)[20;21;22].

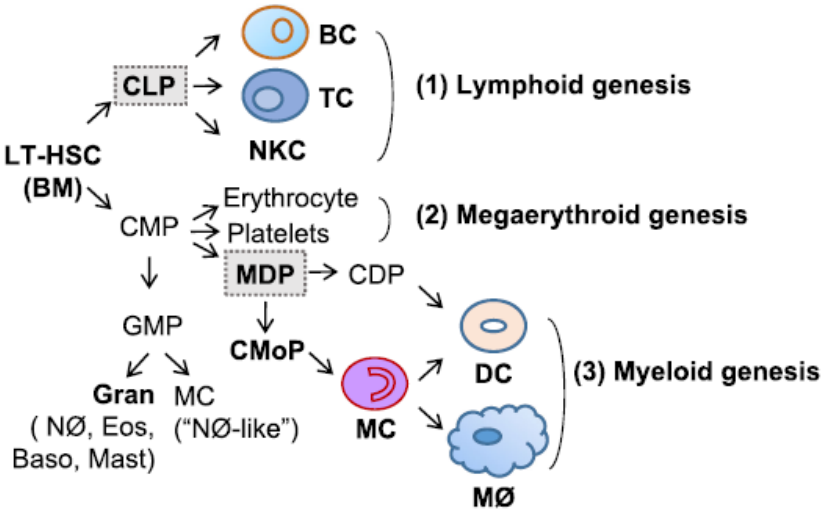

Fig.10[22] Hematopoietic stem cell differentiation. Long-term hematopoietic stem cells (LT-HSC) differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLP or CMP are committed to lymphoid or myeloid lineage genesis, respectively

T-cell (TC) genesis and differentiation

The precursors of T lymphocytes (thymocytes) migrate from BM to the thymus and thymic localization involve chemokines, mainly CC-chemokine ligand 21 (CCL21) and CCL25, and their respective receptors CCR7 and CCR9; the T-cell receptor (TCR) rearrangements during T lymphocytes development is responsible for the antigen receptor repertoire generation.

In the early development phase, double-negative (DN) thymocytes not express the co-receptors CD4 and CD8 or T-cell receptor (TCR). The DN TCs receive signals NOTCH1- and IL-7-mediated from the cortical thymic epithelial cells (cTECs); NOTCH1 will contribute to T lymphocytes lineage commitment by interaction with GATA3, while IL-7 plays a role in T-cell development, lymphoid precursors proliferation and survival. At this stage, $CD4^-$ $CD8^-$ thymocytes gradually lose expression of adhesion molecule CD44, gain expression of IL-2 receptor (CD25) α chain on cell surface and most thymocytes rearrange the V(D)J genes efficiently to express TCRαβ; however, the TCR is not tested for specificity and is termed pre-TCRαβ, which is associated with CD3/ζ for signal transduction.

Pre-TCR expression leads to the double-positive (DP) TC phase, with expression of both CD4 and CD8 proteins ($CD4^+$ $CD8^+$ TC) and the subsequent replacement of pre-TCR with TCR. The DP TC development depends on interactions with peptides via major histocompatibility complex (MHC) molecules expressed by cTECs and dendritic cells. Thymocytes that interact with low avidity are positively selected and receive survival signals, while thymocytes that fail in this interaction or interact with high avidity are selected negatively by apoptotic mechanisms. The positively selected DP thymocytes migrate to the thymus medulla, leaded by CCR7, CCL19 and CCL21 produced by

medullar thymic epithelial cells (mTECs); DP TC that binds to self-peptide–MHCI complexes becomes cytotoxic $CD8^+$ TC and loses CD4 expression, whereas binding to self-peptide–MHCII ligands results in $CD4^+$ TC status, losing CD8 expression. Phenotypically, single-positive (SP) thymocytes express CD62L and CD69 and also acquire the functional capacity of mature T lymphocytes, but are still naïve, not having yet experienced antigens during an adaptive immune response. This thymic phase of differentiation and maturation lasts approximately 12 days and is critical for central tolerance establishment. SP T lymphocytes immunologically competent migrate to peripheral lymphoid sites, remaining in the cell cycle interphase until the interaction with specific antigens presented by antigen-presenting cells (APCs) via MHC. S1P1 (sphingosine-1-phosphate receptor 1) expression is required for outflow of the mature T lymphocytes from the thymus; in addition to S1P1, CCR7, CCL19 and CXCL12 also participate in this process[25].

The subsets differentiation of naïve $CD4^+$ TCs is determined by antigen presented via the antigen presenting cell (APC), immune checkpoints co-signaling, cytokines, and metabolism-associated danger signal (MADS); the $CD4^+$ TCs subsets (Th1, Th2, Th17, Treg, Th9, Th22, and Tfh) differ in their extra and intra cellular markers and each class releases specific cytokines that can have either pro- or anti-inflammatory functions (Fig.11)[22].

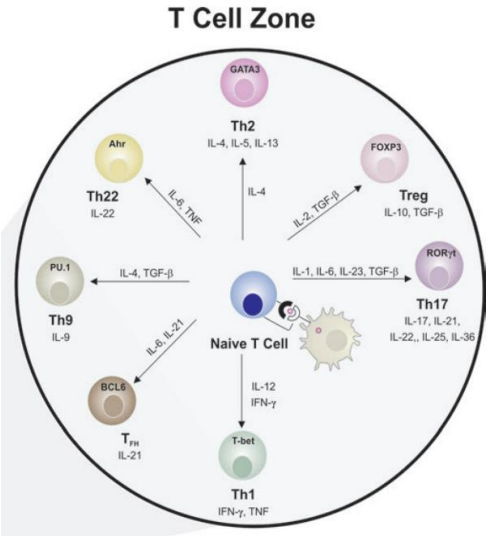

Fig.11[(25)] CD4 subsets differentiation, cytokines and transcription factors

Th1 cells are defined based on the production of pro-inflammatory cytokines such as interferon (IFN)-γ, and tumor necrosis factor (TNF)-α or TNF-β to stimulate innate and T-cell immune responses. This subset is induced by natural killer (NK) and/or dendritic cells through IFN-γ release, which activates signal transducer and activator of transcription (STAT) 1, resulting in activation of lineage-specific transcription factor encoded by T-box transcription factor-TBX21 (*T-bet*) gene. T-bet enhances synthesis of the IL-12 receptor, which activates STAT4 and consequent IFN-γ transcription and production. Th1 type cytokines are responsible for intracellular antigens

death and autoimmune response maintenance, can activate MØ and enhance cell-mediated cytotoxicity.

Instead Th2 cells produce IL-4, IL-5 and IL-13, in association with IgE production and eosinophilic response. Th2 subset differentiation requires IL-4, produced by NOTCH ligand activation, with following STAT6 induction, which in turn activates GATA3, responsible of Th2 lineage cytokines production. Th2 cells control immunity against extracellular parasites and all kinds of allergic inflammatory responses.

Th9 cells produce IL-9 and IL-10 cytokines; functionally enhance antibodies production and increase immune cells infiltration and activity within the respiratory tract, contributing to asthmatic disease. In addition, IL-9 mediates antiparasitic response altering epithelial cells function, augmenting immune cells infiltration into infected sites and enhancing leukocytes immunological function; also, IL-9 can limit tumor growth by stimulating lymphocytes antitumoral activity. Th9 subset development needs of IL-4, inducing STAT6 activation, TGF-β and IL-2-STAT5 signaling expression. In addition, the lineage-specific transcription factor for Th9 development may be BATF, leading to a transcriptional program which results in increased IL-9 and IL-10 production. The IL-9-secreting TCs development involves also TGF-β-induced SMAD proteins and transcription factor PU.1.

Under inflammatory condition, IL-6, IL-23 and TGF-β induce Th17 cells engagement, without Th1 or Th2 cytokines. IL-6 promotes STAT3 activation, which activate retinoic orphan receptor (ROR) transcription factors, RORα and RORγT, leading to production of Th17 cytokines such as IL-17, IL-17F and IL-22. Toll-like receptor pathway is another innate immunological signal promoting Th17 subtype differentiation. Th17 cells play a role in host defense against extracellular pathogens by mediating neutrophils and macrophages recruitment on infected tissues. Moreover, the Th17 subgroup abnormal regulation is implicated in the pathogenesis of several autoimmune and inflammatory diseases, causing tissue injury.

Th22 cells development is promoted by IL-6 and TNF-α, inducing STAT3 and aryl hydrocarbon receptor expression. The Th22 subset exhibits a specific profile of Th1- and Th17-associated genes, such as *IFN-γ*, *IL17a*, *T-bet* and *RORγt*. In addition, several phenotypic markers including CCR6, CCR4, dipeptidyl peptidase IV, CD26 and CD90, are common to Th17 and Th22 subgroups; but differently from Th17 cells, Th22 lymphocytes express CCR10. Th22 cells also produce IL-22 in IL-17 independent manner, unlike Th1 and Th17 subsets.

Follicular helper $CD4^+$ T (Tfh) cells, localized in B-cell follicles and germinal center, enhance immunoglobulin production, facilitating BC-mediated responses. After dendritic cells antigen stimulation, Tfhs up-regulate CXCR5, down-regulate CCR7 and migrate to lymph node

interfollicular regions, where interact with activated B-cells. These interactions result in antibodies production by short-lived plasmablasts in extrafollicular regions or in the germinal center. In both sites, Tfh cells support B-cell maturation, class switch and affinity selection, via cytokines secretion or by surface molecules expression. However, Tfh lymphocytes require a strong TCR signal for induction, in addition to inducible costimulator (ICOS) activation, a CD28-related costimulatory signal provided by activated dendritic cells or B-cells, initiating MAF transcription which induces IL-21 activation; Tfhs development involves also IL-6 and STAT3[22;25].

Tregs are generated by naïve $CD4^+$ T-cells priming with IL-2 and TGF-β; stain positive for forkhead box P3 (FOXP3), CD25 and CD4. CD25 and CD4 are not specific markers because expressed by other T-cell subsets, while FOXP3 is defined the most specific Treg marker playing a cardinal role for cell lineage commitment toward a Treg phenotype. High affinity interactions between TCR and costimulatory molecule CD28 could form $CD25^+$ Treg precursors and are also responsible of *FOXP3* chromatin remodeling; signal transducer and activator of transcription 5 (STAT5) is required for further transition of Treg precursors toward $FOXP3^+$ Treg-cells. In addition, programmed cell death-1 (PD-1) and CTL-associated antigen-4 (CTLA-4) checkpoint receptors contribute to Treg immunosuppressive features. PD-1 is involved in FOXP3 stability maintenance through regulation of the protease asparaginyl endopeptidase (AEP); FOXP3 in turn directs *CTLA-4* transcription. Furthermore, Tregs can express Tim-3 (T-cell immunoglobulin mucin-3) immune checkpoint receptor; Tim-3 is a negative regulator of Th1-cells and $Tim\text{-}3^+$ Tregs exhibits more capability to suppress immune system, exhibiting an active PD-1, CTLA-4 and CD39 expression (Fig.12)[22;23].

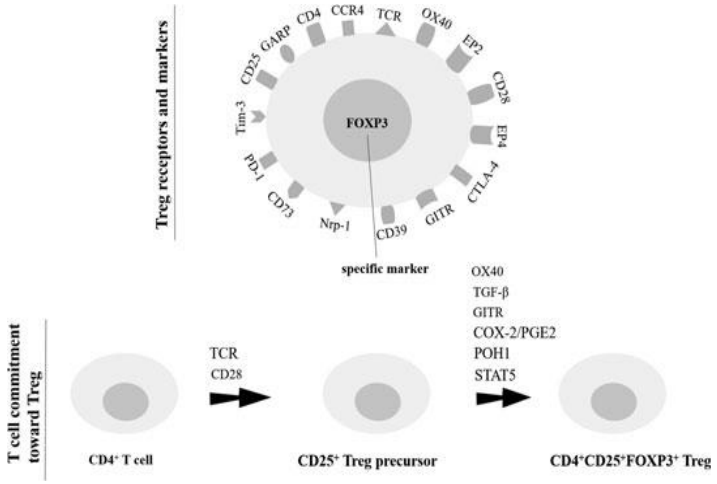

Fig.12[(23)] Regulatory T-cell markers and development

Tregs interact with cancer cells and tumor promoter cells, including cancer-associated fibroblasts (CAFs), macrophage type 2 (M2) cells, myeloid-derived suppressor cells (MDSCs) and regulatory B-cells (Bregs). CAFs enhance Tregs growth, extravasation and infiltration and secrete chemokine (C-X-C motif) ligand 12 (CXCL12) and IL-6 increasing Tregs number in the tumor stroma; also release COX-2/PGE2 and transforming growth factor-β (TGF-β), inducing activity of Tregs which in turn produce TGF-β, the prevalent mediator for trans-differentiation of the normal resident fibroblasts into CAFs. Therefore, the cross-talk between Tregs and CAFs would strengthen their capability to interfere with immunosuppressive and tumorigenic processes.

The pro-tumorigenic macrophages-Tregs interaction is also bidirectional. Tregs, releasing IL-10, STAT3 and VEGF, mediate positively differentiation of monocytes toward protumor M2 phenotype; in turn M2-cells release IL-6 and IL-10 for Tregs activation. In addition, Tregs express CC chemokine receptor 4 (CCR4), known as receptor for CCL22 released by M2-cells: the CCL22/CCR4 binding is useful for IL-10 production by both M2-cells and Tregs, thereby multiplying Tregs activity within the tumor microenvironment (TME). Besides M2 macrophages, MDSCs interact with Tregs and all these immunosuppressive cells are highly related to one another. MDSCs, Tregs and M2-cells express PD-1 receptor on cell surface and release STAT3 in the TME aiming to suppress immune system; MDSCs and M2 macrophages also produce TGF-β to induce Tregs activity and CCL22, released by M2-cells, recruits likewise MDSCs and Tregs. In addition, the role of Tregs along with CAFs and M2-cells, is to dampen cytotoxic T lymphocytes transferring to the tumor core and inhibit their function releasing adenosine and TGF-β. Moreover, Tregs interact with Bregs, which release IL-21, IL-35, TGF-β inducing Tregs activity, and express PD-L1, an immune checkpoint protein functioning via binding to its receptor PD-1 expressed on Tregs cell surface.

The $CD4^+$ TC differentiation into lineages with distinct effector functions is not an irreversible event; among all subsets, Th2, Treg, and Th17 cells are more plastic than Th1 and may not be stable. Indeed, $CD25^-$ $FOXP3^-$ Tregs can be reprogrammed to effector T-cells under inflammatory cues influence, through suppressing receptor/ligand interactions for CD25, neuropilin-1 (Nrp-1) and FOXP3. One of proinflammatory cytokines activated in the reprogrammed Tregs is IFN-γ, related to increased activity of antitumoral M1 and Th1 cells[22;23;24].

Likewise, $CD8^+$ T-cells also acquire different profiles according to costimulatory molecules and cytokines present in the environmental, differentiating into Tc1, Tc2, Tc9, Tc17 or $CD8^+$ T regulatory cells.

Cytotoxic T lymphocytes (CTLs), also named Tc1, are IL-2 and IL-12 dependent and highly cytotoxic, so that are responsible for direct killing of infected, damaged and dysfunctional cells,

including tumor cells. Following activation, Tc1s express rapidly high levels of IFN-γ, TNF-α, perforin and granzymes into immunological synapse.

Instead, Tc2-cells express the transcription factor GATA3 and produce, besides granzymes and perforin, IL-5 and IL-13, but IL-4 limited amounts. Their profile is associated with propagation of Th2-mediated allergy and probably contributes to rheumatoid arthritis. The differentiation of $CD8^+$ T-cells into IL-9 producers (Tc9-cells) occurs mostly in the intestinal epithelium by IL-4 and TGF-β induction, favoring a greater antitumor activity; the transcription factors STAT6 and IRF4 are important for IL-9 production, while FOXP3 affects its inhibition.

The IL-17-producing $CD8^+$ T-cells (Tc17) differentiation is regulated by IL-6 or IL-21 along with TGF-β, while IL-23 stabilizes Tc17 phenotype. Similar to Th17 cells, Tc17s produce IL-17 and IL-21, express the IL-23 receptor and IRF4, RORγt and RORα transcription factors. However, Tc17 subset exhibits impaired cytotoxic activity due to low IFN-γ, perforin and granzyme B production, but enhance antitumor immunity through their proinflammatory properties, which, on the other hand, may contribute to autoimmune processes.

The suppressor $CD8^+$ Treg-cells are defined by $CD44^{hi}$ $CD122^+$ $Ly49^+$ $Foxp3^+$ phenotype; IL-15 exhibits a relevant role for their activity. For suppression, $CD8^+$ Treg-cells rely on diverse effectors including TGF-β, IL-10, granzymes, perforin and indoleamine 2, 3-dioxygenase (IDO).

While most $CD8^+$ T-cells die by apoptosis after antigen clearance, rare cells survive and are identified as long-lived memory T-cells. Memory $CD8^+$ T-cells include central memory ($CD62L^+$ $CD127^+$ $CCR7^+$) and effector memory ($CD162L^-$ $CD127^+$ $CCR7^-$) subsets, distinguished by the relative expression of two homing molecules, CD62L and CCR7. Effector memory T-cells are characterized by a loss of CCR7 expression and intermediate to no CD62 L expression, differentiate readily into effector T-cells that secrete high amounts of IFN-γ and are highly cytotoxic upon re-exposure to cognate antigen. Conversely, central memory T-cells are less differentiated, retain increased proliferative potential and greater self-renewal capability, can produce high levels of IL-2 and acquire effector functions less rapidly[25].

B-cell (BC) genesis and differentiation

In bone marrow (BM), common lymphoid progenitors (CLP) development goes through pro-BC, B1/B2 pre-BC and immature BC stages. In addition, concurrent immunoglobulin gene rearrangements result in the B-cell receptor generation. B-cell differentiation from CLP is regulated by several factors such as E-box binding protein 2A (E2A), early B cell factor-1 (EBF1), purine box factor 1 (PU.1), IKAROS, paired box protein-5 (PAX5) and CXCL12. The first cell committed to B lymphoid differentiation is the pro-B-cell. In the BM microenvironment pro-B-cells begin to

express CD45^{dim}, CD22, CD34, terminal deoxynucleotidyl transferase (TdT) and CD38^{high}; the CD19 expression, one of the most premature B lymphocyte antigens, is activated by PAX5 factor. In the next step of differentiation, pro-B-cells express CD10^{high}, CD38^{high}, CD34, CD79a, TdT, CD19 and are identified as pre-BI-cells. The immunoglobulin gene loci recombination, involving the variable (V), diversity (D), joining (J), and constant (C) regions, starts in this phase. Gene rearrangements are initiated by RAG1 and RAG2, that bind and cleave DNA at specific recombination signal sequences (RSSs). During recombination, D and J gene segments are drawn closer, excluding the intermediary DNA; DJ segment is joined to a V segment originating VDJ rearranged exon. Pre-BI-cells also express TdT, which is responsible for catalysing the random addition of junctional nucleotides. Following the rearrangement, the VDJ segment is adjacent to the constant Cμ region and is an active gene, which codes for the heavy chain, whose synthesis originates the μ intracytoplasmic chain (IgM); developing B-cells become termed pre-BII-cells. During this phase, pre-BII-cells gain heterogeneous CD20 expression, lacking CD34 and TdT expression. Besides, B lymphocytes express the pre-B cell receptor (pre-BCR), formed by the heavy chain μ (Igμ) with λ5 and VpreB joined to a heterodimer Igα (CD79a) and Igβ (CD79b). Immature B lymphocytes immunophenotypic features are CD20 and CD5 expression, enhanced expression of CD45, CD10^{dim} and CD38^{high}, low expression of CD21, and high levels of CD81; immature B-cells undergo a selective process before completing maturation. Indeed, the B lymphocytes that complete, successfully, the immunoglobulin gene rearrangement program are positively selected, receiving survival signals to continue the maturation process, and *RAG* genes are quenched. Though, when B lymphocytes recognize self-antigens, their receptor is modified, the *RAG* genes are reactivated and another V-J rearrangement in the light chain is initiated allowing the development of non-self-reactive BCR; if this rearrangement fails, B-cells undergo a negative selection process through apoptosis. The positively selected cells leave BM and complete the development in the secondary lymphoid organs.

Mature B lymphocytes lack CD10, CD38 and express IgD and IgM on cell membrane; IgD expression results from the VDJ segments transcription with Cδ exon instead of Cμ[22;25].

Mature B-cells include follicular B-cells, marginal zone (MZ) B-cells, B1a and B1b-cells. The follicular B lymphocytes (Fo BCs), located in lymphoid follicles of lymph nodes and spleen, are the mature B-cells majority. For transition from immature BCs to Fo BCs, intermediate level of BCR signal is required. After BCR binding with antigen, BTK5, a TEC-family tyrosine kinase protein, is recruited and activated; an important downstream effector of BCR/BTK5 signaling is nuclear factor-κB (NF-κB). The NF-κB transcription factor family consists of heterodimers or homodimers of subunits p50 (NF-κB1), p52 (NF-κB2), c-REL, p65 (RELA), and RELB; the p50/p65

heterodimer determines Fo BCs fate. BAFF (B-cell activating factor of the tumor-necrosis-factor family) is also required for Fo BCs differentiation. BAFF overexpression in transgenic mice induces Fo BCs production and its engagement activates BTK, which then facilitates BCR-induced activation of NF-κB pathway.

Fo BCs in the extra-follicular zone can be activated by antigens of microbial origin in TC-dependent manner, receiving synergistic signals via BCR, CD40, toll-like receptors (TLRs), and differentiate into short-lived plasmablasts, secreting antibodies. On the other hand, some activated B-cells migrate back to follicles and, under follicular T-helper cells (Tfhs) influence, proliferate vigorously forming the germinal center (GC). In the GC dark zone region, B-cells (called centroblasts) go through a rapid cellular division, somatic hypermutation, and isotypes class switching. Centroblasts, expressing CD10 and BCL6, migrate to the GC light zone region and become centrocytes, which will contact follicular dendritic cells (FDC) and Tfhs through interactions between CD23 and CD40L. In this microenvironment, B-cells with high affinity for antigens will differentiate into plasma cells or memory B-cells and express CD27. Plasma cells return to the bone marrow and exhibit defined characteristics expressing CD19, CD27, CD38, CD45, CD138 and intracytoplasmic Ig[22;25].

Marginal zone B2 BCs (MZ BCs) are innate-like cells, responsible of TC-independent responses, but can also mediate the antigen transport from immune complexes into splenic follicles, which is involved in TC-dependent BC-response; MZ BCs exhibit pan-B markers, weak IgD expression and lack CD10. During differentiation, NOTCH2 interacts with its ligand DL1 (Delta-like 1), initiating NOTCH2 cleavage; the NOTCH2 intracellular domain enters the nucleus where interacts with MAML1 (Mastermind-like 1) and RBP-Jκ transcription factors, inducing the commitment of BC to MZ BC. Also, BAFF/BAFF-R interaction release survival signals through canonical NF-κB activation in MZ BCs.

Conversely, B1 BC population has not been clearly defined in humans, so related knowledge is based on studies in rodents. The phenotype of BTK-deficient mice suggests that a stronger BCR/BTK signaling is required for generation of B1 BCs. Interestingly, B1-cells formation is unaffected by impaired BAFF signaling, raising the possibility that elevated BCR signaling or other microenvironmental factors may be important for BAFF-independent survival. While B1-a BCs contribute to innate-like immune responses, B1-b BCs affect the adaptive immunity[22;25].

Furthermore, recently is identified the regulatory B-cells (Bregs) entity; Bregs promote tumor growth through interactions with Tregs, myeloid-derived suppressor cells (MDSC), tumor-associated macrophages, natural killer (NK) cells, $CD4^+$ and $CD8^+$ T lymphocytes and tumor cells. In human lymphoid malignancies, malignant B-cells appear to act as Bregs inhibiting the antitumor

immune response through expression of suppressive ligands. Phenotypically distinct B-cell subsets, also, exhibit immune regulatory properties through IL-10 dependent or independent mechanisms. IL-10-producing B-cells recur predominantly within a $CD24^{hi}$ $CD27^{+}$ subset and are capable of suppress monocyte cytokine production in vitro. In addition, $CD19^{+}$ $CD24^{hi}$ $CD38^{hi}$ peripheral blood B-cells suppress $CD4^{+}$ T-cell IFN-γ and TNF-α production in vitro, exhibiting inhibitory activity dependent on IL-10, CD80, and CD86; the latter two membrane proteins are key ligands for CTLA-4, a coinhibitory immune checkpoint receptor expressed on activated effector T-cells and Tregs. $CD19^{+}$ $CD25^{hi}$ B-cells also may represent a Breg population in humans with capability of suppress $CD4^{+}$ TCs proliferation and enhance CTLA-4 and FOXP3 expression on Tregs in vitro, through TGF-β dependent mechanisms but not IL-10-mediated. Furthermore, $CD5^{+}$ B-cells are implicated in antitumor immunity suppression through activation of STAT3, a transcription factor that may be involved in IL-10 production.

Moreover, B-cells can express additional surface antigens, which confer regulatory properties. Programmed death 1 ligand (PD-L1), which interacts with PD-1 on T-cells inducing immunological tolerance and limiting effector TCs responses, is expressed on human malignant BCs membrane in several types of lymphoma such as diffuse large B-cell lymphoma (DLBCL), Hodgkin's and follicular lymphomas. Fas-Ligand (FasL), a necrosis factors pathway member, interacts with its receptor FasR (CD95) to initiate the apoptotic signaling cascade; malignant B-cells expressing Fas-L in lymphoid diseases, such as CLL, may inhibit antitumor responses through Th-cells apoptosis induction. In addition, human granzyme B-producing (GrB^{+}) B-cells, induced by IL-21-secreting T-cells, in turn suppress T-cell proliferation via GrB-dependent degradation of TCR-ζ-chain in vitro; furthermore, GrB^{+} BCs localize adjacent to IL-21-producing T-cells in several human cancers including breast, ovarian, cervical, colorectal, and prostate tumors. IL-21-dependent GrB^{+} B-cells may attenuate local antitumor immune responses likewise Tregs, inhibiting $CD4^{+}$ and $CD8^{+}$ effector T-cells proliferation. Moreover, B regulatory cells suppress immune response and promote tumor growth through different mechanisms; a $CD25^{+}$ $CD19^{+}$ $B220^{+}$ B-cell subset expressing constitutively STAT3 may be expanded by tumor and may in turn promote tumor metastasis through TGFβ-dependent conversion of non-Treg T-cells to Tregs. Instead, tumor infiltrating B-cells (TIL-B) exhibit increased expression of LAP/TGF-β, CD80, CD86, and PD-L1 in vivo compared to splenic B-cells; functionally, TIL-Bs demonstrate greater ability to suppress $CD4^{+}$ and $CD8^{+}$ T-cells and NK cells proliferation compared to splenic B-cells. Furthermore, tumor infiltrating B-cells associated with persistently activated STAT3 are identified in several human tumors including melanoma, gastric, lung, liver, and prostate cancers; also, STAT3 activity in human tumors is related to increased TIL-Bs density and tumoral angiogenesis. Lastly, immune

suppressive and tumor promoting functions of MDSCs may be acquired through “education” Breg-dependent, highlighting the Bregs role in modulating myeloid suppressor cell activity, in addition to aforementioned effects on TCs and NK cells, contributing to immune microenvironment (Fig.13)[26].

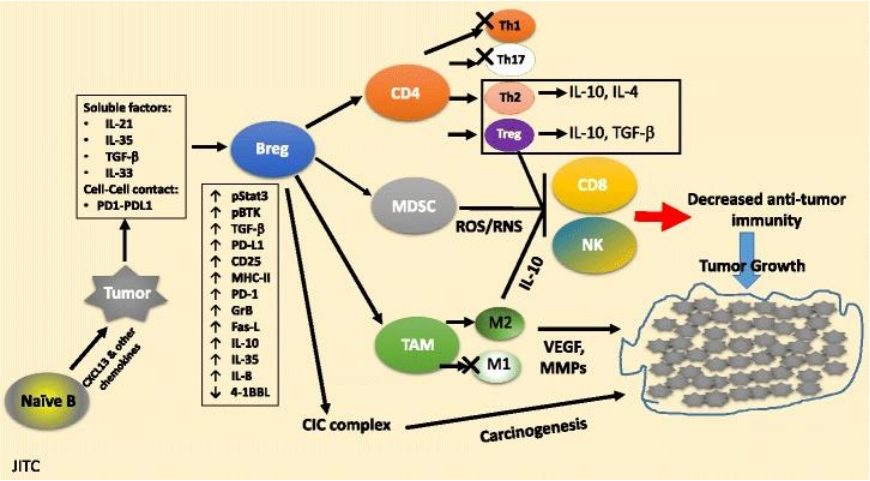

Fig.13[26] The cross-talk between B regulatory cells, Treg and tumor

Natural Killer cell (NKc)

The HSC commitment to NKc differentiation requires several transcription factors and interactions with microenvironmental elements; during differentiation, NK cell precursors express the DNA binding protein inhibitor ID-2 along with PU.1, ETS-1, TOX, and E4BP4. NK cells lack the cluster of differentiation 3 (CD3)/TCR molecules and express low-affinity Fc-receptor γ IIIA (CD16) and neural cell adhesion molecule (NCAM, CD56) on cell surface. Three major human NK cell subsets can be distinguished in peripheral blood: $CD56^{high}$ $CD16^{high/low}$, $CD56^{low}$ $CD16^{low}$, and $CD56^{low}$ $CD16^{high}$; these subsets resemble more NK-cells at different stages of maturation. $CD56^{high}$ $CD16^{high/low}$ cells can be firstly differentiated from BM-derived CLP and localize within lymph nodes[22;25]. $CD56^{high}$ subset exhibits decreased level of CD16 expression and CD94/NKG2A high levels, with lack of killer immunoglobulin(Ig)-like receptors (KIRs); produces plenty of cytokines such as IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and express CCR7. Instead, $CD56^{low}$ cells can translocate to inflamed peripheral tissues expressing CX3CR1, CXCR1 and ChemR23 receptors. Hence, NKs phenotypical hallmarks depend on maturation microenvironment properties and NK cells developmental phase.

NKc, as innate immune system member, is associated with powerful capability of kill tumor cells or prevent metastasis; antitumoral function is regulated by activating and inhibitory receptors. The relevant activating receptors include natural cytotoxicity receptors (NKp30, NKp44, and NKp46), CD16 involved in antibody-dependent cell-mediated cytotoxicity (ADCC), C-type lectin receptors

like NKG2D, SLAM family receptors (2B4, SLAM6, and SLAM7) and some KIRs (KIR2DS2, KIR2DS5, KIR3DS1); in addition, costimulatory proteins like DNAM1 and 41BB regulate activating receptors function. NK cells activation is counterbalanced by inhibitory receptors such as KIRs (KIR2DL1, KIR3DL1, KIR2DL3, KIR2DS4), PD-1, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), TIM-3, and lymphocyte-activation-gene-3 (LAG-3). Moreover, tumor cells recognition by NKs can involve two different mechanisms. The first is defined missing-self recognition: HLA class I molecules absence is associated with disrupted transition of inhibitory signals through KIRs and subsequently activation of NK cells; second mechanism is identified as stress-induced recognition, since upregulated stress-ligands on tumor cells interact with activating receptors and then start triggering of NK cells cytotoxic activity.

Attaching tumor cells, NKs can express apoptotic ligands such as TNF-related apoptosis-inducing ligand (TRAIL) or tumor necrosis factor (TNF) family members like FasL; moreover, NK cells interaction with related receptors on cancer cells is followed by apoptosis induction through caspase cascade. On the other hand, NKs exhibit also a direct mechanism to tumor cells lysis through perforin and granzyme secretion in immunological synapse or ADCC CD16 receptor-mediated[27].

Dendritic cell (DC) genesis and differentiation

Dendritic cells (DCs) are bone-marrow-derived cells, arising from lympho-myeloid hematopoiesis; represent an essential interface between pathogens innate sensing and adaptive immunity activation, with lifespan of days to weeks after entering into periphery, and must be continually replenished by hematopoiesis. Between $CD34^+$ progenitors and mature DCs, exist potential pre-DCs defined as DC-restricted precursors; phenotypically is not a homogeneous multipotent population, rather a cross-section of cells, primed by related but distinct developmental pathways, which exhibit a common, transient phenotype. Thus, potentially the three major DC subsets (plasmacytoid DC, myeloid/conventional DC1, myeloid/conventional DC2) can be traced from hematopoietic stem cells, through lymphoid-primed multipotent progenitors to granulocyte-macrophage DC progenitors (GMDP).

Dendritic cells development is dependent on coordinated action of many transcription factors such as GATA2, PU.1, GFI1, IKZF1 and IRF8. The balance between pDCs and myeloid/conventional DCs development is regulated through antagonism between E2-2 and ID2, an DNA binding inhibitor; E2-2, a lineage-determining factor for pDCs, is negatively regulated by ID2[28;29].

Plasmacytoid dendritic cell (pDC) is characterized by an eccentric nucleus and prominent endoplasmic reticulum and Golgi, resembling a plasma cell. Differently from myeloid/conventional DCs, pDC subset does not express myeloid antigens CD11c, CD33, CD11b or CD13, but retains

GMDP markers CD123 (IL-3R) and CD45RA expression. In addition, pDCs express CD4, though at a higher level than myeloid cDCs, and several cell surface receptors such as CD303, CD304, CD85k (ILT3) and CD85g (ILT7), along with FceR1, BTLA, CD358 and CD300A antigens; additional markers are FAM129C, CUX2 and GZMB. Plasmacytoid DCs are specialized to sense and respond to viral infection through rapid production of type I and III interferons high amounts and cytokines secretion. The binding to surface receptors modulates activation or tolerance through IRF7 and nuclear factor-κB pathways regulation; indeed, IRF7 is the major transducer of type I interferon production, whereas tumor necrosis factor and IL-6 production is dependent upon NF-κB pathway.

Instead, human myeloid cDC1s are DCs associated with high expression of CD141 (BDCA-3, thrombomodulin). Commonly with myeloid cDC2s, express CD13 and CD33, but differ by low CD11c and little CD11b or CD172 expression. Additional markers used to increase identification accuracy are: CLEC9A, the cell adhesion molecule CADM1 (NECL2), the antigen BTLA and also indoleamine 2,3-dioxygenase; DC1 cells are found in blood and among resident DCs in lymph nodes, tonsil, spleen, bone marrow and skin, lung, intestine and liver. Myeloid cDC1 development is dependent upon GATA2, PU.1, GFI1, ID2, IRF8 and Basic leucine zipper transcription factor (BATF3); especially IRF8 acts to preserve DC potential at several hematopoiesis phases through direct or indirect competition with several transcription factors (CEBPA, PU.1, IRF4) that promote other lineages development. Functionally, DC1 subset exhibits a high intrinsic capacity to cross-present antigens via MHC class I, to activate $CD8^+$ T-cells and promote Th1 and natural killer responses through IL-12[28;29;30].

The major myeloid cDCs subtype in human blood, tissues and lymphoid organs is defined as myeloid cDC2 and is characterized by CD1c, CD2, FceR1 and SIRPA expression; myeloid antigens CD11b, CD11c, CD13 and CD33, CLEC10A (CD301a), VEGFA and FCGR2A (CD32A) are identified as consistent cDC2 markers through transcriptional profile, concurrent with cDC1 markers lack. Myeloid cDC2 development is dependent on GATA2, PU.1, GFI1, ID2, ZEB2, RELB, IRF4, NOTCH2 and KLF4 and, differently from pDC and cDC1 subsets, no single transcription factor has exclusive control over their development.

DC2s exhibit a wide range of lectins, TLRs, NOD-like and RIG-I-like receptors; among lectins, CLEC4A, CLEC10A, CLEC12A and CLEC4H1 are highly expressed. In addition, CLEC7A and CLEC6A exhibit high expression level in tissue cDC2s, while CLEC13B and CLEC13D expression is variable. Other receptors include TLRs 2, 4, 5, 6 and 8, NOD2, NLRP1, NLRP3 and NAIP. Human cDC2s can be stimulated to become excellent cross-presenting cells and produce high IL-12 amounts; in addition, cDC2s secrete IL-23, IL-1, TNF-α, IL-8, IL-10 and produce type III interferon

at low level. Moreover, human cDC2s active Th1, Th2, Th17 and $CD8^+$ T-cells in vitro, suggesting that can promote several immune responses in vivo[28;29].

Through appropriate stimuli, DCs mature and express chemokine receptors and costimulatory molecules. The best characterized chemokine receptor upregulated in maturing DCs is CCR7, which is necessary for migration of tumor-infiltrating DCs into tumor-draining lymph nodes (TDLNs). However, CCR7 may also be involved in DCs recruitment into the TME, although its effect may be context specific. Instead, among costimulatory molecules, CD80 and CD86 control T-cells activation or suppression through interaction with CD28 or CTLA4, respectively. Additional costimulatory pathways involved in DC-mediated T-cells priming and reactivation include CD40–CD40L, CD137–CD137L, OX40–OX40L, GITR–GITRL and CD70–CD27 signaling axes. CD137L, expressed on antigen-presenting cells (APCs) membrane, promotes $CD4^+$ and $CD8^+$ TCs activation and survival through CD137; OX40L on DCs and macrophages cell surface also contributes to T-cells survival, and GITRL promotes T-cells resistance to Treg-mediated immunosuppression. CD70 supports $CD8^+$ TCs cross-priming, differentiation and antitumor activity, and CD40 on DCs cell surface interacts with CD40L on T-cells membrane, leading to DC activation. On the other hand, presentation of TAAs in the absence of costimulatory signals leads to TCs anergy, and inhibitory receptors high engagement can limit T-cells effector activity. CTLA4, expressed by T-cells, binds CD80 and CD86 on DCs cell surface with greater affinity than CD28, limiting costimulatory signaling and T-cell activation; in addition, PDL1 and PDL2 expressed by DCs and other cells in the tumor microenvironment inhibit proliferation and cytokine production by PD1-expressing activated T-cells. DCs can also modulate T-cells functions through metabolic substrates availability modification. l-Tryptophan, essential for T-cell-mediated responses, is depleted through conversion to l-kynurenine by indoleamine 2,3-dioxygenase 1 (IDO1); IDO1 production is induced in DCs after recognition of apoptotic cells or CTLA4 binding with CD80 and CD86. Furthermore, tumor-associated DCs can exhibit increased IDO1 expression, and $IDO1^+$ DCs suppress proliferation and effector functions of $CD8^+$ T-cells, NKs and plasma cells, contributing to Treg cells differentiation. In addition to TAAs and endogenous DAMPs, the TME also contains immunosuppressive factors that can inhibit DCs infiltration and lessen their antitumor activity. Cancer cells with active β-catenin reduce CC-chemokine ligand 4 (CCL4) expression, leading to lower cDC1s infiltration and increased tumor growth. Conversely, tumor infiltrating NK cells recruit cDC1s through production of CCL5 and XC-chemokine ligand 1 (XCL1), and promote cDC1s survival via FMS-related tyrosine kinase 3 ligand (FLT3L). On the other hand, tumor cells can reduce NK cells viability, limiting in turn cDCs density and favoring tumor growth; in addition, tumor-derived vascular endothelial growth factor (VEGF) can inhibit FLT3L activity and

negatively impact on cDCs differentiation in vitro. Both tumoral and immune cells can also produce IL-6, a pro-inflammatory cytokine that impairs cDCs differentiation.

Overall, DCs interact with immune system cells and several TME mechanisms perturb their activity, modulating final immune responses and potentially inducing immunological tolerance[29;30].

Monocyte and macrophage genesis and differentiation

Human monocyte cells (MCs) are classified in three subsets by CD14, CD16 surface level expression ($CD14^{++}$ $CD16^{-}$, $CD14^{++}$ $CD16^{+}$, $CD14^{+}$ $CD16^{++}$). A revised gating strategy include CD36, HLA-DR, CD11c, and CCR2 to obtain more phenotypically pure populations of human monocytes and improve the CD14/CD16 subsets definition.

Thus, classical/intermediate/nonclassical monocytes subsets are recently defined as $CD14^{+}$ $CD16^{-}$ $HLA\text{-}DR^{lo}$ $CD11c^{lo}$, $CD14^{+}$ $CD16^{+}$ $CD36^{hi}$ $CCR2^{hi}$ $HLA\text{-}DR^{hi}$ $CD11c^{hi}$, and $CD14^{-}$ $CD16^{+}$ $CD36^{lo}$ $CCR2^{lo}$, respectively. The two surface markers, CD14 and CD16, lends some insight into monocyte function; CD14 acts as a co-receptor for toll-like receptor 4 and mediates lipopolysaccharide (LPS) signaling, while the CD16 antigen is identified as FcγRIIIa and is involved in innate immunity .

$CD14^{++}$ $CD16^{-}$ subpopulation which accounts for 80%–90% of peripheral blood MCs, is identified as classical subset; produces monocyte chemotactic protein-1 (MCP-1/CCL2) and expresses its cognate receptor CCR2. CCR2 is pivotal for monocyte migration out of the bone marrow and homing, while CD62L (SELL) is an important early marker of progenitor cells commitment. During bacterial infection, classical monocytes are recruited to inflammation sites, recognize and phagocytose pathogens, secrete various cytokines, and recruit other immune cells for regulation of the inflammatory response.

$CD14^{++}$ $CD16^{+}$ MCs constitute a very small percentage in circulation under physiological conditions and appear to be transitional MCs exhibiting both phagocytic and inflammatory functions; thus, are classified as intermediate class. Intermediate monocytes produce significantly more tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) compared to the other populations.

$CD14^{+}$ $CD16^{++}$ non-classical MC subtype displays inflammatory characteristics upon activation and antigen presentation; non-classical monocytes exhibit a distinct motility and crawling pattern along the vasculature and hence are defined as patrolling monocytes. However, non-classical monocytes recognize and clear dying endothelial cells in a TLR7-dependent manner to maintain vascular homeostasis, appearing to be sentinels and caretakers of the vascular tissue. Their receptor necessary for migration out of the bone marrow appears to be an S1P receptor, S1PR5.

Myeloid monocyte-dendritic progenitors (MDPs) give rise to MCs within BM. IFN-I signaling in hematopoietic cells is required for generation of Ly6C antigen positive MCs; a critical transcription factor for $Ly6C^{+}$ MC differentiation is PU.1. Differential PU.1 expression has been found within the $Ly6C^{hi}$ population, leading to a spectrum of heterogeneous classical monocytes in the bone marrow and blood compartments. In addition, PU.1 transcription factor level has been shown to dynamically regulate conversion of $Ly6C^{hi}$ monocytes to varied myeloid subsets. PU.1 overexpression leads to IRF8 and Kruppel-like factor 4 (KLF4) activation, which are also key transcription factors involved in $Ly6C^{+}$ MC development; KLF4 is directly regulated by IRF8.
On the other hand, transcription factor nuclear receptor subfamily 4, group A, member 1 (NR4A1) is required for $Ly6C^{-}$ MC development[22;32;33].
Under physiological homeostasis, classical MCs can differentiate into intermediate MCs, and further into patrolling non-classical MCs in circulation; instead, during inflammation MCs migrate to the tissues and further differentiate into macrophages (MØ) according to environmental stimuli. Macrophages can respond to endogenous stimuli that are rapidly generated following injury or infection by innate immune cells but can also respond to signals produced by antigen-specific immune cells. These signals are more prolonged than innate immune stimuli and generally give rise to longer-term alterations in macrophages. In addition, macrophages themselves can produce several factors that influence their own physiology.
Macrophages are classified as M1 (classically activated macrophages) or M2 (regulatory macrophages). The classical macrophages (M1) activation requires both IFN-γ and TNF. Typically, a TLR ligand acting in a MyD88-dependent manner induces the TNF transcription, which can then cooperate with IFN-γ in an autocrine manner to activate this macrophage population. In addition to MyD88, some TLR ligands can also activate TIR-domain-containing adaptor protein inducing IFN-β (TRIF)-dependent pathways; endogenously produced IFN-β can replace the IFN-γ produced by NK cells and T-cells and activate M1 macrophages. Classically activated macrophages produce pro-inflammatory cytokines which are an important component of host defence; for example, IL-1, IL-6 and IL-23 are associated with Th17 cells development and expansion. Instead, IL-4 stimulates arginase activity in macrophages, allowing them to convert arginine to ornithine, promoting extracellular matrix production and wound healing.
On the other hand, the regulatory macrophages (M2) can arise during the later stages of adaptive immune responses, dampening the immune response and limiting inflammation; can also exert indirect regulatory effects on the immune response because produce polyamines that can influence cytokines production and suppress the clonal expansion of neighbouring lymphocytes. The regulatory macrophages generation involves many different pathways, although the MAPK

extracellular-signal-regulated kinase (ERK) emerges as a potential candidate; other factors can provide a signal for regulatory macrophages differentiation, including prostaglandins, apoptotic cells, IL-10 and some ligands for G-protein-coupled receptors (GPcRs). The first signal (e. g. immunological complexes, prostaglandins, adenosine or apoptotic cells) generally has little or no stimulatory function on its own; the combination with a second stimulus, such as a TLR ligand, leads to macrophages reprogramming to produce IL-10, the most important characteristic of regulatory macrophages. In addition to IL-10 production, M2 macrophages also downregulate IL-12 production; therefore, the ratio of IL-10 to IL-12 could be used to define regulatory macrophages. M2 macrophages through IL-10 production can inhibit the production and activity of various pro-inflammatory cytokines, inhibiting inflammation despite retain the capability of produce many pro-inflammatory cytokines. Differently from wound healing macrophages, regulatory macrophages do not contribute to extracellular matrix production, but express high levels of costimulatory molecules (cD80 and cD86) and can present antigens to T-cells[47].

Neutrophils, eosinophils and basophils genesis and differentiation

Neutrophils are terminally differentiated cells that develop in the bone marrow, where are also stored before release into the circulation. Neutrophils are short-lived cells that, in the absence of infection or inflammation, die by a spontaneous apoptosis program, likely within 1-5 d after entering the circulation. During maturation, neutrophils develop several intracellular compartments that are secretory granules and vesicles, storing proteins critical for their antimicrobial and proinflammatory functions. Once released into the circulation, neutrophils are able to sense infection and inflammation through several pattern recognition receptors (PRRs) such as members of the Toll-like receptor (TLR) family with the exception of TLR3, CLEC7A, CLEC1B and cytoplasmic sensors of ribonucleic acids (RIG-I and MDA5), leading to neutrophils migration in interesting tissue.

Following infectious particles encounter, activated neutrophils begin a killing program, including ROI, lytic enzymes and antimicrobial peptides production. Upon acute stimulation, neutrophils release TNF-related apoptosis-inducing ligand (TRAIL), CXCL8, CC-chemokine ligand 20 (CCL20) and IL-1 receptor antagonist (IL-1RA); can also extrude extracellular fibrillary networks termed neutrophil extracellular traps (NETs), composed mainly of DNA, but also of proteins from neutrophil granules. NETs act as a mesh that traps microorganisms and, in turn, facilitates their interaction with neutrophil-derived effector molecules. Importantly, NETs also contain some neutrophil-derived pattern recognition molecules (PRMs) with antibody-like properties. In addition,

ion fluxes triggered by the NADPH oxidase would promote the antimicrobial function of granule proteins.

The infectious particles will be phagocytosed by various neutrophil noxious agents released into the phagosome, resulting in killing and digestion of the microorganisms (Fig.14)[48;50].

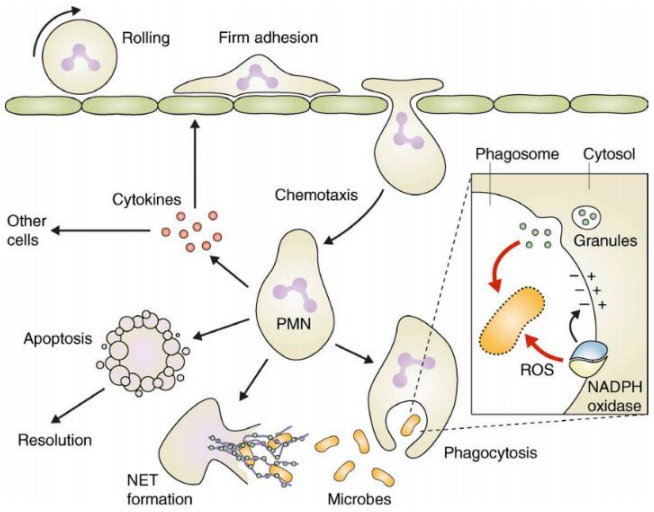

Fig.14[48] Neutrophil functions. After migration to the inflammation site, neutrophils (PMN) phagocytose and digest the invading microbes; release NETs and produce cytokines, which contribute to the inflammatory reaction. Once infection is cleared, neutrophils die by apoptosis and trigger an active program to resolve inflammation

As component of the inflammatory response, neutrophils contribute to recruitment, activation and programming of APCs, generating chemotactic signals that attract monocytes and dendritic cells (DCs), and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state. Neutrophils produce tumour-necrosis factor (TNF) and other cytokines that drive DCs and macrophages differentiation and activation. On the other hand, ectosomes released by human neutrophils inhibit the maturation of both monocyte-derived DCs and monocyte-derived macrophages, possibly increasing their production of the immunosuppressive cytokine TGFβ1 (transforming growth factor-β1). Human neutrophils can also modulate the activation status of NK cells, either by themselves or in cooperation with other cell types. Under physiological conditions, neutrophils are required for the maturation and function of NK cells; in vitro, neutrophils can modulate NK cell survival, proliferation, cytotoxic activity and IFN-γ production through generation of ROI and prostaglandins and/or release of granule components. In turn, culture of neutrophils with NK cells or NK cell-derived soluble factors (such as GM-CSF and IFN-γ) promote neutrophil survival, expression of activation markers, priming of ROI production and cytokine synthesis, suggesting a bidirectional crosstalk between neutrophils and NK cells. Moreover, neutrophils produce cytokines that promote the survival, maturation and differentiation of B-cells

such as BAFF, a proliferation-inducing ligand (APRIL) and TNF-related ligand B-lymphocyte stimulator (BLyS).

Activated neutrophils can attract Th1 and Th17 cells to inflammation sites via the release of CCL2, CXCL9 and CXCL10 or CCL2 and CCL20, respectively. In addition, activated T-cells can recruit neutrophils, although the mechanism used by individual T-cell subsets differs. Indeed, Tregs or Th17 cells, but not Th1 cells, can release CXCL8; in addition, both IL-17A and IL-17F released by Th17 cells stimulate epithelial cells to secrete granulopoietic factors (such as G-CSF and stem cell factor), as well as neutrophil chemoattractant (such as CXCL1, CXCL2, CXCL5 and CXCL8), which thus amplify neutrophil recruitment and activation.

However, neutrophils can also function as powerful suppressors of T-cell activation; in patients with advanced cancer, activated neutrophils can impair T-cell receptor (TCR) ζ-chain expression and cytokine production. The neutrophils capability of augment or inhibit lymphocytes expansion and activation at inflammation sites is counterbalanced by the adaptive immune system's control of the neutrophils production rate in the bone marrow. The cytokine granulocyte colony-stimulating factor (G-CSF) is an essential regulator of neutrophils production through several mechanisms; stromal-cell-derived G-CSF triggers bone marrow neutrophils to release matrix metalloproteinase 9 (MMP9), which solubilizes KIT ligand, helping to mobilize progenitor cells. G-CSF also acts directly on progenitors to increase their proliferation and, suppressing stromal-cell CXCL12 expression, contributes to retain neutrophils in the bone marrow. On the other hand, G-CSF production is regulated by IL-17, which is produced by T-cells; T-cell IL-17 production is controlled through IL-23 release from extravascular macrophages. The release of IL-23 is suppressed when macrophages ingest apoptotic neutrophils (Fig.15) [49;50].

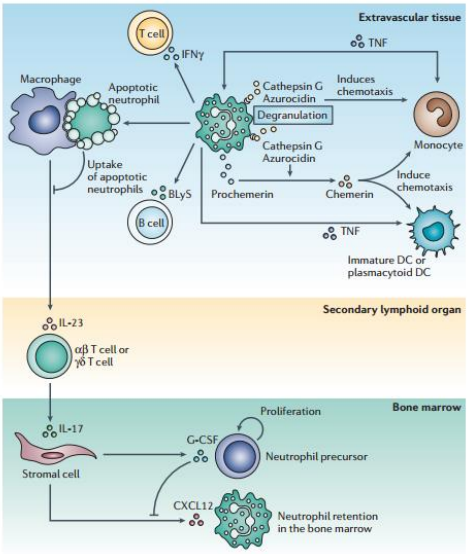

Fig.15[49] Neutrophils interact with monocytes, dendritic cells, T cells and B cells in a bidirectional, multi-compartmental manner

Basophils complete the differentiation and maturation in BM and then circulate in the bloodstream, constituting less than 1% of circulating leukocytes. IL-3 plays a pivotal role by directing granulocyte-monocyte progenitors to differentiate into basophil lineage, especially under pathological conditions. Also, eosinophils differentiate in the bone marrow from IL-5R α-positive progenitors, and then migrate into blood, constituting about 1%–6% of circulating leukocytes. The presence of intracellular granules stained with the acidophilic dye eosin, allow their discrimination from other granulocytes such as neutrophils and basophils. Eosinophils express several surface molecules, whose aggregation can induce eosinophils activation, such as CD32, CD89, complement receptors (CR1/CD35, CR3, and CD88), cytokine receptors (IL-3R, IL-5R, GM-CSF, which promote eosinophil development along with receptors for IL-1 α, IL-2, IL-4, IFN- α, and TNF- α) and chemokine receptors (CCR1 and CCR3). In addition, eosinophils express adhesion molecules (very late antigen 4 (VLA4) and α4 β7), siglec-8 and toll-like receptors (particularly TLR7/8). During inflammatory responses eosinophils could be regulated by IL-33, which can stimulate eosinophils differentiation in IL-5-dependent manner. The integration of positive and inhibitory signals received from microenvironment leads to different secretory pathways: exocytosis, piecemeal degranulation, a form of exocytosis involving the fusion of small and rapidly mobilized secretory vesicles with cell membrane, and cytolysis that is the release of whole and intact granules following the cell membrane rupture. Eosinophil degranulation and tumor-associated tissue eosinophilia have been observed in many solid and hematological cancers with a good and poor prognostic significance, respectively. Some evidences suggest a pro-tumorigenic role for eosinophils through the enhancement of tumor angiogenesis and connective tissue formation. In contrast, it is known that eosinophils may mediate anti-tumor effects by direct cytotoxicity or exerting an IL-4 mediated immunomodulatory function, which regulates the skewing of the immune response from a Th1 to a Th2. Nevertheless, eosinophils activated in tumor microenvironment dominated by Th1 factors have been described to promote $CD8^+$ T-cells cytotoxicity and recruitment also through normalization of tumor vasculature.

On the other hand, in basophils the FcεRI activation triggered by the crosslinking with antigen-specific IgE, results in release of their granule containing inflammatory mediators, which are responsible of early and late-phase anaphylactic reactions. The released mediators comprise histamine, proteases, cytokines and chemokines, which may act locally on other inflammatory cells but also on vessels and smooth muscle to activate protective responses.

The immune responses of type 2 are regulated by several cytokines, namely IL-4, IL-5, IL-9 and IL-13. IL-4 could promote IgE production by B-cells in the presence of a specific antigen, activating allergen specific responses though the link with FcεRI on MCs and basophils. Thus, these cell type

not only participate in the acute phase as effector molecules but are able to initiate Th2 polarization of responses in the late phases interacting with adaptive immune cells. In addition, IL-4 produced by basophils acts on a wide range of immune cells, including type 2 macrophages which activated via IL-4 may play a regulatory role from defence from infections to tissue repair and tumorigenesis[51].

Crosstalk between bone niche, hematopoietic stem cells and immune system

Differently from bone concept as static tissue, it's actually defined that bone is extremely dynamic, undergoing continuous cycles of modeling-remodeling during growth and adulthood. Bone physiology and pathophysiology are regulated by osteoclasts, which resorb bone, osteoblasts, which depose bone, osteocytes, able to resorb and depose bone, affecting bone mechanical properties, and by immune system cells, involved in bone mass maintenance. Overall, bone cells can influence immune system functions, but also immune system components can condition bone health.

Osteoblasts contribute to commitment and differentiation of B lymphocytes from hematopoietic stem cells (HSCs); in addition, a subpopulation of osteoblasts (Spindle-shaped-N-cadherin^{+} CD45^{-} osteoblasts-SNOs) is involved in HSCs proliferation control, thus identifying the bone marrow "endosteal niche". Indeed, osteoblasts can both sustain and negatively regulate HSC proliferation, through granulocyte colony-stimulating factor (G-CSF), Jagged-1/Notch1, and osteopontin signaling. Instead, osteocytes are the main producers of receptor activator of nuclear factor kappa B ligand (RANKL), a cytokine pivotal not only for osteoclasts but also for lymphocyte development; osteoclasts regulate the hematopoietic stem niche both directly and indirectly through osteoblasts. Indeed, osteoclasts can increase HSCs mobilization secreting cathepsin K, which cleaves SDF1, osteopontin (OPN) and stem cell factor (SCF), depriving the bone niche of HSC-binding sites. Consequently, HSCs mobilize and are no longer kept quiescent.

However, among immune system subsets, Th17 and Treg subtypes exhibit emerging role in interactions with bone. Th17 cells may be the most osteoclastogenesis-inducing T-cells, since induce macrophage colony-stimulating factor (M-CSF) and RANKL expression in osteoblasts and stromal cells, produce RANKL and TNF-α and parallelly increase RANK expression in osteoclast precursors; conversely, Tregs are clearly anti-osteoclastogenic through a soluble factors-mediated as well as a contact-mediated mechanisms. In addition, activated neutrophils are osteoclastogenesis inducers, both directly and indirectly; indeed, express RANKL in inflammatory sites and, if site is the synovium, can actively participate in osteoclastogenesis, and through CCL2 and CCL20 secretion recruit Th17 cells which in turn cause bone loss.

Instead, B-cell development relies on the several factors production, such as RANKL, OPG, IL-7, and CXCL12, produced by bone marrow stromal cells and osteoblasts; furthermore, RANKL arising from the bone marrow/bone compartment is crucial for B-cell development, but also B-cells themselves produce RANKL, which acts as autocrine factor. The RANKL production by B-cells suggests that B lymphocytes could influence osteoclasts, supporting their development (Fig.16). Moreover, resident macrophages in bone and bone marrow include osteal macrophages, known as osteomacs, F4/80 positive and TRAcP negative, located close to the bone surface; osteomacs are adaptable cells exhibiting capability of regulate bone mass, become osteoclasts, and actively participate in the immune system homeostasis.

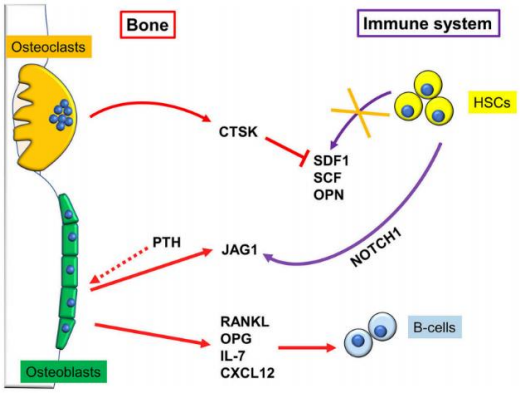

Fig.16[34] Regulation of hematopoietic cells by bone cells. Osteoclasts reduce hematopoietic stem cells (HSCs) homing depriving the bone niche of HSC-binding sites, which causes their mobilization; instead, osteoblasts allow HSCs to engraft and survive into the endosteal niche

Also, IFN-γ produced by several immune system cells affects both osteoblasts and osteoclasts, increasing activity of osteoblast differentiating genes, such as *RUNX2*, *OSX*, *ALP*, osteocalcin, and inhibiting osteoclast differentiation. Furthermore, IFN-γ counteracts M-CSF effect on osteoclast precursors through reduction of its receptor c-fms expression, leading probably to a decreased pool of Rank-positive pre-osteoclasts. However, some evidences suggest a pro-osteoclastogenic effect of IFN-γ in late differentiation phase, depending on NFATc1 and c-fos upregulation, which in turn stimulate DC-STAMP expression, thus promoting osteoclast fusion. In addition, IFN-γ exhibits indirect pro-osteoclastogenic effect due to ability to increase CXCL10 secretion by macrophages; CXCL10 in turn stimulates RANKL and TNF-α production by T-cells. Other pro-osteoclastogenic factors include prostaglandins, IL-6, IL-11, IL-23, IL-1α, IL-1β, IL-7, IL-8 and IL-34[34;53].

BiTE CD19-CD3 immunotherapy in acute lymphoblastic leukemia

The treatment optimization based on novel drug combinations, doses and therapeutic schedules results in a cure rate of 80%-90% in pediatric setting; for adults the cure rates reach 40%-50%, despite high complete remission rates (80%-90%). Even though survival rate is increased, thanks to improvement in treatments due to targeted drugs incorporation (e.g. BCR-ABL1 inhibitors), approximately ~50% of adult ALL patients still relapse. Cytotoxic chemotherapy produces CR rates of 30% to 40% in first salvage and 10% to 20% in later salvages. In addition, few patients have the opportunity to be bridged to allogeneic stem cell transplantation, which offers a chance of long-term remission and cure (< 20%-30%). Thus, the major challenge is a therapeutic strategy that increases cure rate and reduces the need of intensive and prolonged chemotherapy.

Among recent breakthroughs, monoclonal antibody-based therapies represent a promising treatment for adult ALL. Monoclonal antibodies, designed to bind a specific target expressed on leukemic cells, act through several mechanisms, including antibody-dependent and complement-dependent cytotoxicity and induction of apoptosis. ALL cell targets investigated most thoroughly to date include CD19[16;36;37].

CD19 is classified as type I transmembrane protein belonging to the immunoglobulin super-family, with an extracellular N-terminus, a single transmembrane domain and a cytoplasmic C-terminus; its expression is activated by transcription factor paired box 5 (PAX5) and is maintained from the heavy chain rearrangements phase until plasma cell differentiation. The extracellular portion forms a complex with CD21 (CR2, complement receptor 2), CD81 and Leu-13 (CD225) on cell surface; the single transmembrane domain is essential for complex formation, but the most important part of CD19 structure is its cytoplasmic domain, which consists of ~240 amino acids with nine conserved tyrosine residues that play a pivotal role in CD19-mediated signals transduction of CD19-mediated. Indeed, phosphorylated tyrosine residues act as intracellular docking site for signaling molecules that contain Src homology 2 (SH2) domain, such as Lyn, Fyn, Vav, Grb2, PI3K, PLCγ2, and c-Abl. In addition, PI3K involved in pivotal mechanisms of B-cells development is subjected to CD19 modulation; indeed, CD19 could sequester, via its cytoplasmic tail, critical amounts of positive signaling molecules including PI3K, which are activated only when CD19/BCR complex resides into the lipid raft. In the absence of CD19, the CD19 associated PI3Ks are available to bind other adaptor proteins, such as BCAP and TC21, which interact constitutively with BCR and display receptor specific activation properties. Furthermore, CD19-BCR ligation greatly augments BCR-induced $Ca2^{+}$ transport and CD19/CD21 complex can translocate along with BCR into cell membrane, sustaining BCR residency in lipid rafts and resulting in prolonged BCR signaling (Fig.17). CD19 is considered a positive regulator of B-cell development amplifying Src-family

protein tyrosine kinases (PTKs) activation, enhancing mitogen-activated protein kinase (MAPK) activity, promoting cell proliferation. However, CD19 functions related to BCR signaling are complex and may include also regulation of downstream mechanisms. Indeed, in CD19-deficient B-cells after BCR ligation is detected delayed but significantly elevated calcium, suggesting a possible regulation of timing calcium release by CD19[38;39].

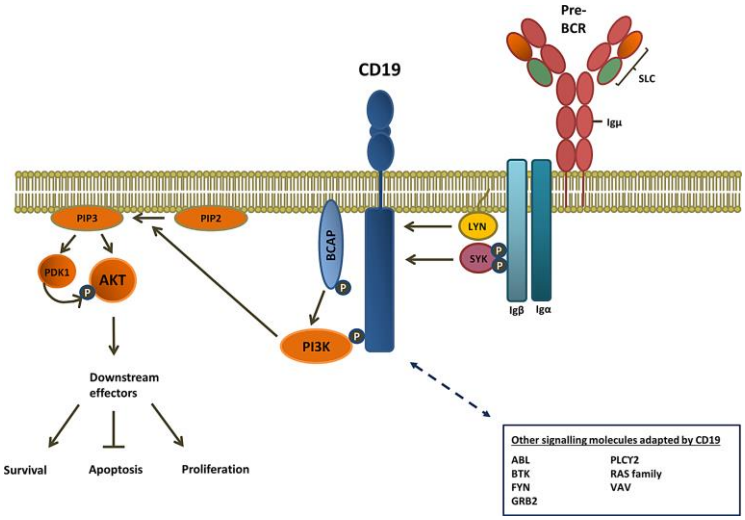

Fig.17

Fig.17[(39)] CD19 signaling. Activated by the pre BCR, the protein kinases LYN and SYK phosphorylate tyrosine residues of CD19, causing an intracellular docking site for multiple signaling molecules

CD19 is associated with malignant B-cell phenotype maintenance through mechanisms involved in cell proliferation, survival and self-renewal, is expressed on B-lineage ALL cells at different developmental stages and high percentages of ALL cells express CD19. Thus, CD19 has been selected as target antigen of T-cell engaging bi-specific (BiTE) single-chain antibody Blinatumomab, the international non-proprietary name given to the CD19-CD3-bispecific BiTE by the World Health Organization.

Blinatumomab is a 55 kDa two single-chain antibody, not glycosylated, that contains both an anti-CD3 arm and an anti-CD19 arm joined by a non-immunogenic linker sequence of 5 amino acids, allowing the flexibility needed for simultaneous binding of two cells. Starting from the binding, Blinatumomab is capable of establish very tight cytolytic synapses between effector cells and $CD19^+$ target cells, followed by perforin/granzyme-mediated cell destruction via proteolytic activation of caspases 3 and 7 and serial lysis in tumor cells. Moreover, the link between Blinatumomab and $CD19^+$ leukemic cells induces polyclonal T-cells activation in association with T-cell compartment high proliferation. Indeed, in response to BiTE CD19-CD3 stimulation, the majority of $CD8^+$ and $CD4^+$ T-cells start to express CD25 and CD69 as well as adhesion molecules on cell surface (e.g. CD2, LFA-1); furthermore, Blinatumomab-activated T-cells transiently release inflammatory cytokines, especially IL-6, IL-10, interferon-γ, in a dose-dependent manner (Fig.18).

BiTE CD19-CD3-based therapy is independent from TCR specificity and peptide antigen presentation through major histocompatibility complex class I molecules[40;41].

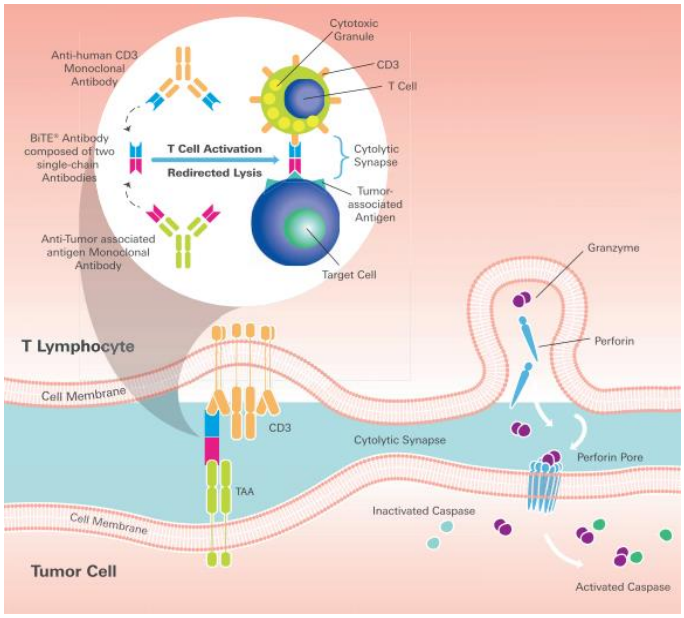

Fig.18[(40)] Mechanism of action of BiTE antibody blinatumomab

Blinatumomab is commonly administrated through a 28-day continuous infusion, using a portable mini-pump in order to maintain steady drug concentration, since pharmacokinetic from preclinical and early clinical studies corresponds to serum half-life of 2–3 hours; after a 14-day rest period, a second cycle is administrated; steady-state concentrations are achieved within 24 hours and persist over the entire treatment course. Blinatumomab pharmacokinetic parameters are not affected by age, sex, weight, body surface area, disease status, and creatinine clearance.

The pharmacodynamic effects of CD19-CD3 BiTE are peculiar: within the first few hours after administration, a rapid transient T-cells decrease occurs, followed by a fast T-cells increase exceeding baseline values. In addition, B-cells rapidly decrease in less than a day, are below the limit of detection in less than 2 days and remain undetectable for the duration of the blinatumomab infusion; this latter phenomenon is attributed to B-cell apoptosis. The low dose (compared with conventional antibodies) of blinatumomab required for response is likely related to the high lytic potential of cytotoxic T-cells. The TCs are activated by engagement of only a few CD3 receptor subunits, can rapidly adopt a serial lysis mode, and can proliferate at site of activation.

The cytokine-release syndrome, characterized by fevers, chills, and hypotension and shortness of breath in severe cases, is a known adverse event during blinatumomab treatment and attributed to rapid malignant cells kill by T lymphocytes during the initial infusion. In addition, CNS adverse events have been reported in 15%–20% of patients treated with BiTE CD19-CD3 and can be severe.

Although not fully explained, the blinatumomab-related CNS toxicity may be associated with activated T-cells adherence to endothelium[42;43].

The early clinical data on Blinatumomab treatment back to around 2000, but confirmatory clinical trials in specific settings are more recent. A phase III trial (TOWER study) has been conducted in more than 400 adult patients with relapsed or refractory Ph-negative ALL randomized (2:1) to blinatumomab (n.271) or standard of care (n.134). The response rates are 44% and 25%, respectively (P<.001); among responders, molecular remission rates are 76% and 48%, respectively; the median overall survival (OS) is 7.7 months (95% CI, 5.6-9.6 months) vs 4.0 months (95% CI, 2.9-5.3 months; P = .01; HR,0.71; Fig.19). 24% of patients in each group proceeded to allo-SCT.

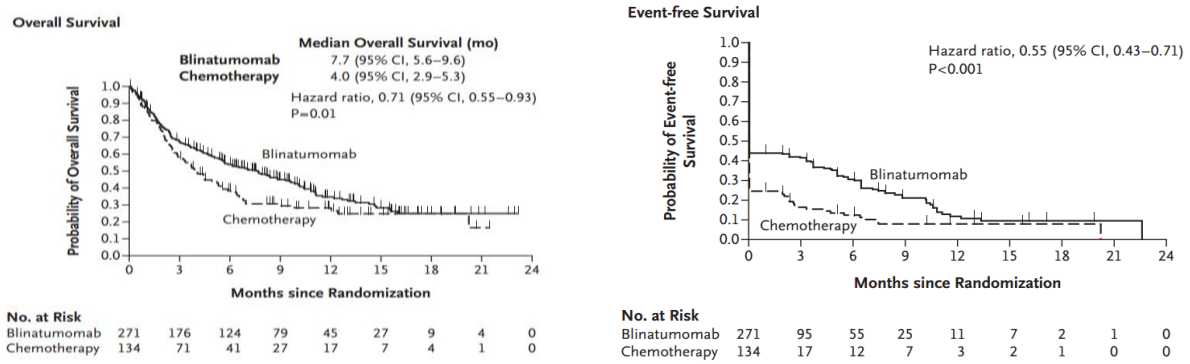

Fig.19[44] Efficacy end points. Overall survival was calculated as the time from randomization to death from any cause; event-free survival was calculated as the time from randomization until relapse after complete remission with full, partial, or incomplete hematologic recovery, or death

In addition, in the phase II ALCANTARA trial 45 adult patients affected by Ph-positive ALL who have progressed after failure of TKI-based therapy have been treated with Blinatumomab. 16/45 (36%; 95% CI) patients achieved CR/CRh during the first two cycles; overall, 14/45 achieved CR. 44% of patients received allogeneic hematopoietic stem-cell transplantation. Median relapse-free survival (RFS) and OS are 6.7 and 7.1 months, respectively. Also, Blinatumomab efficacy has been assessed in 113 patients with ALL in MRD-positive CR. Approximately 78% of patients achieved MRD negativity after one cycle and 80% after 2 cycles. With a median follow-up of 29 months, the median OS is 36.5 months and the RFS 18.9 months; the median OS with achievement of MRD negativity is 38.9 months vs 12.5 months if positive MRD persist. Based on these data and the relevance of MRD status, Blinatumomab has received US Food and Drug Administration approval for patients with positive MRD status[36;44;45;46].

Several studies development includes also the identification of predictive markers associated with CD19-CD3 BiTE treatment. High percentage of Treg (>12.5%) and elevated serum lactate

dehydrogenase levels seem to be independent predictors of response lack in 31 patients with R/R ALL treated with Blinatumomab. Furthermore, the percentage of bone marrow blasts in R/R B-ALL may be predictive for response; indeed, in MT103-211 pivotal trial 73% of patients with less than 50% leukemic cells achieved CR compared with 29% of patients with more than 50% bone marrow blasts at baseline. On the other hand, mechanisms of resistance or escape to blinatumomab treatment are poorly understood. Some evidences suggest that the therapeutic pressure induced by Blinatumomab could select CD19-negative ALL clones as well as clones capable of homing at sites which are less accessible to T-cells. Likewise, causes of primary resistance also remain not clarified[40].

Study design

A retrospective study has been designed to identify biomarkers predictive of response/no response to Blinatumomab treatment. Refractory/relapsed (R/R) B-ALL adult patients treated with BiTE CD19-CD3 from 2014 to 2016 were included in the training set if informed consent and biological samples collected at the baseline and after each treatment cycle were available. Furthermore, according to the same inclusion criteria, R/R B-ALL adult patients under Blinatumomab treatment from 2017 to 2019 represent the study validation set.

The identification of potential predictive biomarkers involved the correlation between gene expression and genomic data and response/no response to therapy.

Materials and methods

The study training set included 18 R/R B-ALL patients (11 male, 7 female, median age 43.5 at treatment start) enrolled in Tower, Alcantara and compassionate protocols (approved by Sant'Orsola-Malpighi EC); 8 R/R B-ALL patients (4 male, 4 female, median age 52 at treatment start) were included in the study validation set. All patients (n.26) received Blinatumomab infusion continuously for 4 weeks (9 μg/d from d1–7 in cycle 1 and 28 μg/d in the following treatment days), and then 2 weeks of suspension; total BiTE CD19-CD3 administration cycles varied, according to the different responses to treatment and to SCT requirement.

26 patients were subdivided in Ph positive group (n.14) and Ph negative group (n.12), if patients were positive for either t(4;11)/*MLL-AF4* (1/12 patients) or t(1;19)/*E2A-PBX1* (0/12 patients) fusion genes or were negative for known molecular rearrangements (11/12 patients). The response to treatment were defined according to the molecular monitoring (BCR-ABL/ABL *ratio* in Ph^+ ALL and Ig qPCR in Ph negative group) and cytofluorimetric assessment; non-responder (NR) patients exhibited minimal residual disease persistence or increase and flowcytometric CD19 positivity,

while the response to treatment was associated with minimal residual disease decrease or negativity and CD19^{-} leukemic cells.

Biological material collection has been performed at baseline and after each treatment cycle, thus obtaining a total of 50 and 24 bone marrow samples in training set and in validation group, respectively. RNA, as extracted by Maxwell® RSC simplyRNA Blood Kit (Promega) from mononuclear cells isolated by Ficoll (GE Healthcare, EuroClone) gradient centrifugation, has been analyzed through gene expression profile using GeneChip Human transcriptome array 2.0 (HTA 2.0, Affymetrix).

The gene expression profiling protocol is the following https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0018137_703174_WTPlus_Reagent kit_Assay_UG.pdf (Fig.20).

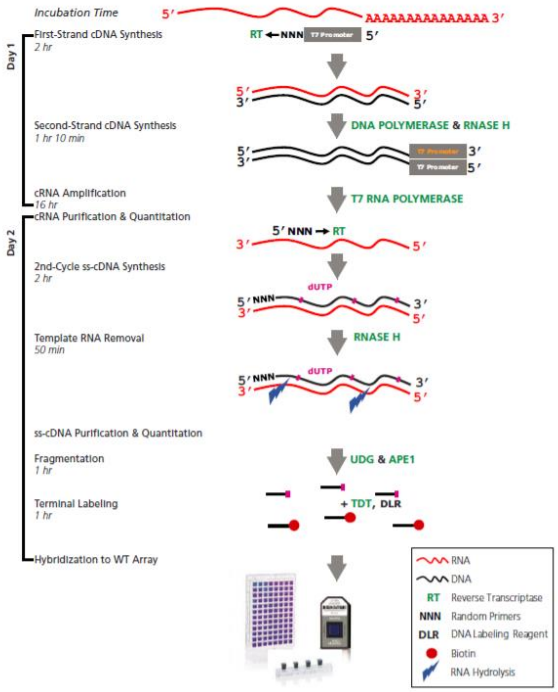

Fig.20 Schematic gene expression profiling (GEP) workflow

The array quality was defined by POS vs NEG AUC >0.7, labeling controls threshold, hybridization controls threshold parameters and has been evaluated by Transcriptome Analysis Console Software (TAC, Affymetrix).

In addition, a bioinformatic analysis pipeline has been designed (in collaboration with Silvia Vitali, DIFA Department-Bologna University), which employed the linear mixed model (LMM) in order to compare responder vs non-responder expression profile, by considering all the possible bias interfering with results. The linear mixed model allows to account whole expression pattern, gene

by gene, taking care of patient classification in terms of therapy response (R or NR), disease classification (Ph positive or Ph negative), blasts percentage, number of treatment cycles performed (time), and of the possible interaction between these factors. The mean, the standard deviation and a p-value of the intercept associated to each gene expression and the effect over it of each label were determined by the model. A random effect over the single patient was also included, which accounts for single sample random variation in the expression.

For each gene the model reads:

$$Y(k, j) = \beta 0 + \beta \bullet C + \Sigma(j)$$

where Y (k, j) is the expression value of the gene in the k-esim sample, associated to patient j; β•C is the scalar product of the effects β={βs} of the condition C={Cs} and Σ (j) is the random effect accounting for single patient variability.

Results

In the training set, 11/18 patients were Ph positive; 8/11 (around 72%) patients did not respond to treatment, while ~27% (3/11) of patients resulted responder to BiTE CD19-CD3 therapy. The other 7 patients were included in the Ph negative group; 5/7 patients were responder, 2/7 not.

Instead, in the validation group 3/3 BCR-ABL positive patients did not respond to treatment. On the other hand, 5/8 patients were classified as Ph negative; 4/5 patients resulted responder, only one not. The analysis through application of Fisher and Pearson's chi-squared tests suggested a significant association between BCR-ABL positivity and unresponsiveness to Blinatumomab therapy (p-values 0.0089 and 0.0063, respectively) (Fig.21).

Condition	Responder	Not Responder	Chi square Pearson test p-value	Fisher exact p-value
Ph+	3	11	0.0063	0.00893 (one tail)
Ph-	9	3		

Fig.21 Analisys of association between BCR-ABL positivity and unresponsiveness to Blinatumomab therapy through Fisher and Pearson's chi-squared tests

All bone marrow samples (74) exhibited an acceptable *ratio* RNA quality (A260/A280 in the range of 1.7 to 2.1), enough to proceed to gene expression profiling; in addition, the quality assessment by TAC software evidenced that 74/74 array were evaluable, since “pass” for POS vs NEG AUC >0.7, labeling controls threshold, hybridization controls threshold parameters.

The bioinformatic analysis trough LMM output allowed to classify the training set patients (R or NR) more significantly, as compared to other analytic methods, such as two way ANOVA; PCAs before and after LMM application is showed in Fig.22-23, respectively.

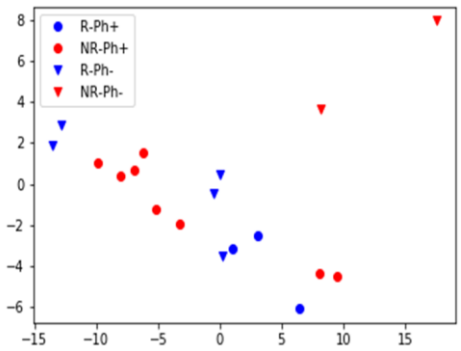

Fig.22 Clustering R vs NR without LMM application

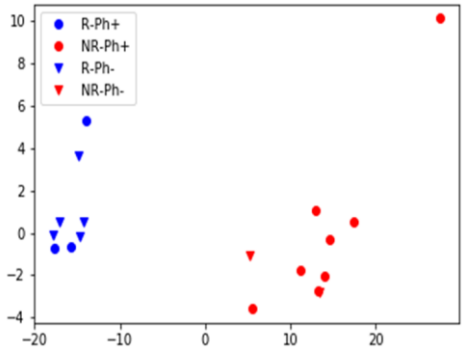

Fig.23 Clustering R vs NR through LMM application

Furthermore, a Leave One (Patient) Out (LOPO) Cross Validation (Fig.24) has been performed to avoid artifacts in the gene signature identification, eventually determined by the intrinsic dataset structure; at each analysis repletion, all the samples associated to a single patient have been excluded to avoid bias.

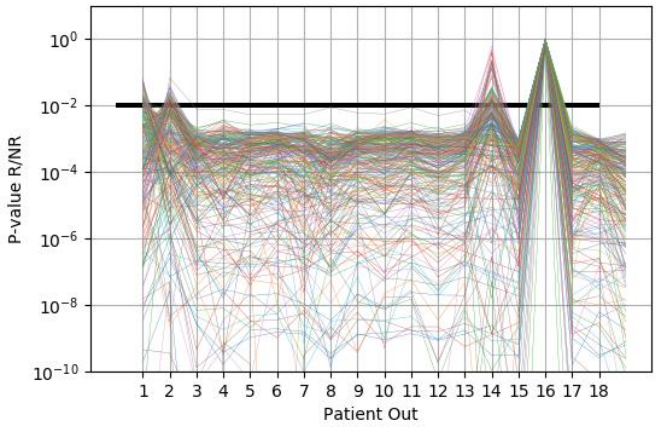

Fig.24 LOPO cross validation graphic. The p-value associated to each gene for each patient is reported on y-axis; on x-axis are reported patient's number

A p-value threshold of ~0.01 has been employed to filter-out genes failing the LOPO; 649 genes passed the filter and were employed to perform a PCA on the GEP values at baseline, corrected for the confounding factors (Fig.25). However, most of genes contributed with a low percentage in the PCA component, thus making hard the selection of smaller, more manageable set of significant genes; therefore, genes that contributed less than 1% to the total variance in the first PCA component were discarded, in order to obtain a smaller set of significant genes (n.11); the PCA was than repeated over this set (Fig.26).

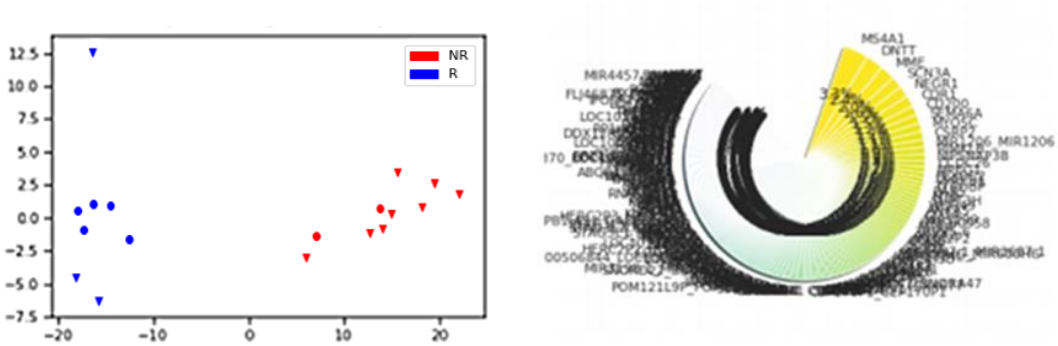

Fig.25 PCA performed at baseline. 649 genes selected through LOPO cross validation differentiate R group from NR group at baseline

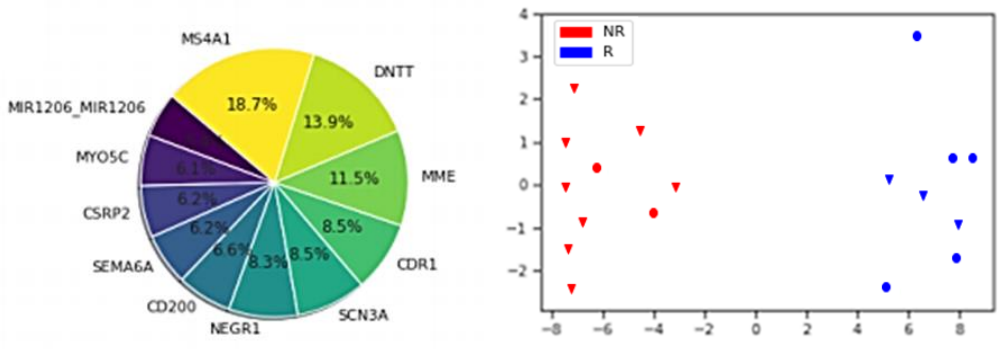

Fig.26 PCA performed at baseline by 11 genes that contributed more than 1% to the total variance

The boxplot of the gene-signature's expression levels, as corrected through the LMM output, highlighted the opposite expression trend between responder and non-responder patients (Fig.27).

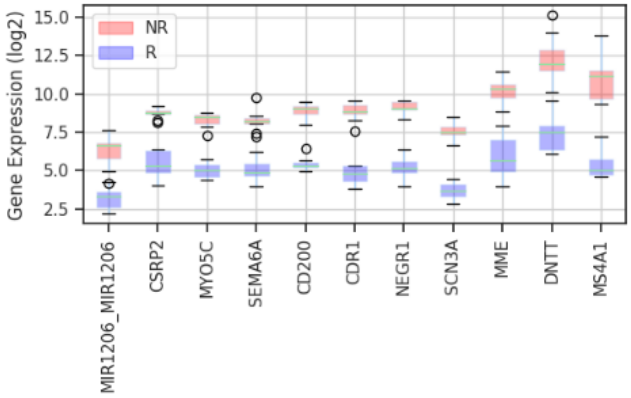

Fig.27 Gene expression level boxplot. On the y-axis, gene expression levels are reported as log2 values; the x-axis shows the genes included in the 11-genes signature

Finally, the LMM power of classify patients as "responder" or "non-responder" has been further confirmed through its output blind application in a validation dataset at baseline; only 1/8 patients was misclassified (Fig.28).

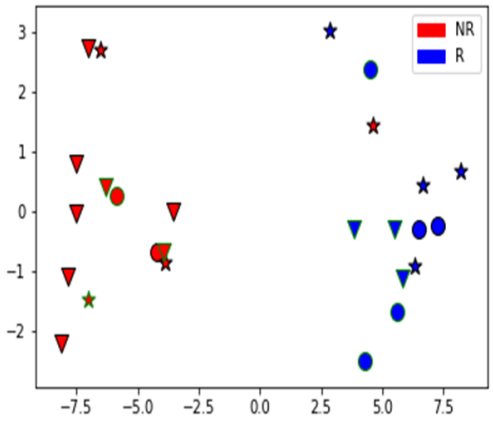

Fig.28 PCA performed at baseline. The 11-genes signature is able to classify as responder or non responder patients included in the training set and the validation set; one misclassified patient is shown in red in the responder group

Discussion

We defined an 11-genes signature (*MS4A1*, *CSRP2*, *MY05C*, *SEMA6A*, *CD200*, *CDR1*, *NEGR1*, *SCN3A*, *MME*, *DNTT*, *MIR1206*), able to classify as "responder" or "non-responder" to BiTE CD19-CD3 treatment a cohort of adult B-ALL patients at baseline, easy-to-use for routine diagnostics.

A gene map including the 11-genes signature was constructed by employing the STRING algorithm. Even if any specific interactions between the 11 genes was highlighted, an overview of the whole map suggested several possible interactions with genes involved in lymphocytes physiology and pathogenesis (*CD10*/*MME*, *DNTT*/*TdT*, *CD20*/*MS4A1*) and immune system response (CD200)[58;59] (Fig.29).

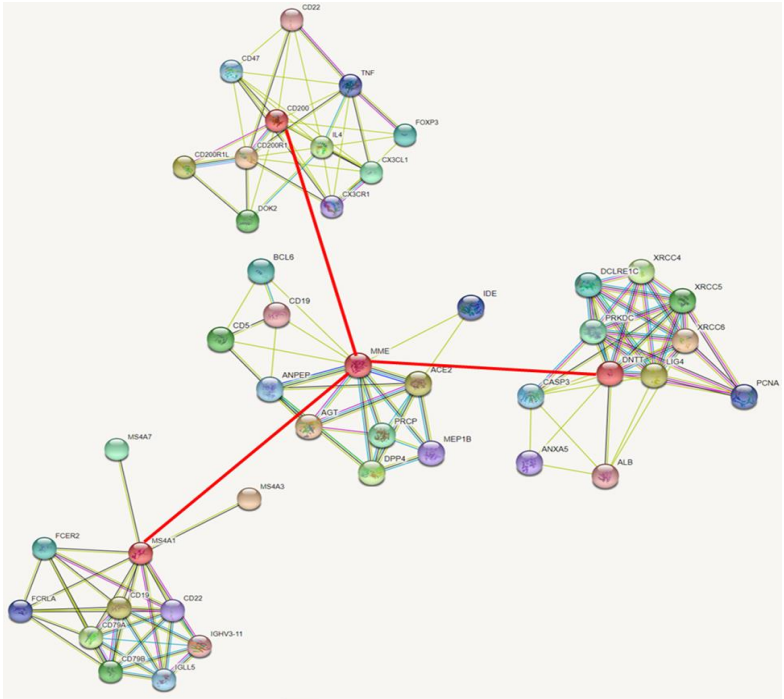

Fig.29 The STRING map

CD10 is a membrane-bound zinc-dependent endopeptidase which regulates the physiology of various peptides by lowering their extracellular concentration available for receptor binding; also, is implicated in the differentiation of immature cells in several tissues (e.g. breast) and in B cell maturation at early-B and pre-B lymphoblastic stages, suggesting a CD10 stem cell regulator role. In addition to its function through enzymatic activity, CD10 could directly mediate signaling events. Indeed, CD10 in GPI-microdomains coimmunoprecipitates with Lyn and p85. This protein complex blocks PI3K (phosphatidylinositol 3-kinase) interaction with focal adhesion kinase (FAK) by competitive binding, leading to decreased FAK phosphorylation and cell migration in the prostatic epithelial model; these findings suggest an indirect negative regulation of cell migration by CD10, independently of its catalytic function. Additional data shown that enzymatic inactive CD10 maintains its ability to recruit the tumor suppressor phosphatase and TENsin homolog (PTEN) that prevents the activation of the Akt signaling pathway implicated in normal and tumor cell growth. Furthermore, CD10 is expressed in B lymphoblastic leukemia/lymphoma and in certain mature B cell lymphomas (plasma cell myeloma, follicular lymphoma, diffuse large B cell lymphoma and Burkitt lymphoma) and very rarely in T cell lymphoma; in some cases CD10 expression has prognostic value. Indeed, CD10 alone can identify patients with higher 5-year survival rates in children with B lineage ALL and indicates a better prognosis than $CD10^{-}$.

However, CD10 interactions with Lyn might be investigated in the CD19-based immunotherapeutic setting. Indeed, CD19 phosphorylated tyrosine residues act as intracellular docking site for signaling molecules that contain Src homology 2 (SH2) domain, among which there is Lyn; and although is not expressed in T cells, Lyn can have a significant impact upon TC function through modulating signaling in cells that interact with them because has important functions in numerous hematopoietic cell types, from early stem/progenitors (signaling via the stem cell factor receptor/c-kit) through to multiple lineages of the lymphoid system (e.g. B cell) and the myeloid system (e.g. macrophages, erythroid cells, platelets, mast cells, eosinophils)[39;55;56].

Furthermore, our results suggest altered expression level of TdT, a member of the X family of DNA polymerases involved in DNA repair; in particular, the TdT is responsible for the random addition of a small number of nucleotides to unpaired DNA regions during the B cell receptor (BCR) and T cell receptor (TCR) gene rearrangements, that is V(D)J recombination. The V(D)J recombination is a complex process that is initiated by the binding of the recombination activating gene (RAG) protein complex onto specific and highly conserved recombination signal sequences (RSSs) followed by the cleavage of these signal sequences. The TdT intervenes in the second phase of V(D)J recombination, in which the DNA ends are recombined by the guidance of various non-homologous end-joining (NHEJ) DNA repair factors. Base pairing of the two strands then triggers

microhomology alignment of the V and D segments, and an exonuclease-mediated cleavage reaction removes the unpaired nucleotides. There are rising evidences that alterations in TdT activity and/or its expression level are involved in cancer initiation, progression, and response to chemotherapy. Indeed, TdT is overexpressed in B cell and T cell ALL and higher levels of TdT activity correlate with a poor prognosis due to sub-optimal response to chemotherapy. In addition, there are several data for inappropriate rearrangements of TCR in B cell ALL, and of both TCR and BCR genes in AML, although these patterns did not always coincide with TdT expression; also, a few data suggest that abnormal TdT polymerase activity may be responsible for extensive junctional N addition in ALL. It is not known whether early and/or abnormal expression of TdT isoforms in hematopoietic progenitors affects normal lymphopoiesis and receptor-driven selection processes in the thymus and the bone marrow, thus giving rise to disordered development. It is assumed that TdT expression is merely a reflection of the stage of development at which a given tumor type arises. However, a reasonable alternative hypothesis is that unchecked or abnormal activity of TdT may have more widespread effects, and it is not known what effects abnormal expression of TdT isoforms might have on the maintenance and exacerbation of lymphoid malignancies[60;61;62;63]. Thus, it would be interesting to explore a possible negative effect of TdT overexpression on TCR functions, given that effective T cells are involved in the therapeutic BiTE CD19-CD3 mechanism.

Also, CD20 is included in the 11-genes signature. CD20, a non-glycosylated phosphoprotein, is a B cell marker expressed during B cell differentiation from the pro-B cell until the plasma cell stage and on the surface of almost malignant B cells. Despite one of the most effective anti-cancer antibody therapies is CD20-based (Rituximab), the CD20 biology is still not completely defined. Several data suggest that CD20 is resident in lipid raft domains of the plasma membrane where it probably functions as a store-operated calcium channel following ligation of the B cell receptor for antigen. Rafts are specific membrane organizations of sphingolipids and cholesterol in the plasma membrane outer leaflet that serve as signaling platforms in lymphocytes and other cells and allow transmembrane propagation of most receptor-mediated extracellular signals, including immune system pathways. Also, CD20 is indirectly associated with src-family kinases Lyn, Fyn and Lck via lipid rafts, regulating apoptotic mechanisms[64;65].

Instead, CD200 expression is an independent prognostic factor associated with reduced overall survival for patients affected by multiple myeloma and acute myeloid leukemia. CD200 is the ligand for CD200 receptor (CD200R), which is an immune inhibitory receptor expressed on myeloid and lymphoid cells and is considered an important immunological checkpoint. Indeed, various studies in *vitro* and in *vivo* have shown that CD200-expressing tumor cells can suppress

responses T cell-mediated, regulating Treg/T effector cell balance; during tumor initiation and progression, $CD200^{+}$ tumor cells can interact with M1 and M2 macrophages through CD200–CD200R-complex, and downregulate IL-10 and IL-12 productions secreted primarily by M2 and M1 macrophages, respectively, suggesting too tumoral microenvironment relevance to define response to therapy[66;67].

The 11-genes signature involves also cysteine and glycine-rich protein 2 (CSRP2), a member of CSRP family encoding a group of short LIM domain proteins which are critical regulators of development and differentiation. CSRP2 transcript levels correlate with leukemia relapse and leukemia-free survival in adults with B cell acute lymphoblastic leukemia and normal cytogenetics: subjects with normal cytogenetics those with high CSRP2 transcript levels had a higher 5-year cumulative incidence of relapse (CIR) and worse relapse-free survival (RFS) compared with subjects with low CSRP2 transcript levels. Functional analyses suggest that CSRP2 promotes cell proliferation, cell-cycle progression, in *vitro* colony formation and cell migration ability, and CSRP2 overexpression is associated with resistance to chemotherapy[68].

Instead, CDR1, a member of SAD-family (synapses of the amphid defective) protein kinases, appears to be the key protein that directly regulates Wee1 activity; in *vitro*, CDR1 phosphorylates and inhibits Wee1 kinase activity, a cell cycle inhibitor. However mechanicistic details are still unknown, these evidences suggest a pivotal role of CDR1 in cell cycle regulation[69].

Furthermore, the membrane-bound protein Semaphorin 6A (SEMA6A), could contribute to regulate vascular development and adult angiogenesis; the mounting data suggest its potential role as prognostic biomarker in glioblastoma cases and to induce apoptosis via FADD binding in lung cancer cells[71;72;73]. While neuronal growth regulator 1 (NEGR1) may serve pivotal functions in the osteoblastic differentiation of mesenchymal stem cells and sodium channel SCN3A is included in a macrophage-related gene signature consisting of twelve genes, useful to predict resistance to targeted therapies and survival of glioma patients[74;75].

Furthermore, our data shown a significant association between BCR-ABL positivity and no response to BiTE CD19-CD3 therapy, suggesting the need for further studies to clarify the underlying mechanisms. An hypothesis might be that the P190 isoform might interact with Lyn, by phosphorylating the Y397 residue involved in kinase activity of Lyn protein; in addition, Lyn might bind and phosphorylate Y177 residue on BCR-ABL, thus leading to GAB-2 (an activator of the PI-3 kinase signaling) recruitment. Finally, Lyn has be shown to have an exclusive role in the regulation of signaling in B lymphocytes in association with CD19 and affects T cells activity because regulates mechanisms in cells that interact with these[19;55].

The one patient misclassification needs more detailed insight. Indeed, either patient-related characteristics or dataset-related causes might be underlying the obtained result and additional data need to be collected in order to clarify.

Conclusions

The Blinatumomab therapy represents one the most treatment innovations in ALL, but resistance mechanisms affect the response to therapy; thus, the identification of predictive biomarkers can improve the Blinatumomab management in ALL. The 11-gene signature (*MS4A1*, *CSRP2*, *MY05C*, *SEMA6A*, *CD200*, *CDR1*, *NEGR1*, *SCN3A*, *MME*, *DNTT*, *MIR1206*), identified through LMM, has been capable to classify patients as responder or non-responder to BiTE CD19-CD3 therapy at baseline.

These data, even if preliminary, suggest that mechanisms underlying unresponsiveness to Blinatumomab treatment are complex and might involve – as expected - the regulation of B and T cells functions. However, a dataset increase would be required, in order to improve both the statistical significance and the accuracy of results; indeed, the definition of responses to therapy might be more in detail classified, by identifying additional categories, such as “early responder” or “late responder”. In addition, the use of deeper, high-throughput molecular methods (e.g. single-cell sequencing) might be evaluated, to unravel the associations between heterogeneous genomic backgrounds, the phenotypic features and the response to BiTE CD19-CD3 treatment.

Finally, more insight within the Blinatumomab biological mechanisms and its possible interactions with TME, which might underlie the lack of response, need to be investigated, in order to optimize the Blinatumomab-based treatment.

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