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ANÁLISE DE CÉLULAS T NÃO CONVENCIONAIS EM PACIENTES COM LEUCEMIA LINFOBLÁSTICA AGUDA DE CÉLULAS B (LLA-B)

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NILBERTO DIAS DE ARAÚJO

ANÁLISE DE CÉLULAS T NÃO CONVENCIONAIS EM PACIENTES COM LEUCEMIA LINFOBLÁSTICA AGUDA DE CÉLULAS B (LLA-B)

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Ata do Exame de Defesa do aluno **Nilberto Dias de Araújo**, em 26 de maio de 2023.

Aos vinte e seis dias do mês de maio de dois mil e vinte e três, às nove horas, no auditório da Fundação HEMOAM, o aluno de doutorado deste Programa, **Nilberto Dias de Araújo**, apresentou à banca examinadora o seu trabalho de tese intitulado: "**Análise de células T não convencionais em pacientes com Leucemia Linfoblástica Aguda de Células B (LLA-B)**". A banca examinadora, composta pelos seguintes membros: **Professor Doutor Allyson Guimarães da Costa** (Presidente), **Professora Doutora Kátia Luz Torres Silva** (Membro), **Professora Doutora Jerusa Araújo Quintão Arantes Faria** (Membro), **Professor Doutor Pedro Rauel Cândido Domingos** e **Professor Doutor Daniel Barros de Castro**, decidiu, após a apresentação e a arguição, pela aprovação de sua tese.

Prof. Dr. Allyson Guimarães da Costa
Presidente

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Prof. Dr. Daniel Barros de Castro
Membro Externo

Dedico este trabalho a meu Pai ***João Eriberto Araújo Silva****, motivo maior da minha felicidade. Tenho certeza que não teria conseguido caminhar sozinho, sem seu amor incondicional quando em vida, sem seu apoio espiritual, que me dá estímulo para seguir em frente.*

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RESUMO

A leucemia linfoblástica aguda (LLA) é uma neoplasia linfo-proliferativa que compromete a produção e maturação celular de origem linfoide na medula óssea. Essa proliferação desregulada resulta em alterações nos mecanismos de ação do sistema imunológico e aumento do número de blastos, ocorrendo um desequilíbrio na produção de várias moléculas imunológicas, entre elas as quimiocinas, citocinas e fatores de crescimento, podendo influenciar a resposta mediada por células. Assim, este estudo foi direcionado para descrever a frequência das populações de células T não convencionais envolvidas na resposta antitumoral de pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) *de novo* durante a terapia de indução a remissão, proposta pelo protocolo terapêutico do Grupo Brasileiro de Tratamento da Leucemia na Infância (GBTLI), versão 2009. Foram realizados dois estudos de revisão bibliográfica sobre as populações de células T não convencionais na LLA-B, em paralelo a um estudo analítico, longitudinal e prospectivo, com coleta de amostras de medula óssea (MO) e sangue periférico (SP) de pacientes pediátricos atendidos na Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM). Uma vez diagnosticados com LLA-B, foi realizada a imunofenotipagem para a classificação em LLA-B comum e para as células T gama-delta (γδ), NKT e células MAIT, com a dosagem de moléculas imunológicas solúveis. Inicialmente, houve a publicação do artigo **"Translating Unconventional T Cells and Their Roles in Leukemia Antitumor Immunity"** abordando o papel das células T gama-delta (γδ), células T natural killer (NKT) e células T invariantes associadas à mucosa (MAIT) no contexto da leucemia e resposta antitumoral. Esta revisão teve como foco o conhecimento atual a respeito do papel destas populações de células T não convencionais na resposta imune antitumoral na leucemia, discutindo a necessidade de mais estudos sobre o potencial imunoterapêutico destas células. Posteriormente foi publicado um segundo artigo de revisão: **"γδT Cells for Leukemia Immunotherapy: New and Expanding Trends"**. Nesta revisão, destacamos diversas características das células Tγδ e suas interações na leucemia. Além disso, exploramos estratégias para maximizar suas funções antitumorais, ilustrando os achados, demonstrando a mobilização das células Tγδ contra o tumor. Delineamos nossas perspectivas sobre sua aplicabilidade terapêutica e indicamos debates pendentes para futuras pesquisas básicas e clínicas sobre leucemia, no intuito de contribuir para o avanço dos estudos sobre células Tγδ na imunoterapia do câncer. Por fim, os dados originais da tese apresentado em formato de artigo para submissão, os resultados e discussões a respeito do estudo intitulado: "**Análise de células T não convencionais e mediadores imunológicos solúveis em pacientes com leucemia linfoblástica aguda de células B (LLA-B)".** Através destes estudos, buscamos explorar a imunobiologia das células T não convencionais ainda pouco exploradas na literatura, destacando seu papel na atividade antitumoral da LLA-B.

Palavras-chaves: Células T não convencionais; leucemia linfoblástica aguda; microambiente tumoral; moléculas imunológicas.

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LISTA DE ABREVIATURAS E SIGLAS

CF	Citometria de Fluxo
CD	*Cluster of differentiation*
CLs	Células leucêmicas
D0	Ao diagnóstico, início da indução a remissão do protocolo quimioterápico
D35	Dia 35 da terapia de indução a remissão do protocolo quimioterápico
D84	Dia 84 da terapia de indução a remissão do protocolo quimioterápico
DC	*Dendritic Cells*
DNAM-1	*DNAX accessory molecule-1*
DRM	Doença Residual Mínima
EDTA	Ácido etilenodiamino tetra-acético
GBTLI	Grupo Brasileiro de Tratamento de Leucemia Infantil
HEMOAM	Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas
IFN	Interferon
LLA	Leucemia linfoblástica aguda
LLA-B	Leucemia linfoblástica aguda de células B
LMA	Leucemia mieloide aguda
MAIT	*Mucosal-associated invariant* T
MHC	Complexo principal de histocompatibilidade
MIC-A/B	*MHC class I polypeptide-related sequence A/B*
MO	Medula óssea
MT	Microambiente Tumoral
NCRs	*Natural cytotoxicity receptors*
NKG2D	*Natural killer group 2 member D*
NKT	*Natural killer* T
SP	Sangue periférico
TCLE	Termo de Consentimento Livre e Esclarecido
TGF	*Transforming growth factor*
TLRs	*Toll-like receptors*
TNF	*Tumor necrosis factor*
TRAIL	*Tumor necrosis factor-related apoptosis-inducing ligand*
ULBPs	*UL16 binding protein*

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1. INTRODUÇÃO

A leucemia linfoblástica aguda (LLA) é uma alteração maligna e proliferativa de células progenitoras de origem linfoide na medula óssea, com migração de células leucêmicas para o sangue periférico e sítios extramedulares. A incidência de LLA segue uma distribuição bimodal, com o primeiro pico ocorrendo na infância e um segundo ocorrendo por volta dos 50 anos (KRUGER et al., 2019; RICHARD-CARPENTIER; KANTARJIAN; JABBOUR, 2019).

Mundialmente, a LLA é o tipo mais comum de câncer infantil, constituindo cerca de um terço de todas as neoplasias malignas de crianças, sendo quatro vezes mais frequente que a leucemia mieloide aguda (LMA) (TERWILLIGER; ABDUL-HAY, 2017). No Brasil, a Região Norte destaca-se por apresentar as maiores taxas de incidência do país, com 39% dos casos. A faixa etária mais acometida são crianças entre 2 a 6 anos, com o sexo masculino sendo o mais afetado, principalmente na primeira infância (SILVA et al., 2019).

A LLA é classificada fenotipicamente em Linhagem B (LLA Pré-B; LLA Pró-B; LLA B Comum e LLA B Madura) e Linhagem T (LLA Pró-T; LLA Pré-T; LLA T Cortical e LLA T Medular). A LLA-B comum é subtipo mais frequente, caracterizado pela proliferação anormal de clones de linfócitos B imaturos na medula óssea (MO) (FARIAS; CASTRO, 2004). O diagnóstico laboratorial é feito, embora não exclusivamente, pela presença mínima de 20% de blastos entre as células nucleadas da MO, incluindo análise de dados citomorfológicos, imunofenotípicos, citogenéticos e moleculares (SWERDLOW et al., 2016).

Os mecanismos imunológicos em pacientes com LLA ainda estão sendo investigados, contudo sabe-se que o processo neoplásico pode provocar várias desordens imunes em consequência da intensa produção de células leucêmicas (CLs), além do processo de evasão da resposta imune. Este descontrole no nicho medular induz um desequilíbrio na produção de várias moléculas, entre elas as quimiocinas e citocinas (BEHRMANN; WELLBROCK; FIEDLER, 2018; CARDOSO, 2020; KARANTANOU; GODAVARTHY; KRAUSE, 2018).

Recentemente, a resposta imune mediada por células em neoplasias malignas se tornou o foco da imunoterapia contra o câncer. Porém, na leucemia, a maioria dos estudos

sobre o potencial citotóxico das células T tem se concentrado apenas nas células T que reconhecem antígenos peptídicos (Ag) apresentados por moléculas polimórficas do complexo principal de histocompatibilidade (MHC). Isso ignora o grande potencial das populações de células T não convencionais, que incluem células T gama-delta (γδ), células natural killers T (NKT) e células T invariantes associadas à mucosa (MAIT). Coletivamente, essas populações de células T podem reconhecer antígenos lipídicos, especialmente peptídeos modificados e metabólitos de pequenas moléculas, além de apresentarem várias outras vantagens, que podem fornecer aplicações mais eficazes na imunoterapia do câncer (EHSANPOUR, 2018).

Portanto, realizar a análise de células T não convencionais no microambiente medular e sangue periférico pode ser uma estratégia eficaz para a compreensão da resposta imune frente as neoplasias hematológicas, possibilitando entender de forma mais detalhada as interações e os aspectos fisiopatológicos entre o sistema imunológico e os blastos leucêmicos na LLA-B.

2. REFERENCIAL TEÓRICO

2.1 Leucemias

A leucemia linfoblástica aguda (LLA) é uma desordem maligna resultante da proliferação clonal de precursores linfoides com maturação interrompida. A doença pode originar-se de células linfoides de diferentes linhagens, dando origem a leucemias de células B ou T ou às vezes com leucemia de linhagem mista (RYTTING et al., 2017).

A LLA foi uma das primeiras neoplasias malignas a responder à quimioterapia e mais tarde foi uma das primeiras que puderam ser curadas na maioria das crianças. Desde então, progressos foram feitos não apenas em novos protocolos de tratamento, mas também na modificação das formas de tratamento com base nos critérios de resposta do paciente (GREAVES, 2016; RICHARD-CARPENTIER; KANTARJIAN, 2019).

Na maioria das vezes, o início da doença é tão abrupto que os sinais e sintomas surgem já nas primeiras semanas da instalação da doença. Até o momento, sabe-se que a leucemia não é hereditária, muito menos contagiosa, no entanto foi considerado um resultado de agravo genético adquirido no DNA de uma única linhagem de células na medula óssea (GREAVES, 2018).

2.2 Epidemiologia e fatores de risco

A nova estimativa do Instituto Nacional de Câncer (INCA) do Ministério da Saúde aponta para 704 mil casos novos de câncer no Brasil para cada ano do triênio 2023 a 2025, com destaque para as regiões Sul e Sudeste, que concentram cerca de 70% da incidência (INCA, 2023). O Instituto Nacional de Câncer (INCA) estima que para cada ano do biênio 2018/2019, sejam diagnosticados 5.940 casos novos de leucemia em homens e 4.860 em mulheres. Esses valores correspondem a um risco estimado de 5,75 casos novos a cada 100 mil homens e 4,56 casos novos para cada 100 mil mulheres (MINISTÉRIO DA SAÚDE, 2023).

A leucemia corresponde ao 15º tipo de câncer de maior incidência no mundo, apresentando 2,4% de todos os casos novos de câncer. No Brasil, nos últimos anos, houve um aumento expressivo na incidência de leucemia, representando atualmente o 9° câncer mais comum. A proporção de casos de leucemia entre as regiões do Brasil é variável e tem aumentado desde os últimos relatórios do INCA.

A região Norte foi identificada como a região com o segundo maior número casos de leucemia, tendo o Amazonas, particularmente sua capital Manaus, classificada em 2010 como a capital com a segunda maior taxa de incidência ajustada à idade (abaixo de 14 anos) de leucemia em crianças e adolescentes. Em 2011, teve a maior taxa de incidência no país, com 76,8 casos de leucemia por milhão de habitantes, aumentando em 2016 para 87,1 (SILVA-JUNIOR, 2019).

A leucemia linfoblástica aguda é a neoplasia mais comum em crianças e adolescentes correspondendo por 70% a 80% em menores de 15 anos e apenas 20% dos casos em adultos. A doença acomete principalmente crianças entre 2 a 6 anos, com discreto predomínio do sexo masculino comparado ao feminino, numa proporção de 2:1 (SILVA et al., 2019). Cerca de 85% dos casos tem fenótipo de células B de células precursoras, 1 a 2% são do subtipo células-B maduras e o restante é do tipo LLA de células T, que apresenta maior resistência ao tratamento padrão (TERWILLIGER; ABDUL-HAY, 2017).

Estudos epidemiológicos de leucemias agudas em crianças examinaram uma série de possíveis fatores de risco (por exemplo, ambientais, genéticos ou infecciosos) em um esforço para determinar a etiologia da doença. Apenas um fator de risco ambiental (radiação ionizante) foi significativamente ligado à LLA ou à LMA. A maioria dos fatores de risco ambientais, tais como campos eletromagnéticos (EMF) e tabagismo, tem associação fraca ou inconsistentemente com qualquer forma de leucemia infantil (GREAVES, 2018; SILVA et al., 2019).

O Amazonas possui a maior extensão territorial de todas as unidades federativas e sua capital, Manaus, foi identificada em 2010 como a capital com a segunda maior taxa de incidência ajustada por idade (menores de 14 anos) de leucemia. Em 2011, apresentou a maior taxa de incidência do país, com 76,8 casos de leucemia por milhão de habitantes, que aumentou ainda mais em 2016 para 87,1. De acordo com a Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), o número de pacientes com diagnóstico de Leucemias aumentou em mais de 10% nos primeiros três meses de 2018. Conforme dados do Departamento de Informática do Sistema Único de Saúde (Datasus), de janeiro a março de 2018, 128 pacientes foram identificados com a doença, enquanto no primeiro trimestre de 2019 houve 152 registros de leucemias agudas (SILVA-JUNIOR, 2019).

2.3 Diagnóstico

Cerca de 90% dos pacientes com LLA apresentam alterações laboratoriais e hematológicas no diagnóstico e estas representam o grau de envolvimento da MO pelos blastos. Anemia normocítica e normocrômica, com contagem de reticulócitos baixa ocorre em aproximadamente 80% dos pacientes. A leucometria está aumentada na grande maioria dos casos, porém pode haver leucopenia, com diminuição significativa de neutrófilos. A contagem de plaquetas inferior a 100.000/mm³ é comum. Em cerca de 5% dos casos a contagem de leucócitos pode ser menor que 2.000/mm³, sem linfoblastos detectáveis no sangue periférico (FARIAS; CASTRO, 2004).

Para a confirmação do diagnóstico, é realizado o mielograma para análise citomorfológica do esfregaço de medula óssea, onde costuma revelar hipercelularidade associada com linfoblastos. Além do mielograma, o refinamento no diagnóstico e classificação das leucemias é obtido através da imunofenotipagem por citometria de fluxo, técnicas de citogenética e biologia molecular (CAMPO et al., 2011; FARIAS; CASTRO, 2004; SWERDLOW et al., 2016).

2.4 Protocolo de tratamento

A grande chance de cura nos protocolos nacionais aconteceu na década de 80 quando iniciaram o primeiro protocolo multicêntrico de tratamento de LLA infantil, formando-se assim o Grupo Brasileiro de Tratamento da Leucemia Infantil (GBTLI-80). Desde então, cinco estudos foram realizados e concluídos em 1982, 1985, 1993, 1999 e 2009, respectivamente (CAZÉ; BUENO; SANTOS, 2010).

Dados brasileiros do último estudo do GBTLI (versão 2009) revelam que, em sete anos de seguimento, a taxa de sobrevida livre de eventos de crianças tratadas pelo protocolo foi de aproximadamente 76,3% para o grupo de baixo risco e 59,8% para o grupo de alto risco (GBTLI-2009). Os pacientes foram estratificados por grupo de risco e analisados durante a terapia de indução em quatro etapas: ao diagnóstico (D0), oitavo dia (D8), décimo quinto dia (D15), trigésimo quinto dia (D35) e octogésimo quarto dia (D84) após o diagnóstico.

A terapia de indução da remissão, segundo o protocolo GBTLI-2009 tem duração de 84 dias e utiliza como critérios de estratificação de risco a idade, contagem

leucocitária, linhagem celular, presença de linfoblastos no SNC e análise citogenética de anormalidades cromossômicas. A resposta precoce à indução é avaliada no D8, com contagem morfológica de blastos em sangue periférico, no D15 e D35, com avaliação da contagem morfológica de blastos e detecção da presença de doença residual mínima (DRM) por citometria de fluxo e testes moleculares (GBTLI-2009).

2.5 Fisiopatologia

A LLA é uma doença multifatorial desencadeada pela interação de fatores genéticos e ambientais, todavia, estudos apontam para uma relação entre o desenvolvimento da doença e a desregulação do sistema imunológico. As células leucêmicas podem modelar funcionalmente seu ambiente através da secreção de diversos fatores que atuam no recrutamento de células imunes como neutrófilos, macrófagos, células dendríticas, linfócitos B e T. Além disso, podem proporcionar um ambiente favorável ao desenvolvimento neoplásico (HUAN et al., 2013).

Os mecanismos imunológicos observados em pacientes com leucemia não são muito conhecidos. No entanto, com os avanços crescentes no campo da imunoterapia, tem havido um grande progresso nas pesquisas relacionadas ao microambiente tumoral na leucemia. Estudos têm demonstrado que as CLs secretam fatores que desregulam nichos de MO saudáveis, reprogramando-os e transformando-os em "nichos leucêmicos", além de induzir uma interrupção na produção balanceada de citocinas e favorecer a persistência leucêmica e o potencial metastático (CHIARINI et al., 2016).

Apesar do microambiente pró-tumoral criado pelas CLs, estudos relatam que uma resposta imune específica pode ser desencadeada e, portanto, contribuir para a defesa contra o tumor. As populações de células T consideradas não convencionais também participam desse processo, exercendo uma atividade imune antitumoral. Coletivamente, essas populações de células T diferem de outras células T principalmente na maneira como reconhecem e respondem a moléculas estranhas (GODFREY et al., 2015).

Ao contrário das células T reativas ao MHC, as células T não convencionais geralmente mostram padrões simplificados da expressão do receptor do antígeno da célula T (TCR) e geralmente têm como alvo moléculas apresentadoras de Ag monomórficas e outros ligantes, onde após sua ativação promovem respostas efetoras

rápidas e fortes. Essas populações de células T incluem células Tγδ, células NKT e células MAIT (GODFREY et al., 2018).

2.6 Células T não convencionais

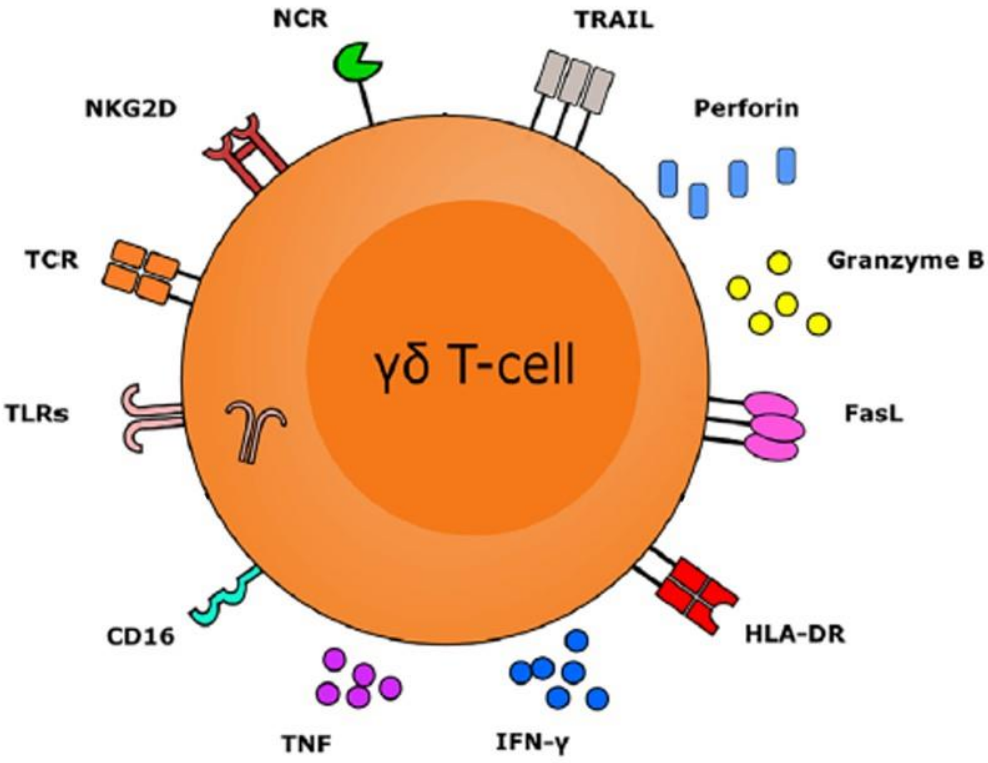
2.6.1 Células T γδ

Embora o papel imunológico das células T convencionais que expressam o receptor de células T (TCR) αβ esteja bem caracterizado, pouco ainda se sabe sobre a função da linhagem de células portadoras de TCRγδ. Esta pequena população de linfócitos possui uma biologia que atua na interface entre a imunidade inata e adaptativa, participando de várias respostas imunes durante a progressão do tumor (ZHAO; NIU; CUI, 2018).

Estas células se originam durante a linfopoiese (a partir de timócitos duplo-negativos) e se destacam pelo reconhecimento e destruição de vários tipos de tumores de forma independente do complexo principal de histocompatibilidade (MHC) (PARKER; CIOFANI, 2020; VANTOUROUT; HAYDAY, 2013). Além disso, essas células constituem até 10% das células $CD3^+$ circulantes e são classificadas de acordo com os rearranjos genéticos da cadeia δ do TCR em três subconjuntos principais: Vδ1, Vδ2 e Vδ3 (em humanos). Esses linfócitos estão presentes na circulação sanguínea, nos tecidos e nas mucosas, locais estratégicos para exercer seu alto poder citotóxico contra infecções e tumores (ZHAO; NIU; CUI, 2018).

As células Tγδ são evolutivamente conservadas e possuem diversas funções pleiotrópicas. Enquanto estas células são naturalmente especializadas na secreção de mediadores pró-inflamatórios **(Figura 1)** (WESCH; GLATZEL; KABELITZ, 2001), estes linfócitos também conseguem adotar fenótipos de resposta do tipo Th2, Th9 e Th17 (CACCAMO et al., 2011; NESS-SCHWICKERATH; JIN; MORITA, 2010; PETERS et al., 2016). No microambiente tumoral (MT), estes linfócitos expressam um diverso repertório de receptores de reconhecimento, que têm recebido um valor prognóstico favorável em diversas malignidades, incluindo leucemias (GENTLES et al., 2015).

Baseado em diversos relatórios, foi confirmado que células Tγδ são capazes de desencadear uma resposta imune através de mecanismos inatos e adaptativos, por exemplo, através da sinapse citolítica com a célula-alvo, além do recrutamento e estimulação de outras células imunes necessárias para o estabelecimento de uma resposta



antitumoral (PRESTI et al., 2018; ZHAO; NIU; CUI, 2018). Esses linfócitos conseguem induzir a regressão neoplásica através da interação célula-célula ou pela secreção de diversas moléculas solúveis (TNF-α e IFN-γ) que inibem a expansão tumoral. As moléculas efetoras induzem um aumento da atividade antitumoral de outras células citotóxicas ou regulam positivamente a expressão de MHC-I pela célula cancerosa (GAO et al., 2003).

Figura 1. Visão geral da célula Tγδ e receptores. A célula Tγδ possui capacidade multifatorial, com interações e vias cruciais, com reconhecimento de moléculas virais, presença de PAMPs, receptores do tipo NK e TLR. As células Tγδ expressam alguns receptores essenciais para o reconhecimento e destruição de tumores, o que lhe dá certa vantagem em relação a outras populações de células T convencionais, seja pelo reconhecimento independente da expressão de MHC ou pela alta expressão dos receptores citados na imagem. O repertório de reconhecimento antileucêmico inclui várias moléculas, como: NKG2D, NCRs, FasL, CD16, DNAM-1 e o próprio TCR γδ.

Fonte: Adaptado de *Canon et al*, 2021.

Além disso, as células Tγδ estimulam a hipermutação somática e a troca de isótipos em células B (CACCAMO et al., 2006; HORNER et al., 1995; PETRASCA et al., 2018), possivelmente induzindo a imunidade mediada por anticorpos. Suas funções efetoras também incluem a ativação de macrófagos e o recrutamento e ativação de células T $CD8^+$ e células NK (BRANDES; WILLIMANN; MOSER, 2005; MANIAR et al., 2010; MAO et al., 2014; MUTO et al., 2015).

O papel imunológico destes linfócitos também inclui o estímulo da maturação de células dendríticas (DCs) e, em troca, observa-se a potencialização das atividades citotóxicas dessas células (MARTINO et al., 2007; VAN ACKER et al., 2015). Notavelmente, as células neoplásicas tendem a expressar diversas moléculas induzidas pelo estresse ou antígenos metabólicos, que são reconhecidos pelo TCR γδ e receptores acessórios, mediando assim uma resposta potente contra o tumor (DE WEERDT et al., 2015; GOMES et al., 2010; GUNDERMANN et al., 2014; SIMÕES; DI LORENZO; SILVA-SANTOS, 2018).

Sem restrições à expressão de MHC, as células Tγδ reconhecem vários antígenos que são expressos em células leucêmicas (CLs) e geralmente incluem moléculas metabólicas e induzidas por estresse (CORREIA et al., 2011; GOMES et al., 2010; SIEGERS, 2012; SIEGERS et al., 2011a, 2011b). Ligantes, como MIC-A / MIC-B e ULBPs, podem ser identificados por meio do receptor NKG2D, que é expresso principalmente em células T γδ (GUNDERMANN et al., 2014; SILVA-SANTOS; STRID, 2018). Além disso, alguns metabólitos da via do mevalonato, conhecidos como fosfoantígenos (pAgs), podem ser reconhecidos diretamente pelos TCRs e são altamente regulados nas CLs (GERTNER-DARDENNE et al., 2012; PANDYRA et al., 2014).

Outras moléculas auxiliam no reconhecimento do tumor e possivelmente suportam a resposta antileucêmica de células Tγδ, como TLRs, DNAM-1, FasL (ligante de Fas), FcγRIII , TRAIL, NCRs (NKp30, NKp44 e NKp46) e o receptor 2B4 (CORREIA et al., 2011; FLAMENT et al., 1996; GERTNER-DARDENNE et al., 2012; KALYAN; WESCH; KABELITZ, 2011; MAMI-CHOUAIB et al., 1991; ZHAO; NIU; CUI, 2018).

As células $V\delta1^+$, que expressam as cadeias de TCR Vγ1 a Vγ11, respondem preferencialmente nos tecidos da pele, epitélio intestinal, pulmão, baço e fígado, onde desempenham papéis cruciais na manutenção do tecido epitelial (VANTOUROUT; HAYDAY, 2013; WU et al., 2017). Sabe-se que esses linfócitos patrulham os diversos

tecidos em busca de células em processo de estresse, derivadas de infecções e tumorigênese, mantendo assim a homeostase tecidual (DAVEY et al., 2018). Para isso, a secreção de citocinas Th1 e Th17 são essenciais para a vigilância imunológica (RAMSTEAD; JUTILA, 2012; SILVA-SANTOS; SERRE; NORELL, 2015; WU et al., 2014; ZHAO; NIU; CUI, 2018).

Essas células, embora incomuns no sangue periférico (~10% de células no sangue), apresentam alta diversidade de reconhecimento de tumor e demonstraram grande potencial contra CLs (CORREIA et al., 2011; KOZBOR et al., 1989; LANÇA et al., 2010). Correia et al. demonstrou que as células Vδ1 que expressaram NCRs conseguiram destruir células tumorais linfoides e mieloides por meio de NKp30 e NKp44, que pareciam reconhecer antígenos que são distintos de seus ligantes clássicos, como a molécula B7-H6, que se liga a NKp30 (CORREIA et al., 2011). No mesmo estudo, a expressão estável desses NCRs foi associada a níveis elevados de granzima B e parecia atuar de forma sinérgica com maior atividade citotóxica contra CLs. Levando em consideração que em algumas leucemias se observa alta expressão de membros da família ULBP, como ligantes NKG2D (POGGI et al., 2004), Lança et al. demonstraram que a expressão de ULBP1 em CLs é importante para o reconhecimento por células Vδ1 (LANÇA et al., 2010). ULBP3 foi confirmado por Poggi et al., que apresentaram resultados semelhantes (POGGI et al., 2004). Portanto, os dados sugerem provável participação imunológica dessas células e indicam uma contribuição significativa para a resposta imune antileucêmica.

Essas células também podem reconhecer antígenos lipídicos ou metabólicos que são apresentados por meio de moléculas semelhantes ao MHC de classe I, como CD1 e MR1 (DAVEY et al., 2018; WILLCOX; MOHAMMED; WILLCOX, 2020). Estas e outras características das células Vδ1 as tornam candidatas em potencial para novas abordagens imunoterapêuticas em vários tumores humanos, sendo exploradas recentemente em diversos ensaios experimentais, incluindo as leucemias (ALMEIDA et al., 2016; DI LORENZO et al., 2019; GARBER, 2020). Embora seus papéis ainda sejam pouco conhecidos, sabemos que as células Vδ1 demonstram funções importantes na atividade antitumoral e merecem destaque por apresentarem características diferentes das células Vγ9Vδ2, seja pela alta expressão de NCRs, seja pela não suscetibilidade a morte celular induzida por ativação (MCIA) (GAN; LUI; MALKOVSKY, 2001; KABELITZ, 1999; MEEH et al., 2006). Os papéis desse subtipo de células T γδ no ambiente da medula

óssea e sangue periférico in vivo, no contexto da leucemia, ainda precisam ser investigados a respeito dos ligantes e receptores de reconhecimento que atuam durante a resposta imune.

O subtipo Vδ2^{+}, que emparelha exclusivamente com a cadeia de TCR Vγ9, atua principalmente no sangue, reconhecendo fosfoantígenos (pAg) derivados de bactérias e células neoplásicas (WILLCOX; DAVEY; WILLCOX, 2018). Uma vez ativados, esses linfócitos secretam moléculas efetoras como IFN-γ, TNF, perforinas e granzimas, exercendo importantes atividades citotóxicas no sangue periférico contra patógenos e tumores (VYBOROVA et al., 2020; ZHAO; NIU; CUI, 2018). Essas células constituem até 95% das células T γδ do sangue (FONSECA et al., 2020; HINZ et al., 1997; KABELITZ et al., 1997; KOZBOR et al., 1989) e geralmente respondem a uma ampla variedade de pAgs, como IPP (isopentenil pirofosfato) e HMB-PP (4-hidroxi-3-metil-but-2-enilpirofosfato), que são intermediários da via do mevalonato em eucariotos e procariotos (GRUENBACHER; THURNHER, 2017; HERRMANN; FICHTNER; KARUNAKARAN, 2020; MOULIN et al., 2017).

O reconhecimento desses pAgs ocorre no contexto da família de moléculas da butirofilina (BTN), como BTN3A1 e BTN2A1 (BLAZQUEZ et al., 2018; KARUNAKARAN et al., 2020; RIGAU et al., 2020; WILLCOX et al., 2019), que podem ser detectados em CLs e medeiam uma resposta imune potente que pode ser usada terapeuticamente (BENYAMINE et al., 2016). Essas moléculas podem ser reconhecidas diretamente através do TCR γδ e são capazes de desencadear uma resposta do tipo Th1 contra as células-alvo (DEBARROS et al., 2011; LAFONT et al., 2001).

O mecanismo de reconhecimento de pAgs ainda não está claro, embora vários estudos tenham expandido recentemente as informações sobre como as células T γδ identificam essas moléculas. Pesquisas recentes apontam que o reconhecimento de pAgs é mediado por moléculas do tipo BTN, que são expressas em células neoplásicas (BENYAMINE et al., 2016; COMPTE et al., 2004) e são capazes de modular as respostas de células T αβ convencionais (CUBILLOS-RUIZ et al., 2010; MESSAL et al., 2011; SARTER et al., 2016; YAMAZAKI et al., 2010), e mais notavelmente, de células T γδ (BLAZQUEZ et al., 2018; DI MARCO BARROS et al., 2016; KARUNAKARAN et al., 2020; PAYNE et al., 2020; RIGAU et al., 2020; WILLCOX et al., 2019). A detecção dependente de pAgs por células Vγ9Vδ2 envolve toda a estrutura do TCR, que interage

com as moléculas de BTN através dos domínios Vγ9 e Vδ2 TCR. Dentre as várias moléculas que compõem a família BTN, as proteínas BTN3A1 e BTN2A1 atuam sinergicamente para a apresentação de pAgs às células T γδ, ligando-se diretamente ao TCR Vγ9Vδ2 (KARUNAKARAN et al., 2020; RIGAU et al., 2020; SANDSTROM et al., 2014; VAVASSORI et al., 2013).

Anteriormente, pensava-se que a expressão unitária de BTN3A1 atuava na ativação desses linfócitos de forma isolada (HARLY et al., 2012), mas agora está claro que a proteína BTN2A1 atua como um fator crítico na ativação das células Vδ2 (KARUNAKARAN et al., 2020). Para que isso ocorra, é necessário que os pAgs se liguem ao domínio intracelular (B30.2) dessas proteínas (GU et al., 2017b; YANG et al., 2019b). Após a ligação de pAgs, os domínios intra e extracelulares de BTN3A1 e BTN2A1 sofrem uma mudança conformacional (GU et al., 2017a; SALIM et al., 2017; YANG et al., 2019a) que permite o contato da cadeia TCR Vγ9 com a molécula BTN2A1, criando sinais de ativação para células T γδ (RIGAU et al., 2020). Além disso, o envolvimento de outras moléculas durante o mecanismo de detecção de pAgs não pode ser ignorado, pois estudos recentes sugerem a colaboração molecular de CDR3, periplakin e GTPase RhoB neste processo (KARUNAKARAN et al., 2020; RHODES et al., 2015; SEBESTYEN et al., 2016).

Além do reconhecimento direto e dependente de TCR, outras moléculas acessórias possivelmente suportam a atividade antileucêmica de células Vδ2 contra CLs, como o receptor DNAM-1 que reconhece o receptor de poliovírus (PVR) e ligantes de Nectina-2, ambos expressos em CLs (GERTNER-DARDENNE et al., 2012) e ULBP4 que podem ser reconhecidos por meio de NKG2D (KONG et al., 2009). As células T γδ também expressam o receptor 2B4 (que reconhece o ligante CD48), uma molécula acessória que fortalece as interações efetoras alvo e possivelmente está relacionada ao aumento da atividade citotóxica contra as células cancerosas (FLAMENT et al., 1996). Uma desvantagem dessas células $V\delta2^+$ é sua forte propensão ao MCIA após exposição prolongada a antígenos e a polarização desses linfócitos em direção a um fenótipo promotor de tumor, que limita a persistência e atuação da resposta imune (GAN; LUI; MALKOVSKY, 2001; MEEH et al., 2006).

Poucos dados estão disponíveis sobre os receptores e ligantes envolvidos nos eventos de imunidade inata e adaptativa mediada por células $V\delta3^+$, que expressam as

cadeias de TCR Vγ2, Vγ3 ou Vγ8, e são um subconjunto específico que responde principalmente no fígado (KENNA et al., 2004; MANGAN et al., 2013). A frequência dessa população de células é baixa no sangue periférico (~ 0,2% do total de células T circulantes), embora seja alta no fígado e na região intestinal (KENNA et al., 2004; MANGAN et al., 2013). Estudos relataram que as células Vδ3 estão relacionadas à imunidade antiviral, respondendo de forma eficiente contra citomegalovírus (KNIGHT et al., 2010), vírus Epstein-Barr (FARNAULT et al., 2013), hepatite (RAJORIYA et al., 2014) e infecção por HIV (KABELITZ et al., 1997).

Até o momento, seus papéis na imunidade antitumoral não são claros, embora alguns estudos tenham identificado uma expansão desses linfócitos no sangue periférico de pacientes com leucemia (BARTKOWIAK et al., 2002). Além disso, as células Vδ3 secretam IFN-γ, expressam NKG2D, FcγRIII e CD161, além de poder responder de forma restrita a CD1d, se assemelhando funcionalmente às células NKT, que reconhecem e destroem células alvo $CD1d^{+}$ (MANGAN et al., 2013; PETRASCA et al., 2018). Posteriormente, foi identificado que essas células também respondem a anexina-2 (ANX-2) (MARLIN et al., 2017), mas seu papel na leucemia ainda é inexplorado, embora tenha sido identificado que essas células se expandem durante a progressão tumoral (BARTKOWIAK et al., 2002).

As células Tγδ têm um enorme potencial para regular a imunidade local e remodelar o nicho tumoral (LO PRESTI et al., 2018; REI; PENNINGTON; SILVA-SANTOS, 2015; WU et al., 2017). As evidências discutidas até agora sugerem que essas células realizam várias atividades antitumorais por meio da identificação direta de CLs por meio do TCR γδ ou de receptores acessórios e pela secreção de moléculas efetoras solúveis contra o tumor. Apesar disso, o conhecimento emergente sobre a interação molecular e celular entre esses linfócitos e as células neoplásicas sugere que, como as células T αβ convencionais, as células Tγδ podem não estar isentas da imunossupressão estabelecida pelo microambiente tumoral (BARYAWNO et al., 2019; HÖPKEN; REHM, 2019; LAMBLE; LIND, 2018; PRESTI et al., 2017). No entanto, a falta de conhecimento sobre essas células no microambiente leucêmico dificulta a elaboração de discussões mais abrangentes sobre a possível comunicação/interação entre essa população de linfócitos e as CLs no compartimento da medula óssea e em sítios extramedulares.

Além disso, outros subconjuntos de células Tγδ que expressam outras cadeias de TCR variáveis (domínios de TCR Vδ5, Vδ6 e Vδ8) foram identificados em outras doenças hematológicas malignas, onde, por exemplo, essas células pareciam se expandir e responder às células cancerosas no sangue de pacientes com linfoma (WANG et al., 2014). No entanto, ainda não está claro se essas células respondem contra CLs e não temos informações disponíveis sobre os possíveis papéis desempenhados por esses subtipos de células Tγδ na imunidade antitumoral da leucemia. Tendo em vista sua enorme aplicabilidade terapêutica, mais pesquisas são necessárias sobre a interação das células T γδ e seus subconjuntos no microambiente leucêmico, e como isso pode impactar no prognóstico dos pacientes.

2.6.2 Células NKT

As células T natural killer (NKT) se apresentam como uma população de células T inatas caracterizadas pela expressão de um TCR semelhantes às das células T convencionais, (ABEL et al., 2018; BENLAGHA et al., 2002). Outra característica marcante dessas células é sua restrição à molécula CD1d, que é uma molécula semelhante ao MHC classe I que apresenta antígenos lipídicos e glicolipídicos (GODFREY et al., 2004; LIAO et al, 2013; WU et al., 2009).

As células NKT constituem aproximadamente 0,001-1% dos linfócitos circulantes (em humanos) e também estão presentes no timo, fígado, intestino e baço (BERZINS et al., 2005; WALDOWSKA et al, 2017). Essas células são divididas em dois subconjuntos principais, as células natural killer T invariáveis (iNKT) e células natural killer T tipo II (NKT-II) (KUMAR et al, 2014). Ambos expressam os seguintes fatores de transcrição: T-bet (ALTMAN et al., 2015; TOWNSEND et al., 2004), PLZF (KOVALOVSKY et al., 2008), RORγt, GATA-3 (ALTMAN et al., 2015) e NF-kB (VAN KAER *et al*, 2011), que juntos possuem alta plasticidade celular e permitem a polarização para fenótipos de perfil Th1, Th2 e Th17 (LEE et al., 2002; MOREIRA-TEIXEIRA et al., 2020; VAN KAER et al, 2011). Além disso, os iNKTs expressam o fator LEF-1 (CHEN *et al*, 2010; WANG *et al*, 2018), que está correlacionado com a regulação da expressão do gene que codifica a molécula CD1d em células apresentadoras de antígeno (APCs) (HASEEB et al., 2019).

Em relação à atividade antitumoral, as células NKT podem atuar diretamente por meio da interação célula-célula, através do receptor Fas e seu ligante (FasL) que

desencadeia a ativação de enzimas das caspases, ocasionando a apoptose nas células-alvo, além da interação de outros receptores como NKG2D, TRAIL, receptores naturais de citotoxicidade (NCRs) e seus ligantes **(Figura 02)** (STRATI, 2020; TOWNSEND et al., 2004; WALDOWSKA et al, 2017).

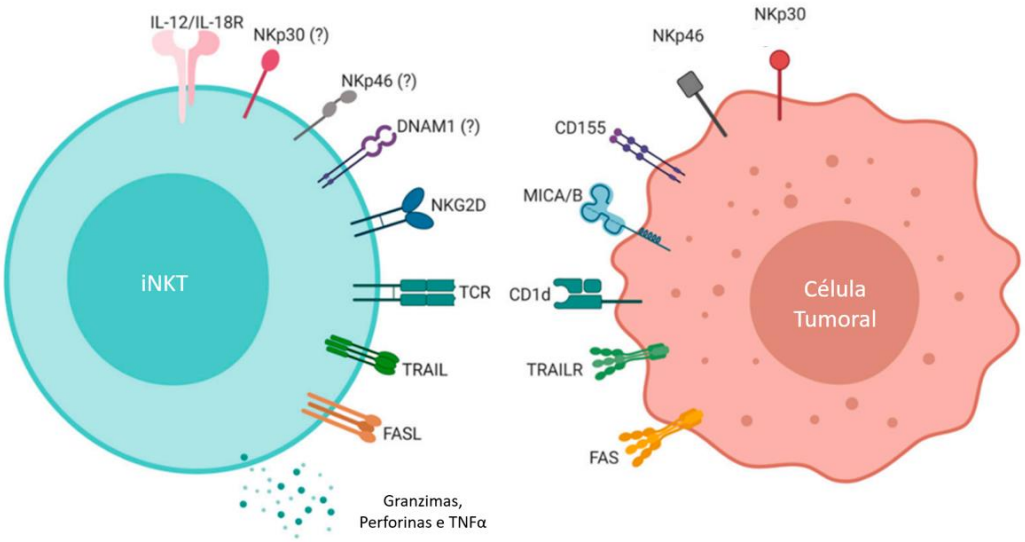


Figura 2. **Célula iNKT e sua citotoxicidade contra células tumorais**. A imagem ilustra como sinais da imunidade inata e adaptativa podem desencadear o potencial antitumoral das células iNKT através de proteínas citotóxicas solúveis e ligadas à membrana.

Fonte: Adaptado de Díaz-Basabe et al, 2020.

Há também a liberação substancial de perforinas e granzimas A e B, além da produção de granusilinas que atuam de forma semelhante às perforinas, formando poros na membrana plasmática e alterando sua permeabilidade, o que resulta na lise de células (SPARROW, 2020; WU et al., 2019).

As células NKT também podem atuar por meio de mecanismos indiretos, liberando uma gama de mediadores, principalmente as citocinas de perfil Th1 ou Th2, que podem variar dependendo do subtipo NKT (BAE et al., 2019). A liberação dos mediadores supracitados pode resultar na imunorregulação de outras células do sistema imune, por exemplo, ativando ou induzindo a maturação de DCs por meio da interação com CD1d ou CD40 / CD40L e IFN, respectivamente. Após a ativação das DCs, elas serão reguladas positivamente, expressando moléculas coestimulatórias como o CD80 e o CD86, além de liberarem citocinas como a IL-12, uma citocina pleiotrópica que desempenha papel essencial na resposta imune do tipo Th1 contra o câncer. As células iNKT também ativam células T citotóxicas CD8 através do IFN-γ, bem como células NK

convencionais e macrófagos que podem agir contra células neoplásicas (BAE et al., 2019; BASSIRI et al., 2014; IL et al, 1999; MCEWEN-SMITH et al, 2015). Além disso, as células NKT também podem estimular os linfócitos B e induzir o aumento da secreção de anticorpos da classe IgG, o que pode resultar em citotoxicidade celular dependente de anticorpos (ADCC) em células neoplásicas que posteriormente sofrerão lise celular citotóxica mediada por células (TAN et al., 2010; WALDOWSKA et al, 2017).

Devido ao seu repertório secretor e a capacidade de ativar e direcionar outras células do sistema imunológico, as células NKT contribuem fortemente para a imunovigilância tumoral. É importante destacar que uma das principais formas de reconhecimento das células cancerosas pelas células NKT ocorre por meio da interação do TCR com o antígeno apresentado pela molécula CD1d, expresso por células B, macrófagos, DCs e diversos tipos de células cancerígenas, em neoplasias sólidas e hematológicas (LEE et al., 2002; VAN KAER et al, 2011). Essas células têm demonstrado contribuir para a vigilância e supressão tumoral, controlando o estágio inicial do processo neoplásico (FIEDLER et al., 2002; GORINI et al., 2017; METELITSA et al., 2003; VIVIER et al., 2012).

As células iNKT, também conhecidas como células natural killer tipo I, apresentando características que diferem de outros subconjuntos, como reatividade a α-galactosilceramida (α-GalCer) e a expressão mais pronunciada do perfil de resposta Th1 (TERABE *et al*, 2007). Outra característica das células iNKT está relacionada ao seu TCR invariante, representado em humanos por uma cadeia TCR-α (Vα24Jα18) e uma cadeia TCR-β (Vβ11). As células iNKT reconhecem antígenos lipídicos e glicolipídios, como análogos α-GalCer, fosfolipídios, diacilgliceróis, gangliósidos (GD3) e glicoesfingolipídios (KUMAR *et al*, 2014; LIAO *et al*, 2013).

Estudos mostram que as células iNKT são fortemente reativas a α-Galcer, um glicolipídeo sintético derivado da esponja marinha *Agelas mauritianus*, que é capaz de induzir um importante efeito imunomodulador em células iNKT, que estimula a resposta antitumoral mediada por células citotóxicas (células NK e linfócitos T CD8) e estão em estado de exaustão ou anergia resultante do TME. A estimulação mediada por células iNKT demonstrou promover o fortalecimento das células citotóxicas a reverter a disfunção apresentada por essas células (BROSSAY et al., 1998; LIAO et al, 2013).

O antígeno α-GalCer apresentado através da molécula CD1d é reconhecido pelo TCR expresso em células iNKT, e induz a produção de citocinas, como IL-2, IL-12 e IL-21, que atuam em células NK convencionais e/ou células T citotóxicas CD8 anérgicas, revertendo assim a disfunção e o caráter hiporresponsivo (BAE et al., 2019; SEO et al., 2017). Além disso, as células iNKT estimuladas por α-GalCer mostraram incitar a ativação de células apresentadoras de antígenos via sinalização de CD40-CD40L e também induzir a produção de IL-12 (BAE et al., 2019; IL et al, 1999; TAN et al., 2010; ZHANG et al., 2019).

Um estudo de Fais et al., em pacientes com leucemia aguda, avaliou a expressão da molécula CD1d nas CLs, bem como a atividade das células iNKT no seu reconhecimento. Por meio da incubação de CLs com 100 ng/ml de α-GalCer por 4 horas, observou-se que os LCs $CD1d^{+}$ associados ao antígeno α-GalCer sofreram apoptose induzida por células iNKT, além de estimular a produção de citocinas INF-γ, TNF-α e IL-5 (FAIS et al., 2005). Resultados semelhantes foram observados por Bojarska-Junak et al, em um estudo com amostras de sangue periférico de pacientes com leucemia crônica, no qual células iNKT estimuladas *in vitro* por 24 horas com 100 ng/ml de α-GalCer mostraram maior expressão intracelular das citocinas INF-γ e IL-4 quando comparadas com voluntários saudáveis. O estudo também apontou que, entre as duas citocinas analisadas, a expressão de IL-4 foi maior que a de INF-γ, indicando que a célula iNKT pode adquirir uma polarização para o perfil de resposta Th2 (BOJARSKA-JUNAK et al., 2018).

Outro estudo mostrou que a frequência de células iNKT foi menor em pacientes com leucemia crônica em comparação com o grupo controle saudável. Além disso, a terapia por 14 dias com IFN-γ e 1 μM de mesilato de imatinibe em combinação com 100 ng/ml de α-GalCer nesses pacientes resultou em um aumento significativo nas células iNKT (ROSSIGNOL et al., 2012). Da mesma forma, Weinkove et al, demonstraram que CLs purificadas e estimuladas in vitro com 200 ng/ml de α-GalCer por 5 dias induziram a proliferação de células iNKT autólogas e alogênicas, mas não em uma quantidade significativa, sendo demonstrado que houve estimulação da produção de IFN-γ por células iNKT em pacientes leucêmicos. No entanto, foi observado que a cultura prolongada dessas células resultou na polarização de células iNKT para um perfil Th2, resultando em altos níveis de citocinas associadas à tolerância ao tumor (WEINKOVE et al., 2013). Investigações adicionais devem ser realizadas para identificar como a

estimulação com α-GalCer pode fornecer melhores resultados, associando o aumento da frequência das células iNKT com a polarização para um perfil de resposta contra CLs.

Finalmente, em uma análise longitudinal realizada ao longo de 18 meses com 22 pacientes com diagnóstico de leucemia ou mielodisplasia e submetidos ao transplante de células-tronco HLA haploidênticas para reconstituição de células iNKT. Nesse estudo foi relatada a existência de correlação entre a frequência de células iNKT e remissão da doença. Os dados observados mostraram que 14 pacientes cujas células iNKT foram completamente reconstituídas apresentaram remissão, e 8 pacientes cujas células iNKT não foram reconstituídas apresentaram recorrência, associando assim a frequência dessas células a um melhor prognóstico (CASORATI et al, 2012; DELLABONA et al., 2011). Além disso, a análise da frequência de células iNKT na circulação e no compartimento da medula óssea de pacientes recém-diagnosticados com leucemia aguda demonstrou que uma baixa frequência dessas células está associada a um pior prognóstico (NAJERA CHUC et al., 2012).

Ao contrário das células iNKT, as células NKT II têm uma variante de TCR αβ e formam rearranjos diversificados. Não são reativos a α-GalCer (BAE et al., 2019; DHODAPKAR et al, 2017; KUMAR et al, 2014), porém são restritos a CD1d (GODFREY et al., 2018) e reconhecem antígenos como β-glucosilceramida (β-GlcCer) e sulfatida, sendo esta última encontrada na membrana plasmática da mielina, no sistema nervoso central, no fígado, no pâncreas e nos rins (ARRENBERG *et al.*, 2010; BLOMQVIST *et al*, 2014; TERABE *et al*, 2018). Outra característica da célula NKT II está relacionada ao seu perfil de resposta, que é mais polarizado para o perfil Th2 (SINGH et al, 2018; TERABE et al, 2007).

Em contraste com os estudos com células iNKT, o número de estudos sobre o papel das células NKT II na leucemia é limitado. No entanto, alguns estudos relatam que o NKT II pode atuar de forma negativa, contribuindo para a supressão da vigilância e da atividade antitumoral e, em algumas situações, contribuindo para a progressão neoplásica (GODFREY *et al*, 2004; KUMAR *et al*, 2014; WALDOWSKA *et al*, 2017). Essa imunossupressão é mediada pela secreção de IL-13, que ativa células supressoras derivadas de mieloides (MDSCs) e estas, consequentemente, suprimem a atividade de células citotóxicas infiltrantes de tumor (SINGH *et al*, 2018).

Também se acredita que esta regulação negativa de NKT II no tumor ocorre devido a uma regulação cruzada entre iNKT e NKT II, onde o efeito das citocinas Th2 produzidas por células NKT II se sobrepõem ao efeito das citocinas Th1 produzidas por iNKT, resultando em um microambiente imunossupressor. Além disso, foi observado que os tumores crescem mais rápido quando a frequência das células NKT II é maior do que a das células iNKT, embora esse mecanismo de supressão ainda precise de mais investigação (TERABE *et al*, 2007; WALDOWSKA *et al*, 2017).

É importante observar que, além das células NKT, existem outras populações de células T não convencionais restritas a moléculas da família CD1, mais especificamente do grupo 1, composto por CD1a, CD1b e CD1c, que possuem antígenos lipídicos ou glicolipídeos, de origem microbiana ou do próprio organismo (LE NOURS et al, 2018). Semelhante às células NKT, essas populações de células restritas a CD1 são consideradas um alvo atraente para estudos na área de imunoterapia do câncer, especialmente no contexto de neoplasias hematológicas, devido à distribuição diversificada de isótipos CD1, onde se demonstrou que são expressos (CD1a, CD1b e CD1c) em 75% dos blastos na leucemia aguda (CONSONNI et al., 2017).

2.6.3 Células MAIT

As células MAIT correspondem a uma população de células T do tipo inatas e são caracterizadas pela expressão de um TCR-α restrito com um padrão de rearranjo gênico único, a saber, *TRAV1-2-TRAJ33 /12 /20 [Vα7.2-Jα33 / 12 / 20* (em humanos)], que emparelham com um repertório limitado da cadeia TCR-β, predominantemente das famílias de genes *TRBV6* e *TRBV20* (LANTZ; BENDELAC, 1994; PORCELLI et al., 1993; TILLOY et al., 1999). Formam um TCR semi-invariante restrito a antígenos não peptídicos apresentados pela proteína relacionada ao MHC-1 (MR1). A MR1 é uma molécula monomórfica altamente conservada ao longo da evolução dos mamíferos (TREINER et al., 2003; YAMAGUCHI et al., 1997), e é capaz de apresentar metabólitos derivados da vitamina B2, sintetizada por uma variedade de microrganismos para as células MAIT (CORBETT et al., 2014; ECKLE et al., 2015).

Como a maioria das células T, as células MAIT se desenvolvem no timo (MARTIN et al., 2009) e são selecionadas positivamente pelos timócitos corticais $CD4^+$

$CD8^+$ $MR1^+$ (MARTIN et al., 2009; SEACH et al., 2013). Após o processo de seleção, eles sofrem maturação extra-tímica e se integram a diferentes tecidos (KOAY et al., 2016). Em humanos, as células MAIT representam até 10% das células T do sangue periférico e são encontradas em abundância em tecidos mucosos, linfonodos mesentéricos e fígado, onde podem representar até 45% de todas as células T (GHERARDIN et al., 2018; GODFREY et al., 2019).

As células MAIT humanas podem ser diferenciadas pelo imunofenótipo, caracterizado pela presença de $CD3^+$ $V\alpha7.2^+$ e $CD161^{high}$ (RAHIMPOUR et al., 2015). Além disso, podem ser categorizadas com base na expressão de correceptores CD4 e CD8 em cinco subconjuntos: $CD4^+$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^-$, $CD4^-$ $CD8\alpha\alpha^+$ e $CD4^-$ $CD8\alpha\beta^+$ **(Figura 3)**; os dois últimos ($CD4^-$ $CD8\alpha\alpha^+$ e $CD4^-$ $CD8\alpha\beta^+$) sendo os mais abundantes e, coletivamente, correspondem a aproximadamente 80% das células MAIT (GHERARDIN et al., 2018). Digno de nota, o desenvolvimento de tetrâmeros MR1 carregados com 5-OP-RU [5- (2-oxopropilidenoamino) -6-d-ribitilaminouracil] marcou um avanço na pesquisa de células MAIT e permitiu a identificação confiável de diferentes subconjuntos fenotípicos e funcionais de células MAIT (REANTRAGOON et al., 2013).

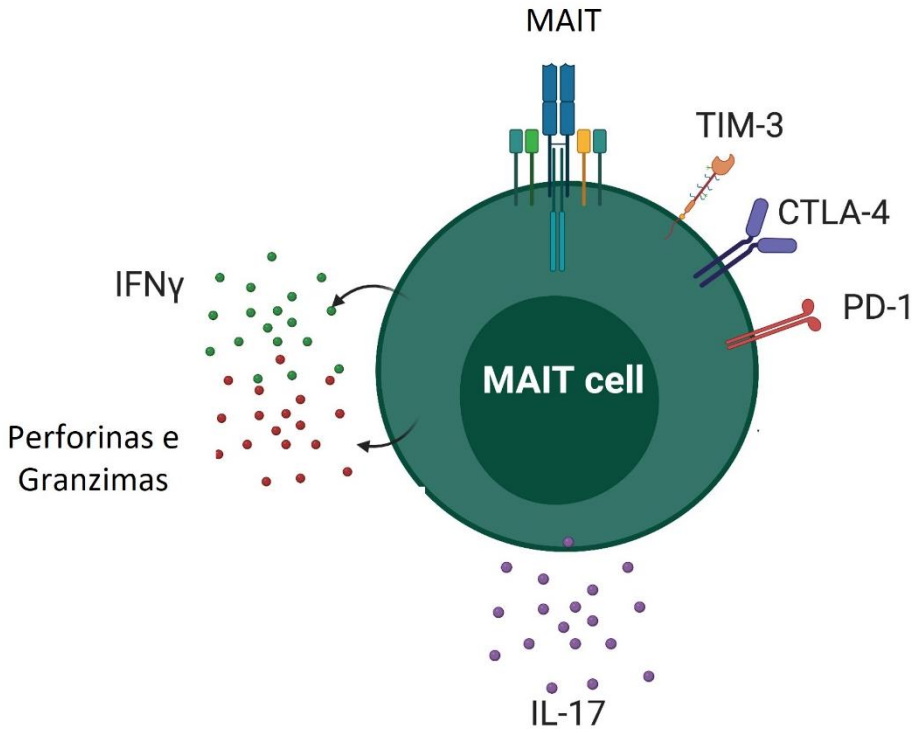


Figura 3: Células MAIT realizam ponte entre os sistemas imunológico inato e adaptativo. As células MAIT com foco no cenário do câncer, se destacam como um novo alvo em potencial para imunoterapia. Após a ativação, as células MAIT tornam-se células efetoras, capazes de produzir uma variedade de citocinas e moléculas. Além de seu papel antimicrobiano, as células MAIT estão relacionadas a respostas imunes ao câncer, com papéis benéficos e ou patogênicos relatados.

Fonte: Adaptado de *O´Neill et al*, 2021

Além das moléculas de superfície mencionadas acima, as células MAIT também expressam CD25, CD26, CD44 e CD69, bem como receptores de citocinas IL-7R, IL-12R, IL-15R e IL-18R e PLZF, fatores de transcrição T-bet e RORγt, proporcionando alta plasticidade e capacidade de secretar mediadores de perfil Th1 e Th17 (TOUBAL et al., 2019). É importante notar que a maioria das células MAIT têm um fenótipo efetor semelhante a um fenótipo de memória (DUSSEAUX et al., 2011), podendo ser rapidamente ativadas por mecanismos que não dependem da estimulação de TCR (USSHER et al., 2014). Isso se deve à alta expressão de vários receptores de quimiocinas e citocinas, sendo considerada uma propriedade que compartilham com outras células T inatas (células T γδ e células iNKT) (HAERYFAR; SHALER; RUDAK, 2018).

Após a ativação, essas células são capazes de liberar quantidades substanciais de perforinas e granzimas, além de produzirem as citocinas pró-inflamatórias TNF, IFN-γ e IL-17A, além de CCL3 e CCL4, que são quimiocinas fundamentais para as respostas imunes à infecção e inflamação (LENG et al., 2019; SERRIARI et al., 2014). A liberação desses mediadores resulta na destruição das células infectadas e na ativação de outras células do sistema imunológico, como as DCs, o que, consequentemente, leva à mobilização das células T convencionais e se reflete em uma cascata de eventos imunológicos (KURIOKA et al., 2015; SALIO et al., 2017).

Devido a algumas características das células MAIT, como sua alta frequência em humanos, além de sua capacidade de secretar rapidamente um repertório de mediadores que induz ativação e regulação de outras células do sistema imunológico, bem como a capacidade de reconhecer antígenos restritos ao MR1, a pesquisa com células MAIT, assim como outras populações de células T restritas ao MR1, tem despertado grande interesse no campo da oncologia. Atualmente, o papel funcional desencadeado por essas células T de tipo inato no câncer não é claro e tem sido objeto de vários estudos. As investigações realizadas em pacientes com neoplasias de mucosa relataram uma redução na frequência das células MAIT circulantes e mostram um acúmulo significativo dessas

células no tecido tumoral (LING et al., 2016; SUNDSTRÖM et al., 2015; WON et al., 2016; SUNDSTRÖM et al., 2015).

Além disso, um estudo *in vivo* recente mostrou que as células MAIT exibiram uma função de promoção de tumor e promoveram metástases de câncer por meio da supressão de células citotóxicas (que eram parcialmente dependentes de IL-17) após interação com moléculas MR1 expressas em células cancerosas (YAN et al., 2020).

Em contraste, foi relatado que células MAIT infiltrantes de tumor não foram comprometidas na produção de citocinas IFN-γ, TNF-α e IL-17. Além disso, no ensaio in vitro, foi observado que as células MAIT, isoladas do sangue periférico de indivíduos saudáveis, não apenas apresentaram atividade citotóxica ativada por linfocina, mas também exibiram citotoxicidade direta em linhagens celulares K562, por meio de degranulação de granzima B e perforina (WON et al., 2016).

Até o momento, o envolvimento das células MAIT na leucemia permanece amplamente inexplorado e, no contexto de outras neoplasias hematológicas, o papel funcional dessas células também é limitado. Um estudo *in vitro* de Gherardin et al. demonstrou que linhagens celulares de mieloma múltiplo (MM) expressam a proteína MR1 e são capazes de apresentar metabólitos da vitamina B para células MAIT isoladas de doadores saudáveis, que, em resposta, induzem a lise dessas células de mieloma com eficiência e cinética semelhantes às células NKT (GHERARDIN et al., 2018).

Outro estudo observou a capacidade funcional desses linfócitos por meio da análise do fenótipo celular em amostras de SP e MO (FAVREAU et al., 2017). Favreau et al. relataram que pacientes recentemente diagnosticados com MM tiveram uma diminuição significativa na frequência de células MAIT quando comparados a controles saudáveis. Eles também destacaram uma redução no fenótipo MAIT $CD8^+$ e duplo-negativo ($CD8^-$ $CD4^-$), além do comprometimento funcional do perfil de resposta Th1, com menos células MAIT produtoras de IFN-γ e TNF (FAVREAU et al., 2017). Este estudo também demonstrou que as células MAIT circulantes exibiam altos níveis de PD-1 e que seu respectivo bloqueio *in vitro* resultou na restauração da função e ativação das células MAIT. Em conjunto, esses resultados destacam a importante atividade antitumoral das células MAIT e as identificam como um potencial alvo na imunoterapia aplicada ao MM.

Da mesma forma, o MR1 também representa um alvo atraente na imunoterapia contra o câncer, devido a características como sua natureza monomórfica e expressão funcional encontrada em diversas neoplasias. A respeito disso, um estudo recente de Crowther et al. demonstraram que um clone de células T humanas potencialmente reconhece um câncer específico ou metabólito associado, restrito a MR1, e medeia a lise de diferentes tipos de células cancerosas, incluindo linhagens de CLs, como tal, mediou a regressão da leucemia *in vivo* e conferiu maior sobrevida em camundongos (CROWTHER et al., 2020).

Além disso, outras células T restritas a MR1 atípicas, que respondem a autoantígenos, foram descritas (GHERARDIN et al., 2016; LEPORE et al., 2017; MEERMEIER et al., 2016). Esse novo grupo de células T foi denominado MR1T e demonstrou reconhecer e eliminar uma ampla gama de células cancerosas que expressam MR1 (LEPORE et al., 2017). No entanto, mais pesquisas são necessárias para elucidar a natureza precisa e função dessas células, bem como sua atividade no contexto do microambiente tumoral (MT).

3. OBJETIVOS

3.1.Objetivo Geral

Analisar o perfil de células T não convencionais e mediadores imunológicos solúveis no sangue periférico e medula óssea de pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) comum.

3.2.Objetivos Específicos

- Realizar revisão bibliográfica sobre a imunobiologia das células T não convencionais e seus mecanismos efetores na resposta antileucêmica;
- Classificar imunofenotipicamente as células dos pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) comum;
- Descrever a frequência de células T não convencionais no sangue periférico e medula óssea de pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) comum;
- Realizar a quantificação de mediadores imunológicos solúveis no sangue periférico e medula óssea de pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) comum;
- Relacionar a frequência de células T não convencionais e de mediadores imunológicos solúveis com uma possível atividade antileucêmica, exercida pelas células T não convencionais circulantes e no compartimento medular de pacientes pediátricos com Leucemia Linfoblástica Aguda de células B (LLA-B) comum.

4. MATERIAL E MÉTODOS

4.1 Aspectos éticos

Este estudo faz parte de um projeto maior intitulado "*Biomarcadores celulares e moleculares envolvidos na resposta imunológica de pacientes com leucemia linfoblástica aguda: novas abordagens aplicadas ao diagnóstico, prognóstico e terapêutica*" aprovado pelo Comitê de Ética em Pesquisa (CEP) da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), sob número de parecer 4.982.395/2021 e CAAE 51257921.2.0000.0009 (**Anexo I**). Todos os indivíduos que participarem deste estudo serão incluídos mediante aceitação do Termo de Consentimento Livre e Esclarecido (TCLE) e Termo de Assentimento Livre e Esclarecido (TALE), para aqueles acima de 06 anos de idade, alfabetizados.

4.2 Revisão Bibliográfica do estudo

Os artigos de revisão bibliográfica desenvolvidos basearam-se na busca em diferentes plataformas de pesquisa científica *on-line* de *open acess* que disponibilizaram o manuscrito na íntegra (*full-text), e para as buscas foram* utilizadas principalmente as seguintes palavras chaves: *"Unconventional T cells", "acute lymphoblastic leukemia", "tumor microenvironment",* e *"soluble immune mediators"*.

4.3 Tipo de estudo e População

Tratou-se de um estudo analítico, longitudinal e prospectivo, com coleta de amostras de medula óssea (MO) e sangue periférico (SP) de pacientes pediátricos atendidos na Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM). Uma população do estudo foi constituída de pacientes atendidos no serviço de hematologia pediátrica da Fundação HEMOAM com diagnóstico de LLA-B, obtidas por demanda espontânea no período de março de 2021 a março de 2023.

Além dos pacientes, foram recrutadas crianças (sem leucemia) atendidas na triagem clínica da Fundação HEMOAM, que realizarem o hemograma de rotina e cujo resultado não apresentou alterações em nenhum dos constituintes sanguíneos, nem infecções detectáveis pelos testes realizados de sorologia.

4.4 Critérios de Inclusão e Exclusão

Os critérios de inclusão utilizados para o grupo caso foram: pacientes recém-diagnosticados com LLA-B comum, admitidos no serviço de hematologia pediátrica da Fundação HEMOAM, com idade >1 e <18 anos, tratados com o Protocolo do Grupo Brasileiro de Tratamento das Leucemias na Infância (GBTLI)-2009.

A participação dos pacientes no estudo foi solicitada para os pais ou responsáveis legais. Após o consentimento da família, foi lido o Termo de Consentimento Livre e Esclarecido (TCLE) para o (s) responsável (eis) e realizado a assinatura do documento, em duas vias e entregue uma via ao (s) responsável (eis). Também foi solicitada à criança sua participação na pesquisa e aquelas acima de 06 anos de idade e alfabetizadas foi lido o Termo de Assentimento Livre e Esclarecido (TALE), seguido de seu consentimento foi solicitado sua assinatura no documento, em duas vias e entregue uma via para a criança/responsável (eis). Para o grupo controle, os critérios de inclusão adotados foram: idade >1 e <18 anos, sem alterações no hemograma e sem infecção prévia há menos de 04 semanas. Não foram incluídos no estudo os indivíduos do grupo caso ou controle com doença inflamatória aguda e crônica conhecida ou com distúrbio autoimune, e recusa do TCLE e TALE.

Como critérios de exclusão, para ambos os grupos, foram adotados os seguintes parâmetros: pacientes diagnosticados com LLA que não sejam classificados em LLA-B comum, material biológico insuficiente ou coagulado.

4.5 Local e obtenção das Amostras

Para este estudo, foram utilizadas amostras de SP e MO de pacientes pediátricos com suspeita de Leucemia Aguda, obtidas através de punção aspirativa da crista ilíaca, esterno ou tíbia e através de punção venosa, respectivamente. A obtenção destas amostras ocorreu paralelamente aos procedimentos médicos habituais, de modo que foram aproveitadas da rotina médica para a pesquisa. As coletas ocorreram em dois *timepoints* da quimioterapia de remissão, sendo estes: dia do diagnóstico (Dia 0, D0) e no 35º dia da terapia de indução da remissão (Dia 35, D35). Foram coletados 2ml de MO e 3ml de SP em tubos com sistema a vácuo, contendo anticoagulante EDTA (ácido etilenodiamino tetra-acético) pela equipe médica da Fundação HEMOAM.

Além das amostras dos pacientes pediátricos utilizadas para Grupo Controle foram obtidas amostras de SP de crianças sem leucemia, obtidas através de punção venosa, para serem utilizadas como valor de referência comparativa nas análises (grupo controle para SP). As amostras obtidas foram então encaminhadas ao Laboratório Multidisciplinar de Pesquisa e foram centrifugadas a 2.000 rpm por 10 minutos. Posteriormente, o sobrenadante/plasma pobre em plaquetas (PPP) foi coletado e transferido para criotubos que, em seguida, foram identificados e condicionados em biorrepositório contendo freezers para armazenamento a -80°C, para posterior dosagem dos mediadores imunológicos solúveis. Com a fração celular restante foi realizada a marcação com anticorpos monoclonais para a imunofenotipagem por citometria de fluxo (**Figura 4**).

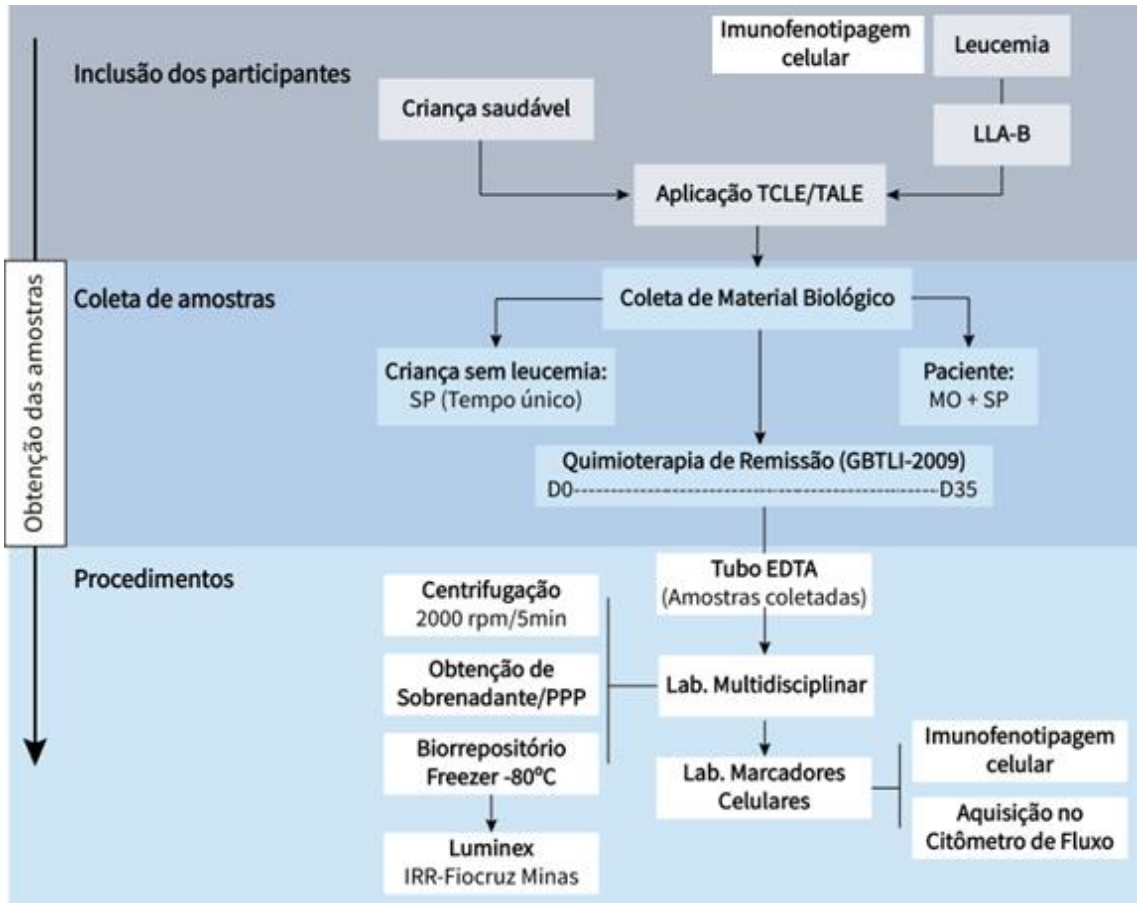


Figura 4. Fluxograma de obtenção das amostras. Demonstração detalhada das etapas de obtenção e processamento das amostras utilizadas no estudo.

4.6 Obtenção dos dados clínicos e laboratoriais

Foi elaborado um banco de dados a partir de registros do laboratório de marcadores celulares da Fundação HEMOAM e fichas de acompanhamento de pacientes que posteriormente foram digitados em planilha eletrônica Excel® 2013 (Microsoft Corporation, Redmond, WA, USA). As variáveis qualitativas e quantitativas analisadas foram: sexo e idade, parâmetros hematológicos e sorológicos, contagem de blastos e presença de Doença Residual Mensurável (DRM).

4.7 Processamento e Análise das Amostras

4.7.1 Sangue Periférico e Medula óssea

A contagem dos leucócitos, série vermelha e índices hematimétricos, bem como a contagem de plaquetas foi realizada em contador de células *ADVIA®2120i Hematology System* (*Siemens Healthcare, Erlangen, Germany*). A contagem diferencial celular (na lâmina do Sangue periférico e da medula óssea) foi feita a partir da contagem de 100 células para contabilizar o percentual de blastos corados pelo método de *May-Graunwald Giemsa*. Segundo a Organização Mundial de Saúde (OMS), o achado de no mínimo 20% de blastos no esfregaço sanguíneo sugerem o diagnóstico de Leucemia Aguda (LA).

4.7.2 Diagnóstico de Leucemia Aguda

De acordo com o resultado do hemograma (tanto de sangue periférico quanto de medula óssea), as amostras que estiveram dentro dos padrões estabelecidos com suspeita de leucemia, foram encaminhadas para confecção das lâminas no Laboratório de Citoquímica e coradas pelo método de *May-Graunwald Giemsa*, para posterior contagem diferencial celular em microscópio óptico, a partir da leitura de no mínimo 20% de blastos encontrados nas lâminas de SP e MO. Decorrente a um número igual ou superior de 20% de blastos visualizados nas lâminas, as amostras de sangue de medula óssea (coletada em EDTA) foram triadas para imunofenotipagem de leucemia aguda e analisada por citometria de fluxo de acordo com o **Quadro 01.**

Quadro 01: Descrição do painel de anticorpos para triagem de leucemias agudas (LA) utilizados na imunofenotipagem e análise posterior por citometria de fluxo.

Tubo	Parâmetros	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
01	**Triagem B / Mielóide**	cMPO (3 µL)	cCD79a (5 µL)	CD34 (5 µL)	CD19 (5 µL)	CD45 (10 µL)	-
02	**Triagem T**	-	cCD3 (2 µL)	CD34 (5 µL)	CD7 (0,5 µL)	CD45 (10 µL)	-

Para a marcação dos antígenos de superfície, foram identificados nos tubos de citometria os respectivos anticorpos monoclonais obedecendo a sequência dos fluorocromos (FITC/PE/Per-CP/PE-Cy7/APC/APC-H7). Em seguida, adicionados em cada tubo os anticorpos monoclonais, foi adicionado 100 µL da amostra de medula óssea.

Em seguida, os tubos foram homogeneizados em vórtex e incubados durante 20 minutos à Temperatura Ambiente (T.A) e protegido da luz. Posteriormente as hemácias foram lisadas com solução de lise 1X, homogeneizadas em vórtex e incubadas novamente durante 10 minutos à Temperatura Ambiente (T.A) e protegido da luz.

Após a marcação, os tubos foram centrifugados por 5 minutos a 1700 rpm (rotação por minutos), desprezado o sobrenadante por inversão, deixando escorrer o excesso em papel absorvente e adicionados 2mL da solução tampão de PBS (8,0g de NaCl, 1,16g de Na_2HPO_4, 0,2g de KH_2PO_4, 0,2 de KCl, 5,0g de BSA e 5,0g de Saponina, qsq 1L H_2O destilada, pH: 7,2), diluído a 1X, para lavagem das células não marcadas e as de não interesse para a análise. Posteriormente, as amostras foram ressuspendidas em 500 µL de PBS 1X para aquisição e leitura no Citômetro de Fluxo *FACSCanto II*® (*Becton, Dickinson and Companhy, San Jose, CA, USA*) da Fundação HEMOAM.

Para a marcação dos antígenos citoplasmáticos (cMPO, cCD79a e cCD3), após a marcação dos antígenos de superfície, foram colocados nos tubos (antes da leitura por citometria), 100 µL da solução de Fixação A *(Kit FIX & PERM Cell Fixation & Permeabilization BD™)*, homogeneizadas no vórtex e incubadas por 15 minutos à Temperatura Ambiente (T.A) e protegido da luz. Em seguida, será adicionado 2 mL de PBS 1X para lavagem, e as amostras centrifugadas por 5 minutos a 1700 rpm.

Posteriormente, o sobrenadante foi desprezado por inversão, deixando escorrer o excesso em papel absorvente. Após essa etapa de fixação celular, foi adicionado nos tubos 100 µL da solução permeabilizante B *(Kit FIX & PERM Cell Fixation &*

Permeabilization BD™), com os anticorpos citoplasmáticos [cMPO (3 µL), cCD79a (5 µL), cCD3 (2 µL)], homogeneizados no vórtex e incubados por 15 minutos à T.A e protegido da luz, para marcação dos antígenos citoplasmáticos.

Após esta fase, foi acrescentado 2 mL de PBS 1X nos tubos para lavagem, homogeneizados no vórtex, centrifugados 5 minutos a 1700 rpm e ressuspendidos com 500 µL de PBS 1X para aquisição e leitura pelo Citômetro de Fluxo *FACSCanto II®* (*Becton, Dickinson and Companhy, San Jose, CA, USA*) da Fundação HEMOAM.

4.7.3 Classificação de Leucemia Linfoblástica Aguda de células B comum

Após identificação de antígenos que caracterizam leucemia aguda de linhagem linfoblástica, com exclusão de outras linhagens ou classificações, foi realizado um painel de ampliação para diagnóstico da linhagem celular específica de Leucemia Linfoblástica Aguda de células B "comum, de acordo com o **Quadro 02.**

Quadro 02: Descrição do painel de anticorpos para Ampliação de leucemia linfoblástica aguda de células B (LLA-B) utilizados na imunofenotipagem e análise posterior por citometria de fluxo.

Tubo	Parâmetros	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
01	**Controle (-)**	-	-	-	-	-	-
02	**AMPLIAÇÃO B**	CD20 (5 µL)	CD66c (5 µL)	CD34 (5 µL)	CD19 (5 µL)	CD45 (10 µL)	CD10 (2 µL)
03	**AMPLIAÇÃO B**	CD15/CD65 (5 µL)	NG2 (5 µL)	CD34 (5 µL)	CD19 (5ul)	CD45 (10 µL)	-
04	**AMPLIAÇÃO B**	cIgM (10 µL)	CD13/CD33 (5 µL)	CD34 (5 µL)	CD19 (5 µL)	CD45 (10 µL)	-
05	**AMPLIAÇÃO B**	CD38 (5 µL)	CD81 (5 µL)	CD34 (5 µL)	CD19 (5 µL)	CD45 (10 µL)	CD9 (3 µL)

Para a marcação dos antígenos de superfície, foram identificados nos tubos de citometria os respectivos anticorpos monoclonais obedecendo a sequência dos fluorocromos (FITC/PE/Per-CP/PE-Cy7/APC/APC-H7). Em seguida, adicionados em cada tudo os anticorpos monoclonais, foi adicionado 100 µL da amostra de sangue de medula óssea.

Em seguida, os tubos foram homogeneizados em vórtex e incubados durante 20 minutos à Temperatura Ambiente (T.A) e protegido da luz. Posteriormente as hemácias

foram lisadas com solução de lise 1X, homogeneizadas em vórtex e incubadas novamente durante 10 minutos à Temperatura Ambiente (T.A) e protegido da luz.

Após a marcação, os tubos foram centrifugados por 5 minutos a 1700 rpm (rotação por minutos), desprezado o sobrenadante por inversão, deixando escorrer o excesso em papel absorvente e adicionados 2mL da solução tampão de PBS (8,0g de NaCl, 1,16g de Na_2HPO_4, 0,2g de KH_2PO_4, 0,2 de KCl, 5,0g de BSA e 5,0g de Saponina, qsq 1L H_2O destilada, pH: 7,2), diluído a 1X, para lavagem das células não marcadas e as de não interesse para a análise. Posteriormente, as amostras foram ressuspendidas em 500 µL de PBS 1X para aquisição e leitura no Citômetro de Fluxo *FACSCanto II*® (*Becton, Dickinson and Companhy, San Jose, CA, USA*) da Fundação HEMOAM.

Para a marcação do antígeno citoplasmático (cIgM), após a marcação dos antígenos de superfície, foram colocados nos tubos (antes da leitura por citometria), 100 µL da solução de Fixação A *(Kit FIX & PERM Cell Fixation & Permeabilization BD™)*, homogeneizadas no vórtex e incubadas por 15 minutos à Temperatura Ambiente (T.A) e protegido da luz. Em seguida, foi adicionado 2 mL de PBS 1X para lavagem, e as amostras centrifugadas por 5 minutos a 1700 rpm. Posteriormente, o sobrenadante foi desprezado por inversão, deixando escorrer o excesso em papel absorvente. Após essa etapa de fixação celular, foi adicionado nos tubos 100 µL da solução permeabilizante B *(Kit FIX & PERM Cell Fixation & Permeabilization BD™)*, com o anticorpo citoplasmático [cIgM (10ul)], homogeneizados no vórtex e incubados por 15 minutos à T.A e protegido da luz, para marcação dos antígenos citoplasmáticos.

Após esta fase, foi acrescentado 2 mL de PBS 1X nos tubos para lavagem, homogeneizados no vórtex, centrifugados 5 minutos a 1700 rpm e ressuspendidos com 500 µL de PBS 1X para aquisição e leitura pelo Citômetro de Fluxo *FACSCanto II*® (*Becton, Dickinson and Companhy, San Jose, CA, USA*) da Fundação HEMOAM.

4.7.4 Imunofenotipagem de células T não convencionais por Citometria de Fluxo

A imunofenotipagem das subpopulações de células T gamma/delta (γδ), células MAIT e células NKT, tanto de amostras de SP quanto de MO, foi realizada pela técnica de Citometria de Fluxo. As subpopulações celulares propostas neste estudo foram marcadas utilizando um painel de anticorpos monoclonais conjugados com fluorocromos, específicos para os marcadores de cada população celular e as citocinas citoplasmáticas de acordo com cada célula, de acordo com o **Quadro 03**.

Quadro 03: Descrição do painel de anticorpos para imunofenotipagem de A imunofenotipagem das subpopulações de células T não convencionais: T γδ, células, iNKT e células MAIT.

Tubo	Parâmetros	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
01	CN	-	-	-	-	-	-
02	T γδ	CD45 (5 µL)	-	CD69 (1 µL)	CD95 (1 µL)	TCRgd	CD3 (1 µL)
03	NKT	CD16	CD161		CD56	CD45	CD3 (1 µL)
04	Células MAIT	TCR Va7.2	CD161	CD69	-	CD45	CD3 (1 µL)

Inicialmente, foram adicionados ±1µl de cada anticorpo monoclonal no fundo dos respectivos tubos de ensaio. Em seguida, 100µl de amostra (MO/SP) foram adicionados em cada tubo e homogeneizados em vórtex e incubados por 30 minutos em temperatura ambiente, ao abrigo da luz. Após a marcação das células e incubação, foram adicionados 2ml de solução de lise 1X (*BD FACS™ Lysing Solution 10X Concentrate*) durante homogeneização em vórtex. Em seguida, os tubos foram novamente incubados por 10 minutos em temperatura ambiente e ao abrigo da luz. Passado a fase de incubação, os tubos foram centrifugados a 1.500 rpm por 5 minutos. O sobrenadante foi desprezado por inversão e o excesso foi brevemente escorrido em papel absorvente. Para a limpeza do pellet celular formado, foram adicionados 2ml de solução PBS-1X (*Phosphate Buffered Saline*) em cada tubo, homogeneizados em vórtex e centrifugados a 1.700 rpm por 5 minutos, desprezando o sobrenadante por inversão ao final, em um ciclo de duas lavagens seguidas. Por fim, após o ciclo de 2 lavagens do pellet celular, a amostra foi ressuspendida em 300µl de PBS-1X para posterior aquisição na plataforma de citometria de fluxo.

A aquisição das amostras foi realizada no citômetro de fluxo FACSCanto™ II (BD Biosciences) da Fundação HEMOAM. Para a identificação morfométrica e imunofenotípica das subpopulações de células T γδ foi utilizado o software FlowJo (v10), com o auxílio de "gates" para a seleção do pool celular de interesse em gráficos que combinam características morfológicas (tamanho e complexidade celular) com características imunofenotípicas através da fluorescência emitida pelos anticorpos monoclonais conjugados com fluorocromos. Os gráficos utilizados foram do tipo "dot plot", "contour plot" e histograma, pois apresentam uma melhor visualização das estratégias de análise.

4.7.5 Dosagem de citocinas, quimiocinas e fatores de crescimento para a quantificação de mediadores imunológicos solúveis

O plasma obtido das amostras de sangue periférico (SP) e medula óssea (MO) nos tempos D0 e D35 da quimioterapia de remissão foram armazenados em biorrepositório para a dosagem de mediadores imunológicos solúveis. Para as quantificações moléculas utilizou-se a plataforma Luminex Bio-Plex Pro™ human cytokines que permitiu a pesquisa dos respectivos analitos: CXCL8, CCL11, CCL3, CCL4, CCL2, CCL5, CXCL10, IL-1β, IL-6, TNF-α, IL-12(p70), IFN-γ, IL-15, CXCL10, CXCL11, IL-1β, IL-1Ra, IL-4, IL-6, IL-7, IL-10, IL-13, IL-17, IL-1Rα, IL-4, IL-5, IL-9, IL-10, IL-13, FGF-Básico, PDGF, VEGF, G-CSF e GM-CSF.

Inicialmente, descongelaram-se as amostras dos soros/plasmas (banho-maria a 37ºC), homogeneizou (vórtex por 5 segundos) e centrifugou a 14.000 x g, por 5 minutos a temperatura ambiente. Subsequentemente, adicionou-se 50 µL das amostras em placas para ensaio Luminex, previamente preparadas através da adição de 50 µL da mistura de beads (diluída 10x), em cada poço, seguida por lavagem (duas vezes com 100 µL de tampão de lavagem). Incubou-se as placas overnight, 4ºC e sob agitação (500±50rpm). No dia seguinte, adicionou-se 25µL do anticorpo de detecção em cada poço (previamente diluído 10x), incubou-se por 30 minutos em agitador (500±50rpm), temperatura ambiente e ao abrigo da luz. Subsequentemente, procedeu-se a lavagem com 100µL de tampão de lavagem, (procedimento realizado por 3 vezes).

Posteriormente, adicionou-se 50 µL da estreptavidina-ficoeritrina (SAPE) em cada poço (previamente diluído 10x) e incubou por 30 minutos em agitador (500±50rpm), temperatura ambiente e ao abrigo da luz. Subsequentemente, procedeu-se a lavagem com 100 µL de tampão de lavagem. Ressuspendeu-se o pellet com adição de 125 µL de tampão de ensaio e homogeneizou-se as amostras por 10 minutos.

Determinou-se as intensidades medias de fluorescência por aquisição das amostras (50 microesferas por analito avaliado) no equipamento Bio-Plex 200 (Bio-Rad Laboratories, California, EUA), utilizando-se o software Luminex xPONENT versão 3.1 (Merck Millipore, Massachusetts, EUA). As concentrações dos biomarcadores foram obtidas a partir da construção de curvas-padrão dos analitos (utilizando uma curva logística de ajuste de 5 parâmetros para transformar as intensidades médias de fluorescência em concentrações (pg/mL), utilizando-se o software Bio-Plex Manager™ versão 6.2 (Bio-Rad Laboratories, California, EUA).

4.7.6 Coleta de dados sociodemográficos e clínicos-hematológicos

A coleta dos dados sociodemográficos (idade, gênero) e clínico-hematológicos (grupo de risco, doença residual mínima, imunofenótipo, mielograma, hemograma) foi realizada a partir dos registros do laboratório de marcadores celulares e prontuários do Setor de Atendimento Médico e Estatístico (SAME), posteriormente foram plotados em planilha eletrônica no Microsoft Excel, onde foi construído o banco de dados do estudo.

4.8 Análise de dados

Os dados demográficos, epidemiológicos e características clínicas dos pacientes foram apresentados em formas de tabelas e gráficos, elaboradas com o programa Excel 2013 (*Microsoft Corporation, Redmond, WA, USA*). As análises estatísticas foram realizadas com os softwares *Graphpad Prism* (v8) e *Stata* (v15). Inicialmente foram realizados testes para verificar normalidade dos dados através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com o teste t student ou Mann-Whitney, enquanto que para as comparações das variáveis com três ou mais grupos, a análise de dados foi por meio dos testes ANOVA One-way ou Kruskal-Wallis, seguido de pós-teste de Dunn's, para múltiplas comparações entre os grupos. Os níveis de significância estatística definidos em ambos os casos foi de $p<0,05$. Por fim, realizamos a análise correlação, para demonstrar as correlações positivas e negativas significativas ($p<0,05$). O índice de correlação (r) será utilizado para categorizar a força de correlação como fraca ($r\leq0,35$), moderada ($r\geq0,36$ a $r\leq0,67$) ou forte ($r\geq0,68$).

5. RESULTADOS

CAPÍTULO I

Publicação – Artigo 1 de revisão

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Review Article

Translating Unconventional T Cells and Their Roles in Leukemia Antitumor Immunity

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Recently, cell-mediated immune response in malignant neoplasms has become the focus in immunotherapy against cancer. However, in leukemia, most studies on the cytotoxic potential of T cells have concentrated only on T cells that recognize peptide antigens (Ag) presented by polymorphic molecules of the major histocompatibility complex (MHC). This ignores the great potential of unconventional T cell populations, which include gamma-delta T cells ($\gamma\delta$), natural killer T cells (NKT), and mucosal-associated invariant T cells (MAIT). Collectively, these T cell populations can recognize lipid antigens, specially modified peptides and small molecule metabolites, in addition to having several other advantages, which can provide more effective applications in cancer immunotherapy. In recent years, these cell populations have been associated with a repertoire of anti- or protumor responses and play important roles in the dynamics of solid tumors and hematological malignancies, thus, encouraging the development of new investigations in the area. This review focuses on the current knowledge regarding the role of unconventional T cell populations in the antitumor immune response in leukemia and discusses why further studies on the immunotherapeutic potential of these cells are needed.

1. Introduction

Leukemia comprises a heterogeneous group of hematological neoplasms, which can be classified into lymphoblastic or myeloid leukemias and divided into acute and chronic types, depending on the affected cell type, maturation stage, and blast count, respectively [1]. While acute leukemias are characterized by a deep block in hematopoietic differentiation and result in an overproduction of immature blasts, chronic leukemias are characterized by the excessive production of partially mature differentiated cells, for example, lymphocytes in chronic lymphocytic leukemia (CLL) and granulocytes in chronic myeloid leukemia (CML) [2, 3]. The hallmark of these neoplasms is the increase in leukemic cells (LCs) in the bone marrow (BM) and their release in the peripheral blood (PB) and in extramedullary sites [1].

The immunological mechanisms in patients with leukemia are not very well known. However, with the increasing

Table 1: The table indicates some functions triggered by unconventional T cells in the immune response against the tumor.

Subsets	Role played in the immune response against cancer cells	Reference
$\gamma\delta$ T cells	Mediate tumor regression by recognizing MIC-A/MIC-B and ULBPs through TCR and NKG2D	[34, 35, 51, 52, 90]
	Antitumor potential increased by expression of NCRs	[36]
	Positive regulation of pAgs in LCs mediates the immune response through $\gamma\delta$ TCR	[40, 41, 72]
	BTN3A/BTN2A1 increases the antitumor functions of $\gamma\delta$ T cells in blood	[72–74]
	Mediate tumor regression by recognizing PVR and nectin-2 through TCR and DNAM-1	[41]
	BTN3A-expressing LCs are recognized and destroyed by $\gamma\delta$ T cells in blood through TCR	[69]
	Induce the maturation of DCs, which consequently enhance their activity against LCs	[29, 30]
NKT cells	Mediates tumor regression by recognizing $CD1d^+$ LCs through TCR	[133]
	Induces direct destruction of tumor cells through granulysin	[124, 125]
	Induces direct tumor lysis through FasL	[111]
MAIT cells	They act in the immunovigilance during the initial phase of the neoplastic process	[131–134]
	Induces direct cytotoxicity mediated by granzyme B and perforin in cancer cells that express MR1	[162, 175]

advances in the field of immunotherapy, there has been great progress in research regarding the tumor microenvironment in leukemia. Studies have shown that LCs secrete factors that disrupt healthy BM niches, reprogramming and transforming them into "leukemic niches," as well as inducing a disruption in balanced cytokine production, and favoring leukemic persistence and metastatic potential [4, 5]. However, despite the protumor microenvironment created by LCs, studies have reported that a specific immune response can be triggered and, therefore, contribute to the defense against the tumor, although not sufficient enough to control the neoplasia [6].

Several studies have described the immunotherapeutic potential of CD8 and CD4 T cells that recognize peptide antigens (Ag) presented by polymorphic major histocompatibility complex (MHC) class I and MHC class II molecules, respectively [7, 8]. Leaving aside, the populations of T cells are considered "unconventional," which are also implicated in tumor immunity, although their role in them is not well understood. Collectively, these T cell populations differ from their conventional counterparts mainly in the way they recognize and respond to foreign molecules. Unlike MHC-reactive T cells, unconventional T cells generally show simplified patterns of the T cell antigen receptor (TCR) expression and usually target monomorphic Ag-presenting molecules and other ligands, where after their activation, they promote rapid and strong effector responses [9, 10]. These T cell populations include $\gamma\delta$ T cells, NKT cells, and MAIT cells.

In this review, we describe the main characteristics of these T cell populations and explore their activities during the neoplastic process, as well as their relationship with the establishment of an antitumor immune response or tumor-favorable response, as described briefly in Table 1. A better understanding of the participation of these underexplored cells in tumor dynamics may provide a basis for the development of potential immunotherapeutic strategies in the field of leukemias.

2. Subsets of Unconventional T Cells in Antileukemic Response

2.1. Gamma-Delta ($\gamma\delta$) T Cells. $\gamma\delta$ T cells are developed in the thymus during lymphopoiesis and, by the expression of the TCR (T-cell receptor), composed of gamma (γ) and delta (δ) chains [11]. These cells are capable of providing a potent and lasting immune response through innate and adaptive mechanisms and stand out for their recognition and destruction of several tumors, regardless of major histocompatibility complex (MHC) expression [12]. In addition, these cells constitute up to 10% of circulating $CD3^+$ cells and are classified according to the genetic rearrangements of the δ chain of the TCR into three main subsets: Vδ1, Vδ2, and Vδ3 (in humans) [13]. These lymphocytes are present in blood circulation, tissues, and mucosal membranes, which are strategic places to exercise their high cytotoxic power against infections and tumors [14].

$\gamma\delta$ T cell lineage is evolutionarily conserved and has several pleiotropic functions. While these cells are naturally specialized in the secretion of proinflammatory mediators (Figure 1) [15], these lymphocytes can also adopt Th2-, Th9-, and Th17-like response phenotypes [16–18]. In the tumor microenvironment (TME), these lymphocytes express a diverse repertoire of recognition receptors that have received favorable prognostic value in several malignancies, including leukemia [19]. Based on several reports, it has been confirmed that $\gamma\delta$ T cells are capable of triggering an immune response by direct and indirect mechanisms, for example, through the cytolytic synapse with the target cell, and the recruitment and stimulation of other immune cells necessary for the establishment of an antitumor response [12, 20].

These lymphocytes can induce neoplastic regression through cell-cell interaction or by secreting several soluble molecules (such as IFN-γ and TNF) that inhibit tumor expansion. These effector molecules induce an increase in the antitumor activity of other cytotoxic cells or positively regulate the expression of MHC-I by cancer cells [21]. In addition, $\gamma\delta$ T cells stimulate somatic hypermutation and isotype switching in B cells [22–24] and possibly induce antibody-mediated immunity. Their effector functions also include the activation of macrophages and the recruitment and activation of $CD8^+$ cytotoxic T cells and NK cells [25–28]. The immune role of these lymphocytes also includes stimulating the maturation of dendritic cells (DCs), and, in turn, DCs are able to potentiate their cytotoxic activity

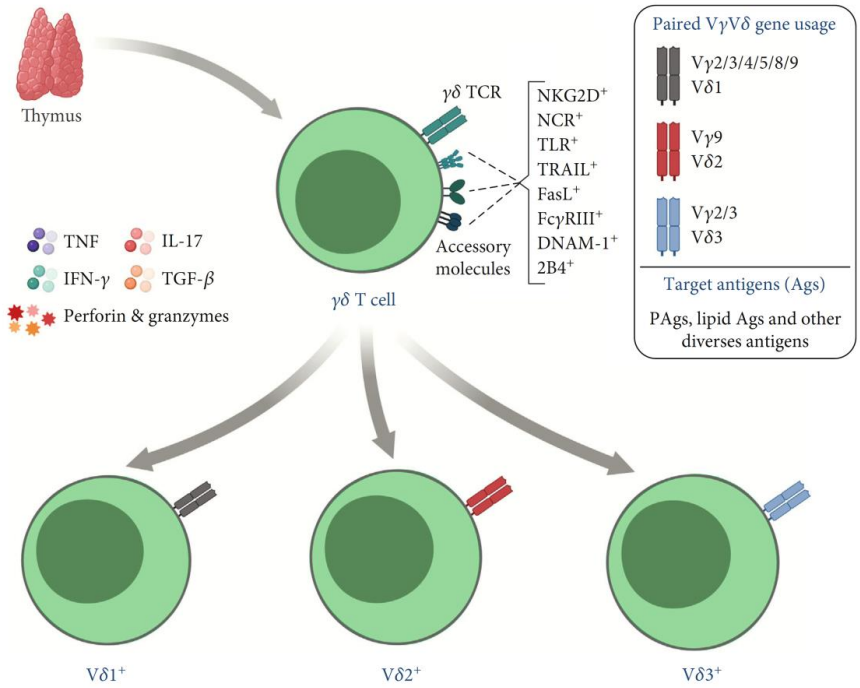


FIGURE 1: Overview of subpopulations, important receptors, and cytokines produced by γδ T cells. γδ T cells express some receptors that are essential for the tumor recognition and destruction, which gives a certain advantage when compared to other conventional T lymphocyte populations, either due to MHC independence or due to the high expression of the receptors mentioned in the image. The antileukemic recognition repertoire includes several molecules, such as NKG2D, NCRs, FasL, CD16, DNAM-1, and the TCR γδ itself.

[29, 30]. Notably, cancer cells tend to express several stress-induced molecules or metabolic antigens that are recognized through γδ TCRs and accessory receptors, thereby, mediating a potent response against the tumor [31–34].

Without restrictions on MHC expression, γδ T cells recognize several antigens that are expressed in LCs, and generally include metabolic molecules and stress-induced molecules [32, 35–38]. Ligands, such as MIC-A/MIC-B and ULBPs, can be identified through the NKG2D receptor, which is expressed mainly in γδ T cells [34, 39]. Furthermore, some metabolites of the mevalonate pathway, known as phosphoantigens (pAgs), can be recognized directly through the TCRs and are highly regulated in LCs [40, 41]. In addition, other molecules assist in tumor recognition and possibly support the antileukemic response of γδ T cells, such as TLRs (toll-like receptors), DNAM-1 (DNAX Accessory Molecule-1), FasL (Fas ligand), FcγRIII, TRAIL (TNF-related apoptosis-inducing ligand), NCRs (Natural Cytotoxicity Receptors) such as NKp30, NKp44, and NKp46, and the 2B4 receptor [12, 36, 41–44].

Vδ1$^+$ cells, which express TCR chains Vγ1 to Vγ11, respond preferentially in skin tissues, intestinal epithelium, lung, spleen, and liver, where they play crucial roles in maintaining epithelial tissue [14, 45]. It is known that these lymphocytes patrol the several tissues in search of stressed cells, derived from infections and tumorigenesis, thus, maintaining tissue homeostasis [46]. For this, the secretion of Th1 and Th17 cytokines is essential for immune surveillance [12, 47–49].

These cells, although uncommon in peripheral blood (~ 10% of blood γδ T cells), have a high diversity of tumor recognition and have demonstrated great potential against LCs [36, 50, 51]. Correia et al. demonstrated that Vδ1$^+$ cells that expressed NCRs managed to destroy lymphoid and myeloid cancer cells through NKp30 and NKp44, which seemed to recognize antigens that are distinct from their classic ligands, such as the molecule B7-H6, which binds to NKp30 [36]. In the same study, the stable expression of these NCRs was associated with elevated levels of granzyme B and seemed to synergize greater cytotoxic activity against LCs [36]. Taking into account that in some leukemias, a high expression of members of the ULBP family is observed, as NKG2D ligands [52], Lança et al. demonstrated that the expression of ULBP1 in LCs is important for recognition by Vδ1 cells [51]. ULBP3 was confirmed by Poggi et al. who presented similar findings [52]. Therefore, the data suggest possible immunological participation of these cells and indicate a significant contribution to the antileukemic immune response.

These cells can also recognize lipid or metabolic antigens that are presented through MHC class I-like molecules, such as CD1 and MR1 [46, 53]. These and other characteristics of Vδ1 cells make them potential candidates for new immunotherapeutic approaches in several human tumors, and these

have recently been explored in several experimental trials, including some against leukemia [54–56]. Although their roles are still poorly known, we know that Vδ1 cells demonstrate important functions in antitumor activity, and these deserve to be highlighted since they present characteristics that are different to Vγ9Vδ2 cells, either due to their high expression of NCRs or the nonsusceptibility to activation-induced cell death (AICD) [57–59]. The roles of this subset of $\gamma\delta$ T cells in the environment of bone marrow and peripheral blood *in vivo*, in the context of leukemia, still need further investigation regarding the ligands and receptors of recognition that are engaged during the immune response.

The V$\delta2^+$ subset, which pairs exclusively with the Vγ9 TCR chain, responds mainly in the blood, where it recognizes pAgs derived from bacteria and cancer cells [60]. Once activated, these lymphocytes secrete effector molecules such as IFN-γ, TNF, perforins, and granzymes and exert important cytotoxic activities in peripheral blood against pathogens and tumors [12, 61]. These cells make up as much as 95% of blood $\gamma\delta$ T cells [50, 62–64] and generally respond to a wide variety of pAgs, such as IPP (isopentenyl pyrophosphate) and HMB-PP (4-hydroxy-3-methyl-but-2-enylpyrophosphate), which are intermediates of the mevalonate pathway in eukaryotes and prokaryotes [65–67]. The recognition of these pAgs occurs in the context of the butyrophilin (BTN) family of molecules, such as BTN3A1 and BTN2A1 [68–71], which can be detected in LCs, and mediates a potent immune response that can be used therapeutically [72]. These molecules can be recognized directly through the $\gamma\delta$ TCR and are capable of triggering a Th1-like response against the target cells [73, 74].

The mechanism for recognizing pAgs is not yet clear, although several studies have recently expanded the information about how $\gamma\delta$ T cells identify these molecules. Recent reports have pointed out that pAgs recognition is mediated by BTN-like molecules, which are expressed in cancer cells [72, 75] and are able to modulate the responses of conventional $\alpha\beta$ T cells [76–79], and most notably, of $\gamma\delta$ T cells [68–71, 80, 81]. The dependent detection of pAgs by Vγ9Vδ2 cells involves the entire structure of the TCR, which interacts with BTN molecules through the Vγ9 and Vδ2 TCR domains. Among the various molecules that make up the BTN family, the proteins BTN3A1 and BTN2A1 synergize the presentation of pAgs to $\gamma\delta$ T cells, binding directly to the TCR Vγ9Vδ2 [68, 70, 82, 83].

Previously, it was thought that the unit expression of BTN3A1 performed the activation of these lymphocytes alone [84], but it is now clear that the BTN2A1 protein acts as a critical factor in the activation of Vδ2 cells [70]. For this to occur, it is necessary that pAgs bind to the intracellular domain (B30.2) of these proteins [85, 86]. After binding of pAgs, the intra- and extracellular domains of BTN3A1 and BTN2A1 undergo a conformational change [85–87] that allows the contact of the TCR Vγ9 chain with the BTN2A1 molecule, sending activation signals to $\gamma\delta$ T cells [68]. In addition, the involvement of other molecules during the pAgs detection mechanism cannot be ignored, as recent reports suggest the molecular collaboration of CDR3, periplakin, and GTPase RhoB in this process [70, 88, 89].

In addition to direct and TCR-dependent recognition, other accessory molecules possibly support the antileukemic activity of Vδ2 cells against LCs, such as the DNAM-1 receptor that recognizes the PVR (poliovirus receptor) and Nectin-2 ligands, both expressed in LCs [41], and ULBP4 that can be recognized through NKG2D [90]. $\gamma\delta$ T cells also express the 2B4 receptor (which recognizes the CD48 ligand), an accessory molecule that strengthens target effector interactions and is possibly related to increased cytotoxic activity against cancer cells [44]. One disadvantage of these V$\delta2^+$ cells is their strong propensity for AICD upon prolonged exposure to antigens and the polarization of these lymphocytes towards a tumor-promoting phenotype, which limits the persistence and efficiency of the immune response [57, 59].

Little data is available regarding the receptors and ligands involved in the events of innate and adaptive immunity mediated by V$\delta3^+$ cells, which express the TCR chains Vγ2, Vγ3, or Vγ8 and are a specific subset that responds mainly in the liver [91, 92]. The frequency of this cell population is low in peripheral blood (~0.2% of total circulating T cells) though it is high in the liver and intestinal region [91, 92]. Studies have reported that V$\delta3^+$ cells are related to antiviral immunity, responding efficiently against cytomegalovirus [93], Epstein-Barr virus [94], hepatitis [95], and HIV infection [64]. So far, their roles in antitumor immunity are not clear, although some reports have identified an expansion of these lymphocytes in the peripheral blood of patients with leukemia [96]. Also, Vδ3 cells secrete IFN-γ, express NKG2D, FcγRIII, and CD161, and appear to respond in a restricted way to CD1d, functionally resembling iNKT cells, which recognize and destroy CD1d$^+$ target cells [24, 91]. Subsequently, it was identified that these cells also respond to ANX-2 (annexin-2) [97], but their role in leukemia is still unexplored, although it has been identified that these cells expand during tumor progression [96].

$\gamma\delta$ T cells have an enormous potential to regulate local immunity and remodel the tumor niche [45, 98, 99]. The evidence discussed so far suggests that these cells realize various antitumor activities through the direct identification of LCs through the $\gamma\delta$ TCR or accessory receptors and by secreting soluble effector molecules against the tumor. Despite this, emerging knowledge about the molecular and cellular interaction between these lymphocytes and cancer cells suggests that, like conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells may possibly not be exempt from the immunosuppression established by the TME [100–103]. However, the lack of knowledge about these cells in the leukemic microenvironment makes it difficult to elaborate larger and more comprehensive discussions about the possible crosstalk between this population of lymphocytes and the LCs in the bone marrow compartment and in extramedullary sites.

In addition, other subsets of $\gamma\delta$ T cells that express other variable TCR chains (TCR domains Vδ5, Vδ6, and Vδ8) have been identified in other hematological malignancies, where, for example, these cells seemed to expand and respond to cancer cells in the blood of patients with lymphoma [104]. However, it is still unclear whether these cells respond against LCs and not have information available on the possible roles played by these $\gamma\delta$ T cell subsets in

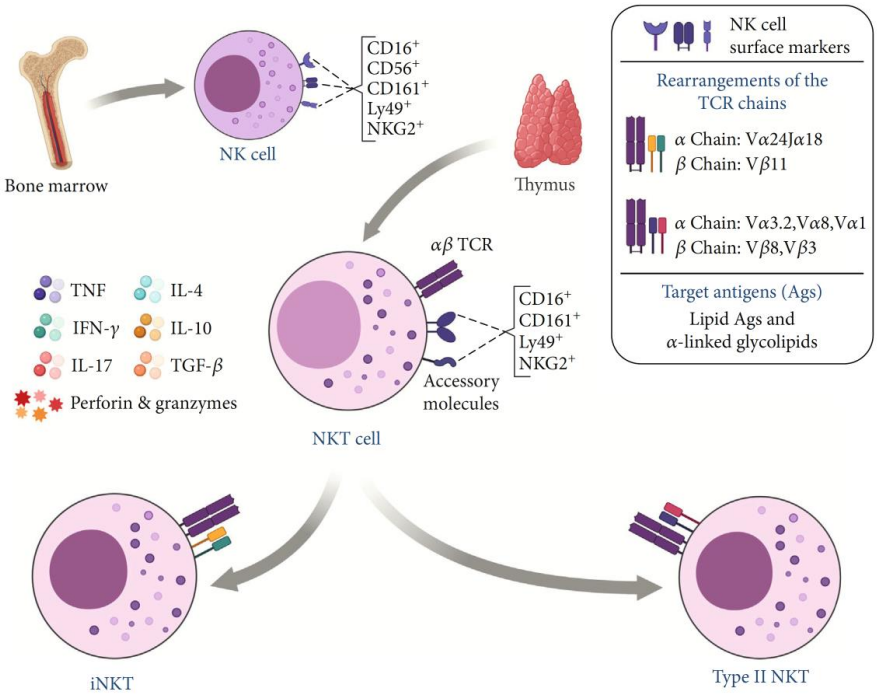


FIGURE 2: Overview of subpopulations, important receptors, and cytokines produced by NKT cells. Unlike conventional NK cells that mature in the bone marrow, NKT cells develop in the thymus and acquire an invariant or variant TCR, through which their subgroups are stratified. Recent studies show that these lymphocytes participate in the antitumor immune response by recognizing CD1d+ tumors and secreting Th1, Th2, and Th17 profile cytokines.

leukemia antitumor immunity. In view of its enormous therapeutic applicability, further research is needed on the interaction of $\gamma\delta$ T cells and their subsets in the leukemic microenvironment and on how this can impact the prognosis of patients.

2.2. Natural Killer T (NKT) Cells. Natural killer T cells (NKT) correspond to a population of innate-like T cells characterized by the expression of a TCR composed of alpha (α) and beta (β) chains similar to those of conventional T cells, in addition to specific surface markers of natural killer cells (NK), such as $CD16^+$, $CD56^+$, $CD69^+$, $CD161^+$, $NKG2D^+$, and $Ly49A^+$ [105, 106]. Another striking feature of these cells is their restriction to the CD1d molecule, which is an MHC class I-like molecule that presents lipid and glycolipid antigens [107–109].

NKT cells constitute approximately 0.001-1% of circulating lymphocytes (in humans) and are also present in the thymus, liver, intestine, and spleen [110, 111]. These cells are divided into two main subsets, the invariant natural killer T (iNKT) and natural killer T type II (NKTII) cells [112]. Both express the following transcription factors: T-bet [113, 114], PLZF [115], RORγt, GATA-3 [114], and NF-kB [116], which together grant high cellular plasticity and allow polarization for phenotypes of profile Th1, Th2, and Th17 [116–118]. In addition, iNKTs express the LEF-1 factor [119, 120], which is correlated with the regulation of the expression of the gene that encodes the CD1d molecule in antigen presenting cells (APCs). The LEF-1 factor also plays a crucial role as a regulator of the Wnt pathway, and it is possible that it influences the growth, development, differentiation, and functions of NKT cells [121].

In regards to their antitumor activity, NKT cells can act directly through cell-cell interaction, through the Fas receptor and its ligand (FasL) that trigger the activation of caspase enzymes and cause apoptosis of the target cells, in addition to the interaction of other receptors such as NKG2D, TRAIL, natural cytotoxicity receptors (NCRs), and their ligands (Figure 2) [111, 113, 122]. There is also the substantial release of perforins and granzymes A and B, in addition to the production of Granulysin that act in a similar way to perforins, forming pores in the plasma membrane and altering their permeability, which results in cell lysis [123, 124].

NKT cells also can act through indirect mechanisms by releasing a range of mediators, especially Th1 or Th2 profile cytokines, which can vary depending on the NKT subtype, a fact that will be discussed later [125]. The release of the aforementioned mediators can results in the immunoregulation of other cells of the immune system, for example, activating or inducing the maturation of DCs through interaction with CD1d or CD40/CD40L and IFN, respectively. After activation of DCs, they will be regulated positively, expressing costimulatory molecules such as CD86 and CD80, in addition to release cytokines such as IL-12, a

pleiotropic cytokine that plays an essential role in Th1-type immune response against cancer. iNKT cells also activate CD8 cytotoxic T cells through the IFN, as well as conventional NK cells and macrophages that can act against cancer cells [125–128]. In addition, NKT cells can also stimulate B lymphocytes and induce increased secretion of IgG class antibodies, which can result in antibody-dependent cell cytotoxicity (ADCC) in cancer cells that will subsequently undergo cytotoxic cell-mediated cell lysis [111, 129].

Due to their secretory repertoire and the ability to activate and target other cells of the immune system, NKT cells contribute strongly to tumor immunovigilance. It is important to highlight that one of the main forms of recognition of cancer cells by NKT cells occurs through the interaction of the TCR with the antigen presented by the CD1d molecule, expressed by B cells, macrophages, DCs, and several types of cancer cells, in solid and hematological neoplasms [116, 117]. These cells have been shown to contribute to tumor surveillance and suppression, controlling the initial stage of the neoplastic process [130–133].

iNKT cells, also known as natural killer type I cells, have characteristics that differ from other subsets, such as reactivity to ∝-galactosylceramide (α-GalCer) and the more pronounced expression of the Th1 response profile [134]. Another characteristic of iNKT cells is related to its invariant TCR, represented in humans by a TCR-α chain (Vα24Jα18) and a TCR-β chain (Vβ11). iNKT cells recognize lipid antigens and glycolipids such as α-GalCer analogs, phospholipids, diacylglycerols, gangliosides (GD3), and glycosphingolipids [107, 112].

Studies show that iNKT cells are strongly reactive to α-GalCer, a synthetic glycolipid derived from the marine sponge *Agelas mauritianus*, which is capable of inducing an important immunomodulatory effect in iNKT cells and which stimulates the antitumor response mediated by cytotoxic cells (NK cells and TCD8$^+$ lymphocytes) that are in a state of exhaustion or anergy resulting from the TME. Stimulation mediated by iNKT cells has been shown to promote the invigoration of cytotoxic cells and reverse the dysfunction presented by these cells [107, 135].

The α-GalCer antigen presented through the CD1d molecule is recognized by the TCR expressed in iNKT cells and induces the production of cytokines, such as IL-2, IL-12, and IL-21, which act on conventional NK cells and exhausted or anergic CD8 cytotoxic T cells, thus, reversing the dysfunction and the hyporesponsive character [125, 136]. In addition, α-GalCer-stimulated iNKT cells have been shown to incite the activation of APCs via CD40-CD40L signaling and also induce the production of IL-12 [125, 126, 129, 137].

A study by Fais et al., in patients with acute leukemia, evaluated the expression of the CD1d molecule in LCs, as well as the activity of iNKT cells in their recognition. Where, through incubating LCs with 100 ng/ml of α-GalCer for 4 hours, it was observed that the CD1d$^+$ LCs associated with the α-GalCer antigen underwent apoptosis induced by iNKT cells, in addition to stimulating the production of cytokines INF-γ, TNF-α, and IL-5 [138]. Similar results were observed by Bojarska-Junak et al. in a study with BP samples from patients with chronic leukemia, in which iNKT cells stimulated *in vitro* for 24 hours with 100 ng/ml of α-GalCer showed greater intracellular expression of the INF-γ and IL-4 cytokines when compared with healthy volunteers. The study also pointed out that, among the two cytokines analyzed, the expression of IL-4 was greater than that of INF-γ, indicating that the iNKT cell may acquire a polarization for the Th2 response profile [139].

Another study showed that the frequency of iNKT cells was lower in patients with chronic leukemia compared to the healthy control group and that therapy for 14 days with IFN-γ and 1 μM IM (imatinib mesylate) in combination with 100 ng/ml of α-GalCer in these patients resulted in a significant increase in iNKT cells [140]. Similarly, Weinkove et al. demonstrated that LCs purified and stimulated in vitro with 200 ng/ml of α-GalCer for 5 days incited the proliferation of autologous and allogeneic iNKT cells, but not in a significant amount it was also demonstrated that there was a stimulation of IFN-γ production by iNKT cells in leukemic patients. However, it was observed that the prolonged culture of these cells resulted in the polarization of iNKT cells to a Th2 profile and resulted in high levels of cytokines associated with tumor tolerance [141]. Additional investigations should be carried out to identify how stimulation with α-GalCer can provide better results, associating the increased frequency of iNKT cells with polarization for a response profile against LCs.

Finally, in a longitudinal analysis carried out over 18 months with 22 patients that had been diagnosed with leukemia or myelodysplasia and who underwent HLA haploidentical stem cell transplantation for iNKT cell reconstitution, the existence of a correlation between the frequency of iNKT cells and disease remission was reported. The observed data showed that 14 patients whose iNKT cells were completely reconstituted showed remission, and 8 patients whose iNKT cells were not reconstituted showed recurrence, thus, associating the frequency of these cells with a better prognosis [142, 143]. Furthermore, the analysis of the frequency of iNKT cells in the circulation and bone marrow compartment of newly diagnosed patients with acute leukemia demonstrated that a low frequency of these cells is associated with a worse prognosis [144].

Unlike iNKT cells, NKT II cells have an $\alpha\beta$ TCR variant and form diversified rearrangements. They are not reactive to α-GalCer [112, 125, 145]; however, they are restricted to CD1d [146] and recognize antigens such as β-glucosylceramide (β-GlcCer) and sulfatide, the latter being found in the plasma membrane of myelin, in the central nervous system, in the liver, in the pancreas, and in the kidneys [147–149]. Another characteristic of the NKT II cell is related to its response profile, which is more polarized for the Th2 profile [134, 150].

In contrast to studies on iNKT cells, the number of studies on the role of NKT II cells in leukemia is limited. However, some studies report that NKT II can act in a negative way by contributing to the suppression of surveillance and antitumor activity and, in some situations, contributing to neoplastic progression [111, 112, 151]. Such immunosuppression is mediated through the secretion of IL-13, which activates myeloid-derived suppressor cells (MDSCs), and these, consequently, suppress the activity of tumor-infiltrating

cytotoxic cells [150]. It is also believed that this negative regulation of NKT II in the tumor occurs due to a cross-regulation between iNKT and NKT II, where the effect of Th2 cytokines produced by NKT II cells overlap the effect by the Th1 cytokines produced by iNKT, resulting in an immunosuppressed microenvironment. In addition, it has been observed that tumors grow faster when the frequency of NKT II cells is higher than that of iNKT cells, although this suppression mechanism still needs further investigation [111, 134].

It is important to note that, in addition to NKT cells, there are other populations of nonconventional T cells restricted to molecules of the CD1 family, more specifically group 1, composed of CD1a, CD1b, and CD1c, which have lipid antigens or glycolipids, of microbial origin or from the organism itself [152]. Similar to NKT cells, these CD1-restricted cell populations are considered an attractive target for studies in the field of cancer immunotherapy, especially in the context of hematological neoplasms, due to the diversified distribution of CD1 isotypes, where they have been shown to be expressed (CD1a, CD1b, and CD1c) in 75% of acute leukemia blasts [153].

2.3. Mucosal-Associated Invariant T (MAIT) Cells. MAIT cells correspond to a population of innate-like T cells and are characterized by the expression of a restricted TCR-α with a unique gene rearrangement pattern, namely, TRAV1-2-TRAJ33 / 12/20 (Vα7.2-Jα33 / 12/20 in humans), which pair with a limited repertoire of the TCR-β chain, predominantly from the TRBV6 and TRBV20 gene families [154–156], and form a semi-invariant TCR restricted to nonpeptide antigens presented by MHC-related protein 1 (MR1). MR1 is a monomorphic molecule that is highly conserved throughout the evolution of mammals [157, 158] and is capable of presenting metabolites derived from vitamin B2 that is synthesized by a variety of microorganisms for MAIT cells [159, 160].

Like most T cells, MAIT cells develop in the thymus [161] and are positively selected by the cortical thymocytes $CD4^+$ $CD8^+$ $MR1^+$ [161, 162]. After the selection process, they undergo extrathymic maturation and integrate with different tissues [163]. In humans, MAIT cells represent up to 10% of peripheral blood T cells and are found in abundance in mucous tissues, mesenteric lymph nodes, and liver, where they can represent up to 45% of all T cells [164, 165].

Human MAIT cells can be immunophenotyped as $CD3^+$ Vα7.2$^+$ $CD161^{HI}$ [166] and can be categorized based on the expression of CD4 and CD8 coreceptors in five subsets: $CD4^+$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^-$, $CD4^-$ CD8$\alpha\alpha^+$ e $CD4^-$ CD8$\alpha\beta^+$ (Figure 3); the last two ($CD4^-$ CD8$\alpha\alpha^+$ and $CD4^-$ CD8$\alpha\beta^+$) being the most abundant and collectively correspond to approximately 80% of MAIT cells [164]. Of note, the development of MR1 tetramers loaded with 5-OP-RU [5-(2-oxopropylideneamino)-6-d-ribitylaminouracil] marked a breakthrough in MAIT cell research and have allowed for the reliable identification of distinct phenotypical and functional MAIT cell subsets [167].

In addition to the surface molecules mentioned above, MAIT cells also express CD25, CD26, CD44, and CD69, as well as IL-7R, IL-12R, IL-15R, and IL-18R cytokine receptors and PLZF, T transcription factors T-bet and RORγt, providing high plasticity and the ability to secrete mediators of Th1 profile and/or Th17 profile [168]. It is important to note that most MAIT cells have a memory-like phenotype effector [169] and can be quickly activated by mechanisms that do not depend on TCR stimulation [170]. This is due to the high expression of various cytokine and chemokine receptors, a property they share with other innate-like T cells ($\gamma\delta$ and iNKT cells) [171].

After activation, these cells are able to release substantial quantities of perforin and granzymes, in addition to producing the proinflammatory cytokines TNF, IFNγ, and IL-17A, as well as CCL3 and CCL4, which are chemokines that are crucial for immune responses to infection and inflammation [172, 173]. The release of these mediators results in the destruction of infected cells and the activation of other immune cells, such as DCs, which consequently leads to the mobilization of conventional T cells and reflects in a cascade of immunological events [174, 175].

Due to some characteristics of MAIT cells, such as their high frequency in humans and their ability to rapidly secrete a repertoire of mediators that induces activation and regulation of other cells of the immune system, in addition to the ability to recognize antigens restricted to MR1, research on MAIT cells, as well as other populations of T cells restricted to MR1, has aroused great interest in the field of oncology. Currently, the functional role triggered by these innate-like T cells in cancer is unclear and has been the subject of several studies.

Investigations performed on patients with mucosal neoplasms have reported a reduction in the frequency of circulating MAIT cells and show a significant accumulation of these cells in the tumor tissue [176–178]. However, it is important to note that tumor-infiltrating MAIT cells were less able to produce IFN-γ in response to factors secreted by the TME [178]. In addition, a recent *in vivo* study by Yan et al. showed that MAIT cells exhibited a tumor-promoting function and promoted cancer metastasis through the suppression of cytotoxic cells (which was partly IL-17 dependent) after interaction with MR1 molecules expressed on cancer cells [179].

In contrast, in the study by Won et al., tumor-infiltrating MAIT cells were not compromised in the production of cytokines IFN-y, TNF-α, and IL-17. In addition, in the *in vitro* assay, it was observed that MAIT cells, isolated from PB from healthy individuals, not only had lymphokine-activated killer activity but also exhibited direct cytotoxicity in K562 cell lines through the degranulation of granzyme B and perforin [177].

To date, the involvement of MAIT cells in leukemia remains largely unexplored, and, in the context of other hematological neoplasms, the functional role of these cells is also limited. An *in vitro* study by Gherardin et al. demonstrated that multiple myeloma (MM) cell lines express the MR1 protein and are capable of presenting vitamin B metabolites to MAIT cells isolated from healthy donors, which, in response, induce the lysis of these myeloma cells with efficiency and kinetics similar to NK cells [164].

Another study observed the functional capacity of these lymphocytes by analyzing the cell phenotype in samples of

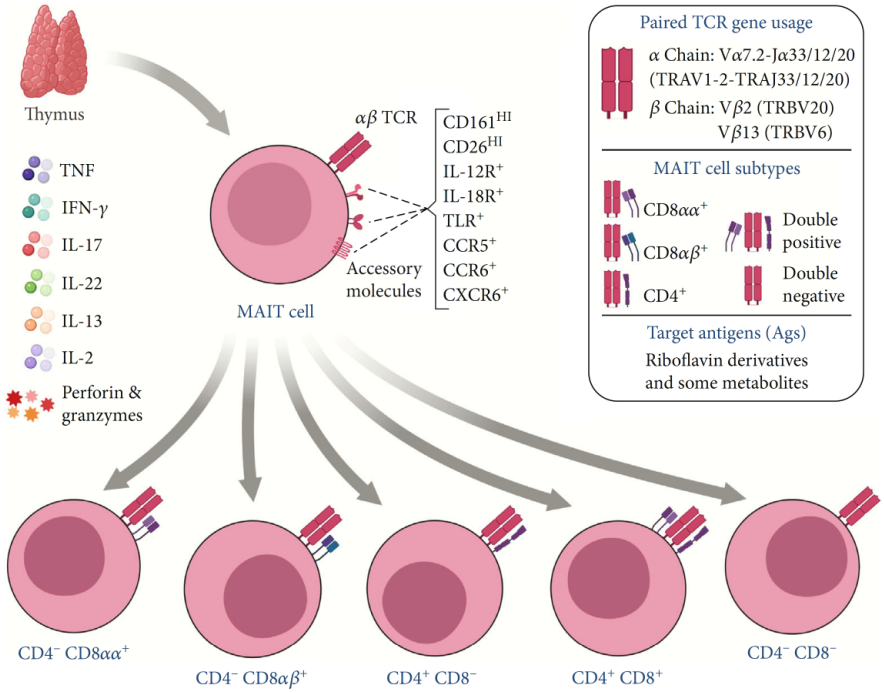


FIGURE 3: Overview of subpopulations, important receptors, and cytokines produced by MAIT cells. MAIT cells develop in the thymus, where they acquire a semi-invariant TCR, restricted to MR1. In humans, they can be categorized into five subsets based on the expression of the CD4 and CD8 coreceptors, with $CD4^-$ $CD8\alpha\alpha^+$ or $CD4^-$ $CD8\alpha\beta^+$ being the most abundant, collectively corresponding to approximately 80% of MAIT cells. They produce a repertoire of Th1 and Th17 cytokines (IFN-γ, TNF, IL-2, IL-17A, and IL-22), in addition to perforins and granzymes B, and express various cytokine, chemokine, and homing molecules.

PB and BM [180]. Favreau et al. reported that patients recently diagnosed with MM had a significant decrease in the frequency of MAIT cells when compared to healthy controls. They also highlighted a reduction in the MAIT $CD8^+$ and double-negative ($CD8^-$ $CD4^-$) phenotype, in addition to the functional impairment of the Th1 response profile, with fewer IFN-γ and TNFα producing MAIT cells [180]. Favreau et al. also demonstrated that circulating MAIT cells exhibited high levels of PD-1 and that their respective *in vitro* blockade resulted in the restoration of MAIT cell function and activation [180]. As a whole, these results highlight the important antitumor activity of MAIT cells and identify them as a potential immunotherapeutic target in MM.

Likewise, MR1 also represents an attractive target in immunotherapy against cancer, due to characteristics such as its monomorphic nature and functional expression found in several types of cancer cells [181]. In this regard, a recent study by Crowther et al. demonstrated that a human T cell clone potentially recognizes a specific cancer or associated metabolite, restricted to MR1, and mediates the lysis of different types of cancer cells, including LCs lineages, as such, it mediated *in vivo* leukemia regression and conferred longer survival in mice [182]. Moreover, other atypical MR1-restricted T cells, which respond to autoantigens, have been described [181, 183, 184]. This new group of T cells has been named MR1T and has been shown to recognize and eliminate a wide range of cancer cells that express MR1 [181]. However, more research is needed to elucidate the precise nature and function of these cells, as well as their activity in the context of TME.

3. Concluding Remarks and Perspectives

Unconventional T cells can promote tumor rejection and offer advantages that indicate them as being potential targets for T-cell-based immunotherapy. Although effective, it is important to note that unconventional T cells are not exempt from the influence of checkpoint receptors, since these cells positively regulate the inhibitory PD1 receptor on their cell surface after activation [185–187]. However, checkpoint blockade therapy using drugs based on anti-PD1 and anti-CTLA-4 is proving to be a powerful approach for preventing effector cells from entering into a state of anergy caused by cancer cells, thereby, providing a persistent immune response [188].

In the context of hematologic neoplasms, among the promising alternatives to conventional chemotherapy are the upcoming immunotherapies, in particular, the transfer of chimeric antigen receptor (CAR) T cells [189]. These autologous T cells, which are designed to express a CAR receptor against the CD19 antigen, are at the forefront of contemporary oncohematological therapies and lead to high

rates of remission in B cell malignancies [190, 191]. It is important to highlight the obstacles, such as the complex and expensive individualized manufacturing process and the loss or modulation of the target CD19 antigen, that lead to resistance and relapse after therapy with CAR T cells.

In this scenario, unconventional T cells present themselves as possible solutions to overcome these obstacles, since they have a series of specific biological characteristics that can significantly expand and diversify the repertoire of CAR-based therapies, although they also have their limitations [192]. Few preclinical studies have investigated unconventional T cells as alternative platforms for CAR engineering, and, given this limitation, it is not surprising that there is a very low number of clinical trials that evaluate the viability of CAR $\gamma\delta$ T therapies (B cell lymphoma, NCT02656147; and acute myeloid leukemia, NCT03885076) and CAR NKT (refractory B cell neoplasm, NCT03774654) in hematologic neoplasms that are in progress.

The fact is that the role of unconventional T cells during the neoplastic process is usually related to better immune surveillance or antitumor response in patients with leukemia, and that, to date, unconventional T cells are still largely underexplored. Factors, such as the absence of barriers related to histocompatibility, since the molecules that present Ag for unconventional T cells are monomorphic; activation by TCR dependent and independent mechanisms; ability to gather quick and powerful responses; in addition to the high frequency in specific tissues in humans, all demonstrate the need for further studies on the immunotherapeutic potential of these cells and, mainly, the translation of these studies into clinical trials.

Abbreviations

AICD: Activation-induced cell death
ALL: Acute lymphoblastic leukemia
AML: Acute myeloid leukemia
ANX-2: Annexin-2
APCs: Antigen-presenting cells
BM: Bone marrow
BTN: Butyrophilin
BTN2A1: Butyrophilin subfamily 2 member A1
BTN3A: Butyrophilin 3A
CAR: Chimeric antigen receptor
CD: Cluster of differentiation
CDR3: Complementarity-determining region–3
CTLA-4: Cytotoxic T-lymphocyte–associated antigen 4
CLL: Chronic lymphocytic leukemia
CML: Chronic myeloid leukemia
DCs: Dendritic cells
DNAM-1: DNAX accessory molecule-1
Fas: Fas Cell Surface Death Receptor
FasL: Fas ligand
FcγRIII: Fc-gamma receptor type III
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HIV: Human immunodeficiency virus
HMB-PP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
IFN: Interferon
IL: Interleukin
iNKT: Invariant NKT
IPP: *Isopentenyl pyrophosphate*
LCs: Leukemic cells
MAIT: Mucosal-associated invariant T
MHC: Major histocompatibility complex
MIC-A: MHC class I polypeptide-related sequence A
MIC-B: MHC class I polypeptide-related sequence B
MM: Multiple myeloma
MR1: MHC class I-related protein
NCRs: Natural cytotoxicity receptors
NK: Natural killer
NKG2D: Natural-killer group 2 member D
NKT: Natural killer T
PB: Peripheral blood
PD1: Programmed cell death protein 1
pAgs: Phosphoantigens
PVR: Poliovirus receptor
TCR: T-cell receptor
TGF: Transforming growth factor
TLRs: Toll-like receptors
TME: Tumor microenvironment
TNF: Tumor necrosis factor
TRAIL: TNF-related apoptosis-inducing ligand
ULBP: UL16-binding protein
α-GalCer: α-Galactosylceramide
$\gamma\delta$ T: Gamma-delta T.

Additional Points

Cell-mediated immune response in malignant neoplasms is becoming the focus in immunotherapy against cancer. Unconventional T cells utilize a range of receptors and proinflammatory or regulatory profiles that directly contribute to the immune response in the leukemic microenvironment. The role played by unconventional T cells found in the leukemic medullary microenvironment may be related to the patient's prognosis. Cytokines and chemokines secreted by unconventional T cells include IL-1β, IL-4, IL-6, IL-10, IL-13, IL-17, IL-22, IL-23, IFN-γ, TNF-α, and TGF-β.

Disclosure

The funders had no role in study design, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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CAPÍTULO II

Publicação – Artigo 2 de revisão

γδ T Cells for Leukemia Immunotherapy: New and Expanding Trends Translating Unconventional T cells and their roles in Leukemia Antitumor Immunity, January 2021, **Frontiers of Immunology.** 21 Sep 22; 12:729085. DOI**:** 10.3389/fimmu.2021.729085.

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γδ T Cells for Leukemia Immunotherapy: New and Expanding Trends

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Recently, many discoveries have elucidated the cellular and molecular diversity in the leukemic microenvironment and improved our knowledge regarding their complex nature. This has allowed the development of new therapeutic strategies against leukemia. Advances in biotechnology and the current understanding of T cell-engineering have led to new approaches in this fight, thus improving cell-mediated immune response against cancer. However, most of the investigations focus only on conventional cytotoxic cells, while ignoring the potential of unconventional T cells that until now have been little studied. γδ T cells are a unique lymphocyte subpopulation that has an extensive repertoire of tumor sensing and may have new immunotherapeutic applications in a wide range of tumors. The ability to respond regardless of human leukocyte antigen (HLA) expression, the secretion of antitumor mediators and high functional plasticity are hallmarks of γδ T cells, and are ones that make them a promising alternative in the field of cell therapy. Despite this situation, in particular cases, the leukemic microenvironment can adopt strategies to circumvent the antitumor response of these lymphocytes, causing their exhaustion or polarization to a tumor-promoting phenotype. Intervening in this crosstalk can improve their capabilities and clinical applications and can make them key components in new therapeutic antileukemic approaches. In this review, we highlight several characteristics of γδ T cells and their interactions in leukemia. Furthermore, we explore strategies for maximizing their antitumor functions, aiming to illustrate the findings destined for a better mobilization of γδ T cells against the tumor. Finally, we outline our perspectives on their therapeutic applicability and indicate outstanding issues for future basic and clinical leukemia research, in the hope of contributing to the advancement of studies on γδ T cells in cancer immunotherapy.

Keywords: gamma-delta T cells, leukemic microenvironment, off-the-shelf cell therapy, clinical trials, cell transplantation

INTRODUCTION

The leukemic microenvironment is composed of a complex and distinct network of factors that strongly support the growth and clonal dissemination of leukemic cells (LCs), thus impacting the patient's clinical outcome (1–4). In this context, whereas conventional T cells ($CD4^+$ or $CD8^+$) and natural killer cells (NK) have been reported as "cytotoxicity mediators" capable of inducing tumor regression *in vivo* and controlling leukemic proliferation, several reports pointed to the fact that other T cells considered "unconventional" also have a high potential for coordinating the immune system and play complex and promising roles in cancer immunity (5–9). These antitumor responses are generally mediated by individual molecules with high or low diversity, such as the alpha-beta (αβ) or gamma-delta (γδ) T cell receptor (TCR) (10, 11).

In contrast to the αβ TCR, which is highly reactive to polymorphic molecules of the major histocompatibility complex (MHC), γδ TCR-expressing T cells perform their functions through recognition of antigens (Ags) presented by several monomorphic molecules, which in turn, promote a strong, rapid and effective response (12–14). In addition to being evolutionarily conserved, γδ T cells are important effectors, since they link innate and adaptive immune responses (11, 15, 16), and are highlighted as promising targets in cancer immunotherapy, especially for leukemias. These hematological malignancies are highly heterogeneous and are defined based on blast count, maturation stage and flow cytometry immunophenotyping, which allows them to be generally classified in acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (4, 17–23).

A potential therapy against these malignancies may depend on the mobilization and targeting of effector immune cells capable of producing antitumor factors and effectively killing LCs in different compartments with the absence of toxicity or alloreactivity. In this context, γδ T cells have unique attributes that support the promising development of an off-the-shelf cell therapy, as these lymphocytes provide a lasting and efficient response through mechanisms that include a higher cytotoxicity, functional plasticity, the production of several soluble molecules and responsiveness independent of MHC/HLA expression (24, 25). Although the tumor microenvironment (TME) and the adjacent LCs may develop several strategies to escape from γδ T cell-mediated immune surveillance, *ex vivo* or *in vivo* activation, the expansion and the genetic modification of these lymphocytes may increase their antileukemic reactivity and overcome suppression and resistance established by the TME (1, 26).

There is emerging evidence that γδ T cells exhibit persistent antitumor responses in different compartments in patients with leukemia and preserve healthy tissues; however, the adjacent mechanisms are still poorly understood (27–31). Therefore, γδ T cells are being translated into several clinical and therapeutic strategies targeting these hematological malignancies. Herein, we integrate the current knowledge regarding the diversity of γδ T cells and their associated potential in leukemia immune surveillance. Several approaches to improve their antitumor functions allow effective targeting against LCs and, therefore, will be discussed here. Finally, we emphasize open questions about γδ T cells and their subtypes, and also highlight their therapeutic applicability against leukemia. A better understanding of the functional relevance of γδ T cells in these malignancies has important implications, as we may be close to the unprecedented ascension of T cell-based therapies and their positioning as key-components for improving immunotherapy against cancer.

UNTANGLING THE RIDDLE OF γδ T CELL DIVERSITY

γδ T cells make up a lymphoid lineage that has relevant functions in tissues and blood circulation. Their development is regulated in the thymus, where they undergo maturation in different stages of thymic ontogeny (32–34). In this process, genetic rearrangements define the compromise and differentiation of double-negative thymocytes ($CD4^-$ and $CD8^-$) for the T cell lineage-expressing γδ TCR (35–38). Subsequently, these cells migrate to peripheral blood (PB) and mucosal tissues, where they play key roles in the host's immunity as primary effectors in the response against infections and cancer (15, 39, 40), preceding the responses of the αβ T cell lineage (41).

Currently, four major subtypes of human γδ T cells have been documented, which are defined by the TCR δ chain (i.e., Vδ1, Vδ2, Vδ3 and Vδ5) according to the Lefranc & Rabbits's system nomenclature (42). Vδ1 and Vδ2 subtypes are the most predominant (11, 43–45). Vδ3 cells make up the majority of $Vδ1^-/Vδ2^-$ subtypes and are rarely found in PB, although they are found in large numbers in the liver (46). Vδ5 cells also can be found in PB or tissues, but their functions are not entirely clear (43, 47–50). Here, we will focus on Vδ1, Vγ9Vδ2 and Vδ3 cells that are primarily thought to be involved in antileukemic responses.

Overall, γδ T cells constitute up to 10% of circulating $CD3^+$ cells, though predominate among all tissue-resident T cells (51–54). Vδ1 and Vγ9Vδ2 subtypes represent ~10% and 90% of blood γδ T cells, respectively (51, 55, 56). While polyclonal Vδ1 cells are distributed throughout tissues, and exhibit adaptive-like behavior after detection of metabolic Ags and stress-induced molecules, Vδ2 cells predominate in blood and exhibit innate-like behavior after detecting molecules named phosphoantigens (pAgs) and other non-peptide antigens (11, 39, 57–59). A minor subtype of Vδ3 cells makes up ~0.2% of total circulating T cells and recognize CD1d and annexin-A2 (ANX2) (49, 60). In addition, little-known subtypes include Vδ5 cells, which detect the endothelial protein C receptor (EPCR), and other distinct clonal populations such as Vδ4, Vδ6, Vδ7 and Vδ8 (43, 61–63). Nonetheless, the enigma of the combinatorial and functional diversity of γδ TCRs has been partly revealed only for the Vδ1 and Vγ9Vδ2 subtypes **(Table 1)**.

TABLE 1 | Diversity of human γδ T cells.

Subtype	Paired Vγ gene usage	Tissue distribution	Major secreted effector molecules	Major recognition receptors	Activation stimulus or TCR ligand	Ref.
Vδ1	Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, Vγ10, Vγ11	Skin, gut, liver, spleen, lung, PB and BM	IFN-γ, TNF, IL-4, TGF-β and IL-17	TCR, TRAIL, FasL, NKG2D, NCR, FcγRIII and 2B4	Lipid Ags, MIC-A/B, ULBP, NCRL, CD1, MR1 and BTNL	(13, 29, 64–70)
Vδ2	Vγ9	PB, spleen, BM and LN	IFN-γ, TNF and IL-17	TCR, TRAIL, FasL, NKG2D, DNAM-1, TLR, FcγRIII and 2B4	pAgs, BTN, BTNL, N-BPs, MICA/B, ULBP, PVR and Nectin-2	(71–75)
Vδ3	Vγ2, Vγ3, Vγ8	Liver, gut, PB, BM and LN	IFN-γ, TNF, IL-4 and IL-17	TCR, FcγRIII and NKG2D	CD1d and ANX2	(46, 49, 60)
Vδ4	Vγ6	PB	ND	ND	ND	(61–63)
Vδ5	Vγ4	PB	IFN-γ and TNF	TCR	EPCR	(43)
Vδ6	ND	PB	ND	ND	ND	(61–63)
Vδ7	ND	PB	ND	ND	ND	(61–63)
Vδ8	ND	PB	ND	ND	ND	(61–63)

An expanded view of human γδ T cell subtypes allow us to observe that their diversity is principally dictated by the individual variations of γδ TCRs and the diversity of their co-receptors. The TCR repertoire of Vγ9Vδ2 cells is the best known and targets butyrophilin (BTN) proteins, for example, which undergo a spatial and conformational change in the target cell membrane, and activate these lymphocytes in a phosphoantigens (pAgs)-dependent fashion. In contrast, non-Vδ2 TCRs are still poorly studied, although some ligands have been discovered, namely, CD1, MHC class I related protein (MR1) and the endothelial protein C receptor (EPCR), which can be expressed in cancer cells. Additionally, cell activation is not mediated only by γδ TCR binding to their cognate ligand, but optionally requires the engagement of co-receptors, such as DNAX accessory molecule-1 (DNAM-1) and natural cytotoxicity receptors (NCR), which results in the high production of effector molecules.

ANX2, annexin A2; BM, bone marrow; BTNL, butyrophilin-like; FasL, human apoptosis-related factor ligand; FcγRIII, Fc gamma receptor III; LN, lymph node; MICA / B, MHC class I chain-related antigens A and B; N-BPs, aminobiphosphonates; NCRL, NCR ligand; ND, not determined; NKG2D, natural killer group 2 member D; PB, peripheral blood; PVR, polyoma virus receptor; TCR, T cell receptor; TLR, toll-like receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ULBP, UL16-binding proteins.

γδ T CELLS AND LEUKEMIA: THE LEUKEMIC MICROENVIRONMENT MATTERS

Basic scientific discoveries regarding leukemia have revealed that LCs adopt numerous mechanisms for evading immune surveillance (1, 76, 77). This cancer cell hallmark involves a heterogeneous group of components i.e., stromal and/or immune cells, specific receptors and soluble molecules that are present in the leukemic microenvironment, and which reprogram the hematopoietic niche and promote the clonal expansion of LCs in the bone marrow (BM). The subsequent tumor overload in this compartment results in the release of LCs into the blood, constituting two important sites of high leukemic clonal proliferation (1, 4, 26, 78). This is because LCs can bypass antitumor responses and, consequently, develop a high potential for making the environment extremely tolerogenic (79–81). For this, they adopt intrinsic and extrinsic strategies that impair the immune response of T cells and NK cells (26, 77, 82). Among these strategies, the negative regulation of HLA expression, high expression of inhibitory ligands for programmed cell death 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4) or lymphocyte activation gene 3 (LAG3) and the production of regulatory factors (i.e., cytokines, chemokines and inhibitory enzymes) are important changes that contribute to the inhibition of antitumor cells and the recruitment of suppressor cells that support the survival of LCs (83–99).

These established modifications in the leukemic microenvironment have great capacity for modifying cellular functions and for suppressing antileukemic responses – a consequence of the increase in components, such as regulatory T (Treg) cells, immunosuppressive myeloid cells (IMC), mesenchymal stromal cells (MSC) and inhibitory proteins (e.g., PD1 and CTLA4), which have a high regulatory influence (1, 26). γδ T cells are not exempt within this context, since they are susceptible to the effects of several molecules such as interleukin (IL)-4, IL-6, IL-13, IL-17, IL-23 and transforming growth factor beta (TGF-β) (100–105). These factors can play synergistic or pleiotropic roles, and can induce γδ T cell exhaustion or their polarization into a tumor-promoting phenotype (**Figure 1**), thus contributing to malignant progression (24, 106–109).

Although LCs can escape the immune surveillance of αβ T cells and NK cells, they have several molecular targets that can be detected by γδ T cells; however, the crosstalk between these lymphocytes and the leukemic microenvironment is still poorly understood (**Figure 1**). Initially, γδ T cell responsiveness does not depend on MHC expression by LCs, whereas conventional αβ T cells require the MHC-Ag axis for activation to occur. The restricted specificity of conventional αβ TCR is also an important factor to be considered, as it is restricted to the detection of peptide antigens. In contrast, γδ TCR can identify stress-induced molecules, pAgs, lipid Ags and many other non-peptide molecules (110). In the context of leukemias, these attributes may offer an unconventional response pathway against these hematological malignancies.

Mobilization and Recruitment of γδ T Cells Into the TME

The pattern of γδ T cell migration and recruitment has not yet been fully characterized in the context of cancer and, therefore, represents an important question to be investigated. In humans,

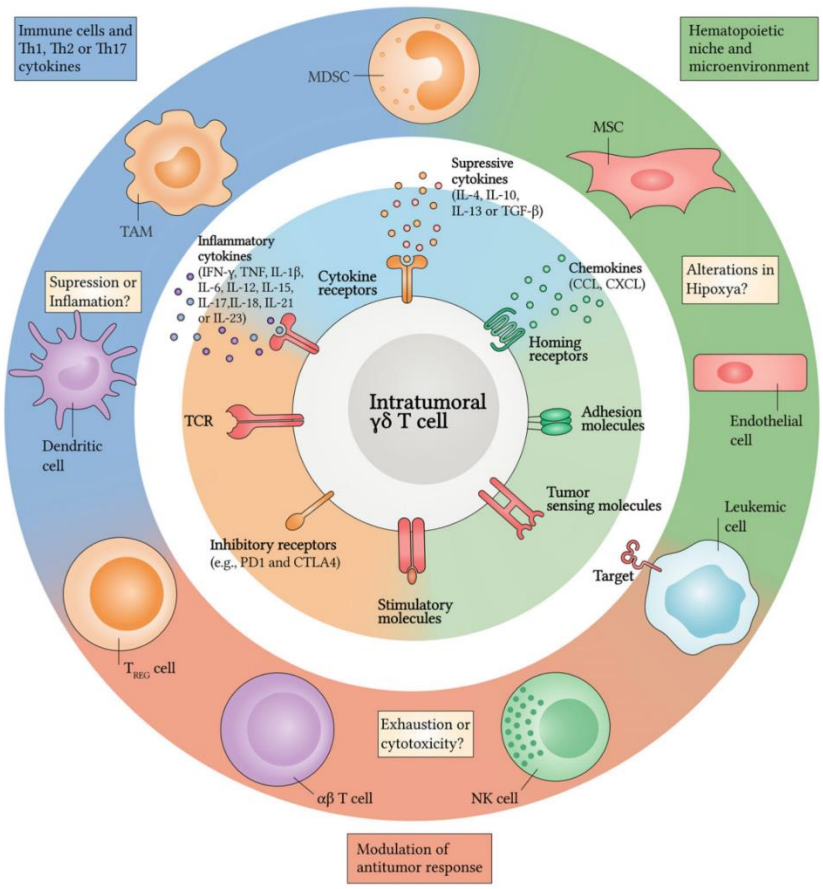


FIGURE 1 | Crosstalk between γδ T cells and the leukemic microenvironment. Upon infiltrating the TME, γδ T cells are exposed to several persistent inflammatory and/or suppressive signals. Pathways implicated in crosstalk with the leukemic microenvironment can be classified into three general categories (center and inner circle): (i) cell-to-cell signals including antigen recognition by γδ T cell receptor (TCR), stimulatory or inhibitory molecules and/or tumor-sensing molecules; (ii) soluble factors such as cytokines and chemokines that will drive changes in expression levels of (iii) homing receptors and adhesion molecules. Several stromal and/or immune cells could be the source of many of these changes (outer circle). Among these, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), regulatory T (Treg) cells and dendritic cells (DC) retain their reprogramming potential into the TME by regulating inflammation or suppression through Th1, Th2 and Th17 cytokines. In addition, the hematopoietic niche can regulate hypoxia, responsible for supporting leukemic cells (LC) survival. Mesenchymal stromal cells (MSC) and endothelial cells can also express many factors that attract antitumor cells, such as γδ T cells, αβ T cells and NK cells which can exert cytotoxicity or undergo cell exhaustion after infiltrating the leukemic microenvironment. CCL, CC-chemokine ligand; CTLA4, cytotoxic T lymphocyte antigen 4; CXCL, CXC-chemokine ligand; PD1, programmed cell death protein 1.

Vδ1 cells up-regulate the expression of CC-chemokine receptor 2 (CCR2) and CXC-chemokine receptor 3 (CXCR3) and infiltrate the TME. They are also activated by CC-chemokine ligand 12 (CCL2) and CXC-chemokine ligand 10 (CXCL10) and exhibit higher IFN-γ production (111, 112). Furthermore, Vδ1 cells express CXCR1 strongly and CCR5 weakly, whereas their Vγ9Vδ2 counterpart only exhibit strong expression of CCR5 (113). Interestingly, the CCR4/CCR8–CCL17/CCL22 pathway has also been shown to be an additional axis of chemoattractant signaling that recruits Vδ1 cells to the TME (114). It is important to note that Vγ9Vδ2 cells, besides retaining a high expression of CCR5, also express CCR3 and CXCR3, and can trigger antitumor responses in peripheral tissues during metastasis (115, 116).

A more accurate analysis of the profile of homing receptors expressed by γδ T cells would reveal how these cells migrate to the bone marrow microenvironment, for example. It is known that the mobilization of immune cells in this compartment is mediated mainly by the CXCR4-CXCL12 pathway, and it has been shown that $CXCR4^+$ γδ T cells (preferably Vδ1 cells) respond to CXCL2 *in vitro*, but their intramedullary homing abilities have not yet been evaluated in the *in vivo* context of leukemia (117–119).

Despite this, many *in vitro* studies have shown that γδ T cells recognize and destroy leukemia blasts, but the complex network of interactions with the tumor environment *in vivo* remains poorly elucidated (120–122). A comparative analysis suggested that Vδ1 TCR-expressing γδ T cells were the most frequent subtype in the BM of pediatric patients with ALL (123). Subsequently, a low circulating γδ T cell frequency was detected in patients with AML before chemotherapy. Patients who regressed to minimal residual disease exhibited higher γδ T cell frequencies, whereas patients with a high leukemic burden exhibited decreased counts (27).

Transcriptomic analyses revealed an abundance of tumor-infiltrating Vγ9Vδ2 cells in cohorts of patients with leukemia (124). This high frequency was positively correlated with the survival of these patients. Although these results are encouraging, the method used to determine the relative proportions of these cells has failed to differentiate them correctly from αβ T cells and NK cells. As a result, this may have contributed to a higher γδ T cell count.

Vδ1 cells have been reported to have increased percentages in patients with CLL (28–31). A high frequency of these cells has been shown to be directly proportional to leukemic progression, that is, patients in more severe states exhibited higher Vδ1 cell counts when compared to healthy patients. This allows these lymphocytes to constitute the major γδ T cell subtype in the PB of these patients, where Vγ9Vδ2 cells generally predominate. This finding was also accompanied by cytotoxic Vδ1 cells with high granzyme (Gzm) B expression (28). Taken together, these data suggest that leukemia affects the γδ T cell frequency and that these cells have some influence during disease regression or progression.

On the other hand, a higher Vγ9Vδ2 cell frequency was associated with a poor prognosis in patients with untreated CLL (125). These lymphocytes showed a dysfunctional phenotype with reduced expression of NKG2D, although the derived LCs showed a high pAgs synthesis. This suggests that Vγ9Vδ2 cells expand in patients with leukemia and may exhibit functional exhaustion, apparently after long-term exposure to pAgs produced by LCs. Based on these reports, it becomes clear that the precise frequency of these cells and their clinical significance during the progression of leukemia is still controversial. In addition, the few studies carried out again suggest that the microenvironment of these malignancies has a strong influence on γδ T cells.

The Leukemic Cell–γδ T Cell Interactome

The sensing of LCs and γδ T cell activation are attributed to antigen recognition by γδ TCR and/or NK cell receptors (NKR), which include the natural killer group 2 member D (NKG2D) receptor, for example **(Figure 2)**. Several reports have shown that LCs express several NKG2D ligands, which include stress-induced molecules, such as MHC class I chain-related protein A (MIC-A), MHC class I chain-related protein B (MIC-B) and UL16-binding proteins (ULBP) (71, 126, 127), while the lack of expression of these ligands is high related to immune evasion of LCs (128, 129). Besides this, some γδ T cell subtypes have a well-documented role in promoting NKG2D-mediated antileukemic responses.

Vδ1 cells recognize and destroy ULBP3$^+$ MIC-A$^+$ LCs and produce higher concentrations of interferon (IFN)-γ and tumor necrosis factor (TNF) in response to the tumor (29). In parallel, Vδ2 cells detect high regulated ULBP1 in LCs and this is indicative of tumor susceptibility to the cytotoxicity of these lymphocytes (130–132). It has also been established that Vδ1 and Vδ2 cells can destroy ULBP2$^+$ LCs (133). Although an almost undetectable ULBP4 expression has been reported in leukemias (129, 134), remarkably, it has been shown that Vδ2 cells detect this molecule in LCs and respond with potent cytotoxicity (135). Therefore, the NKG2D receptor plays a key-role in γδ T cell-mediated immune surveillance in leukemia.

In addition to the expression of stress-induced molecules, an uncontrolled synthesis of metabolic molecules by cancer cells has emerged as a target that can be detected exclusively by reactive γδ T cells, such as the pAgs identified by Vγ9Vδ2 TCR. The pAgs detection mechanism involves butyrophilin (BTN) molecules, which are proteins related to the B7 family of co-stimulatory molecules. BTNs are essential prerequisites in γδ T cell activation, as they perform the intracellular capture of pAgs, undergo spatial and conformational changes in the membrane surface of target cells and consequently bind to the Vγ9 and Vδ2 TCR chains, sending strong stimulatory signals (72, 73). Thus, BTN3A2 has been shown to mediate the recognition of leukemic blasts even though it does not have the B30.2 intracellular domain, important in the internal pAgs uptake (136, 137). This suggests that BTN3A2 can recruit other isoforms, such as BTN3A1 or BTN3A3, and send activation signals through their intracellular domains (138). It is important to highlight that the presentation of pAgs by BTN proteins is highly regulated in LCs, whereas in normal cells the opposite occurs (139).

γδ T cells can also identify specific Ags in the context of monomorphic MHC class I molecules, such as the CD1 protein family (64). These proteins can mediate endogenous or exogenous lipid Ags recognition by γδ TCR and can be detected without loading with lipid Ags (140–142). Two major subtypes of CD1-reactive γδ T cells have been identified, namely Vδ1 and Vδ3 cells (60, 143). It is well established that these molecules are expressed in LCs and exhibit different expression patterns that are related to the leukemia subtype (144). In this context, γδ T cells may play important roles against LCs through the recognition of CD1 proteins and their isoforms.

In fact, CD1 proteins have established themselves as mediators of γδ T cell antitumor responses (145). It is important to note that the Vδ1 subtype represents a large proportion of these reactive cells (143), therefore it is suspected that Vδ1 cells can contribute to antitumor immunity through a CD1-dependent pathway. Recently, it was discovered that these cells with Vδ1 TCR, specifically Vγ4Vδ1 cells, detected CD1b in transfected LCs while they producing IFN-γ after recognition (146). These cells also recognized BTN-like (BTNL) proteins, such as BTNL3 and BTNL8, which suggests that CD1b-reactive

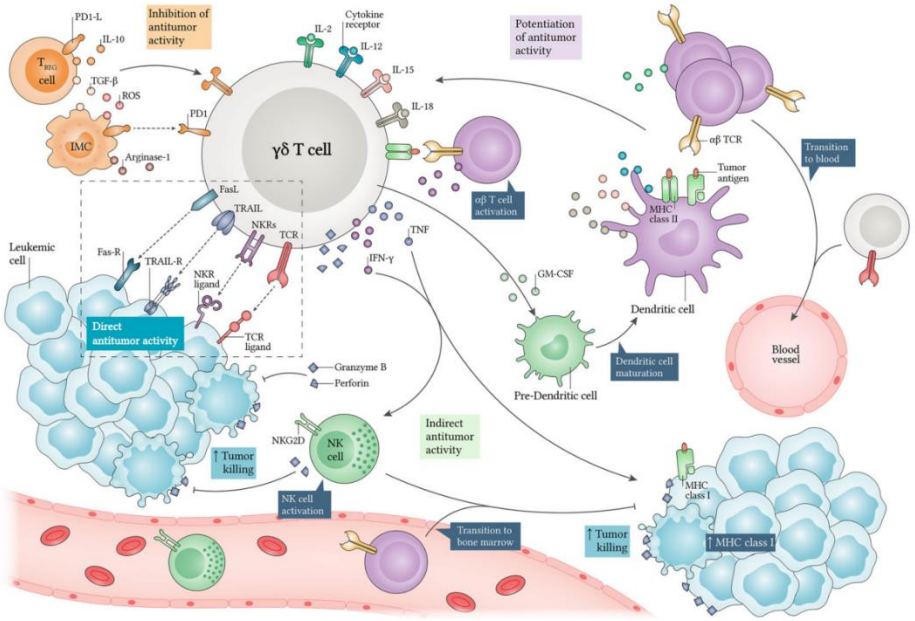


FIGURE 2 | Antileukemic roles of γδ T cells and their regulation. γδ T cells kill leukemic cells (LC) *via* direct and indirect mechanisms. When identifying LCs through γδ TCR and co-receptors such as natural killer cell receptors (NKR), they secrete high levels of perforins and granzymes, mediating direct target killing. Additionally, γδ T cells produce interferon (IFN)-γ and tumor necrosis factor (TNF), which can increase MHC class I expression in LCs, and enhance αβ T cell-mediated cytotoxicity. IFN-γ release also allows NK cell activation, which can enhance tumor killing *via* NKG2D. Alternatively, γδ T cell-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce dendritic cell (DC) maturation, which in turn potentiates antitumor responses *via* interleukin (IL)-2, IL-12, IL-15 and IL-18. Thus, αβ or γδ T cells and NK cells can be recruited for exerting cytotoxicity in many compartments. Moreover, γδ T cells display APC functions and support αβ T cell and NK cell polarization towards an antitumor phenotype. In contrast, their cytotoxicity can be decreased by regulatory T (Treg) cells and immunosuppressive myeloid cells (IMC), since they produce several inhibitory factors such as IL-10, transforming growth factor β (TGF-β), reactive oxygen species (ROS) and Arginase-1. Finally, PD1-PD1L axis expression can regulate γδ T cell antitumor activities. APC, antigen-presenting cell; FasL, Fas ligand; Fas-R, Fas receptor; PD1, programmed cell death protein 1; PD1-L, PD1 ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor.

γδ T cells may respond through the engagement and bispecific combination of CD1b and BTNLs (13).

CD1c recognition has also been investigated and although it does not yet have a well-defined description, it has been shown that this isoform can be recognized by γδ T cells (147). Their involvement in detection of LCs has not yet been reported, although it is clear whether CD1c is positively regulated in LCs (144), thus hypothesizing a possible role for CD1c in γδ T cell activation. In contrast, CD1d has been extensively investigated and the molecular insights about its recognition by γδ T cells have helped us significantly to understand its participation in immune surveillance (148). Interestingly, a high expression of CD1d has been associated with a poor prognosis in leukemia (149–152), but it should be noted that Vδ3 cells can expand and respond against $CD1d^+$ target cells through a CD1d-restricted reactivity and with a potent secretion of effector molecules, such as IFN-γ (60, 153). Although initial studies suggest a CD1 protein-mediated cytotoxicity, questions regarding γδ T cell subtypes and their reactivity to these ligands, in the context of leukemia, still remain.

Monomorphic MHC class I-related protein (MR1) has gained prominence after many discoveries about its regulatory role in mucosal-associated invariant T (MAIT) cell biology and its expression in cancer. This protein can mediate the recognition of folate and riboflavin derived small metabolites (154, 155). In addition, recent reports support that MR1 can present not yet defined specific tumor Ags for MR1-restricted T cells (156, 157). As expected, it was also recently established that γδ TCR recognizes this molecule (65), although direct evidence for $MR1^+$ LCs detection has not yet emerged. The identification of this protein by MR1-reactive T cells may mean a new therapeutic target for cancer immunotherapy and clearly places γδ T cells on the map as a promising and important T cell population.

As discussed above, detection of LCs appears to involve many Ags and stimulatory receptors and is not driven solely by the binding of γδ TCRs to their cognate ligands, but optionally requires the involvement of additional co-receptors and targets. Other NKRs, such as DNAX accessory molecule-1 (DNAM-1), can identify their ligands, such as the polyoma virus receptor (PVR) and nectin-2 molecules, in LCs (74, 158). Although a negative role has been reported for DNAM-1 expression in leukemia (159), this co-receptor is involved in the activation of γδ T cell cytotoxicity after interaction with their ligands in leukemic blasts. This is evidenced when Vγ9Vδ2 cells kill LCs

in a TCR and DNAM-1 dependent fashion, with robust secretion of perforins and granzymes (74).

Notably, Vδ1 cells can lyse LCs *via* NKp30 and NKp44, which are highly regulated *via* the synergistic signal of cytokines and TCR (66). The expression of these natural cytotoxicity receptors (NCR) is related to higher granzyme production and cytotoxicity (66). It is important to highlight that NKp30 has been proven to be crucial for Vδ1 cell-mediated antitumor response. However, NKp30 and NKp44 are bound to an as yet undetermined target (66), ignoring their classic ligands, such as B7-H6 and MLL5 that bind to NKp30 and NKp44, respectively (67), suggesting an as yet unknown additional ligand. In addition, NKp46-expressing Vδ1 cells showed higher cytotoxic activity against LCs and IFN-γ and Gzm B production, while $NKp46^-$ γδ T cells showed reduced antileukemic activity (68). Despite this, the target ligand recognized by $NKp46^+$ γδ T cells in LCs has not yet been demonstrated, although it is well established that cancer cells express ligands for this protein (69, 70).

HARNESSING γδ T CELLS AGAINST LEUKEMIA: FROM MARROW TO BLOOD

γδ T cells are loaded with effector weapons of great potential for cancer immunotherapy (160). Findings in recent years point to important roles for these cells, highlighting them as potential predictive biomarkers, which justifies the current focus of studies on the nature of these cells and the TME (14, 25, 161). It is important to remember that several characteristics discussed here make γδ T cells potential candidates for innovative therapies against tumors and include: (i) activation in a TCR-independent manner; (ii) the ability to recognize Ags regardless of MHC/HLA expression; (iii) effector molecules production and direct and indirect cytotoxicity potentiation against cancer cells; and (iv) their role as antigen-presenting cells (APC) that induce the proliferation of antitumor cells **(Figure 2)**.

Given the high responsiveness against LCs and the absence of toxicity or alloreactivity against the host (162, 163), the application of γδ T cells in leukemia treatment may mean a new advance in cancer immunity and immunotherapy. To make this possible, several strategies for γδ T cell handling have been developed and tested and have presented interesting data **(Figure 3)**. The following subsections will focus on clinical trials and findings, as well as the activity of these cells in response to applied methods. Afterwards, we will discuss potential therapies that may specifically target γδ T cells and their subtypes, while summarizing the main approaches that are being explored to reach their clinical potential.

Expanding γδ T Cells With pAgs, Drugs, Cytokines and Feeder Cells

Intrinsic synthesis of pAgs in cancer cells can be manipulated through pharmacological blockade mediated by aminobiphosphonates (N-BP), such as zoledronate (ZOL) and pamidronate (PAM), which interfere metabolically in the mevalonate pathway (164). The mechanism involved causes these compounds to block the enzymatic activity of farnesyl pyrophosphate synthase, which is present in this metabolic pathway. N-BP–induced interruption results in the intracellular accumulation of pAgs in cancer cells or APCs with subsequent recognition by γδ T cells and activation after cell-cell interaction (165–168). Cancer cell sensitizing with these compounds increases the tumor's susceptibility to γδ T cell cytotoxicity, and this also applies in leukemia (132).

Some experimental evaluations took advantage of the γδ T cell recognition mechanism (directed to pAgs) to obtain a better *in vitro* or *in vivo* expansion of these lymphocytes and test their therapeutic efficacy. To date, these approaches have focused on ZOL, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) and synthetic pAgs, such as bromohydrin pyrophosphate (BrHPP) (169, 170). These compounds are generally administered in combination with low cytokine doses such as IFNs, IL-2, IL-12, IL-15, IL-18 and IL-21. In addition, these approaches can induce an antitumor phenotype and the pronounced expression of associated receptors (171–176).

Vγ9Vδ2 cell expansion has become more accessible because, in addition to being the most prevalent subtype in PB (55, 56), it can also recognize a diversity of relatively well-defined target molecules (177). When these cells are treated with ZOL + IL-2 + IFN type I, their cytotoxic activity is increased and Vγ9Vδ2 cells may be able to efficiently destroy lymphoid and myeloid lineage LCs, as proposed by Watanabe et al. (171). In their study, γδ T cells were generated *in vitro* with ZOL + IL-2 for 14 days, and after this period they were activated with IFN type I for up to 3 days. Thus, the resultant γδ T cells were well expanded in the culture and showed a significant expression of CD69, TNF-related apoptosis-inducing ligand (TRAIL), IFN-γ and TNF, which suggests the acquisition of an activated phenotype and antileukemic reactivity.

In the same vein, sensitization with ZOL + Imatinib has also been shown to increase the cytotoxic synapse between Vγ9Vδ2 cells and LCs (178). Initially, Imatinib resistant or sensitive LCs had low susceptibility to γδ T cells, but *in vitro* treatment with ZOL + Imatinib was able to reverse this situation. The lysis of these LCs was mediated by TCR, NKG2D, TRAIL and perforins. This high cytotoxicity was dependent on ZOL, since it was observed that Vγ9Vδ2 cells exerted low antitumor activity that was slightly increased after sensitization of LCs. To validate these findings, it was further demonstrated that when Vγ9Vδ2 cells, ZOL and IL-2 are infused in a leukemia mouse model, they mediate tumor regression *in vivo* and confer greater survival in these mice (178).

The ability of N-BPs to invigorate exhausted Vγ9Vδ2 cells has also been reported in other investigations (179, 180) and appears to be a promising alternative for their use, given that a higher exhausted γδ T cell frequency has also observed in leukemia (27, 125). It is important to note that, in this context, these cells exhibit a low expression of CD107a, FcγRIII (CD16), IFN-γ and TNF, while B and T lymphocyte attenuator (BTLA), LAG3 and PD1 proteins are more highly regulated on their cell surface (179, 180). When cultured with allogeneic LCs, these lymphocytes had low cytotoxic activity, while γδ T cells from healthy patients

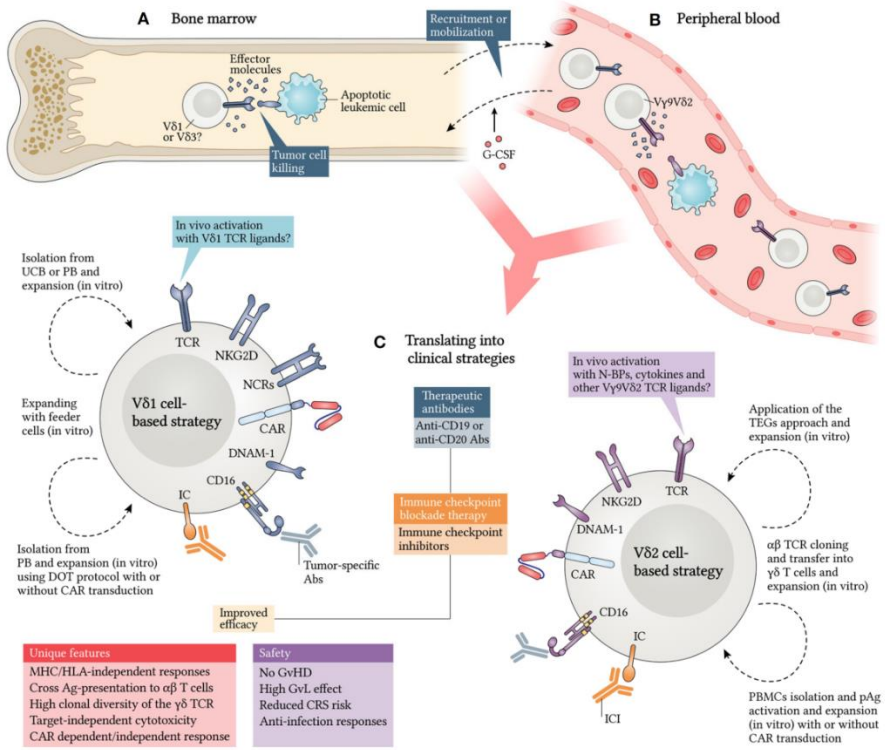


FIGURE 3 | Translating γδ T cells into clinical strategies against leukemia. γδ T cells exert antitumor responses in different compartments and expressing distinct TCR patterns. Vδ1 and Vδ3 subtypes have been implicated as cytotoxic mediators in bone marrow **(A)**, while Vγ9Vδ2 cells have been shown to respond mainly in peripheral blood **(B)**. However, strategies are directed towards Vδ1 and Vδ2 subtypes, as they are the best known **(C)**. Granulocyte colony-stimulating factor (G-CSF) was shown to be a potential adjuvant to mobilize γδ T cells for peripheral blood and enrich the graft. Additionally, Vδ1 cells can be isolated from UCB or PB and expanded *in vitro* using some approaches, such as the DOT protocol, already reviewed here. *In vivo* stimulation with Vδ1 TCR ligands may be a good alternative, but it remains poorly investigated. In parallel, Vδ2 cells can be isolated from PB and activated and/or expanded *in vitro* using pAgs. A new therapeutic concept consists in the cloning and transfer of γδ TCRs into αβ T cells (TEGs) and can enhance antileukemic responses. The fact is that many of these strategies give rise to γδ T cells that express several recognition receptors and have a higher capacity to target leukemic cells (LC), which can be further improved with chimeric antigen receptor (CAR) transduction. Moreover, the use of therapeutic antibodies (Abs), such as immune checkpoint inhibitors (ICI), anti-CD19 and anti-CD20 Abs, can also provide improved efficiency in potential approaches, since γδ T cells have unique features and an attractive degree of safety for their translation into clinical trials. CRS, cytokine release syndrome; DOT, Delta One T; PB, peripheral blood; IC, immune checkpoint; TEGs, T cells engineered to express a defined γδTCRs; UCB, umbilical cord blood.

responded efficiently (179). Notably, when Vγ9Vδ2 cells that were considered dysfunctional were cultured *ex vivo* with mature monocyte-derived dendritic cells (Mo-DC) and N-BPs for 8 days without the presence of LCs, the observed functional impairments could be reversed (179).

Ibrutinib has also been shown to activate γδ T cells against LCs, since Weerdt et al. reported that it was able to induce an antitumor phenotype (180). In their study, allogeneic and autologous γδ T cells were cultured with LCs. As already seen, γδ T cells from patients with leukemia proved to be dysfunctional in terms of cytokine production and cytotoxicity, while those from healthy patients had a strong antitumor activity (179). When Vγ9Vδ2 cells from both cases are treated with Ibrutinib, an effector Th1 phenotype and memory cells are induced. Overall, their antitumor properties can be recovered after *ex vivo* stimulation and after treatment with Ibrutinib, which binds to the IL-2–inducible T cell kinase molecule and promotes activation against LCs (180).

Other investigations have presented a new alternative: the combination of IL-15 plus N-BPs or pAgs promotes significantly greater expansion, high cytotoxicity and a more pronounced Th1 phenotype in γδ T cells, when compared to expansion methods using only IL-2 (174, 181, 182). IL-15 is a powerful growth factor for γδ T cells (183, 184) and can synergize with other molecules and enhance the antileukemic capacity of these cells **(Figure 2)**, as we will highlight below.

The *ex vivo* tests carried out by Van Acker et al. (174) demonstrated that the administration of IL-15 + isopentenyl pyrophosphate (IPP) is able to improve γδ T cell cytotoxicity against LCs. In contrast, γδ T cells stimulated with IL-2 + IPP were more likely to deviate to a Th2 and Th17-like response phenotype when interacting with LCs (174). It is important to

highlight that stimulation by IL-15 promoted a more robust IFN-γ and TNF secretion when compared to IL-2 stimulation. In addition, culturing these lymphocytes with IL-2, IL-15 and ZOL for 14 days critically enhanced the expansion rates to almost 1000-fold the total yield of viable cells, which showed a 590-fold increase in the γδ T cells cultured only with IL-2 + ZOL (174).

Interestingly, when IL-2 + IL-15 and ZOL are administered to γδ T cells isolated from patients with leukemia, during 14 days of culture, they assume different phenotypic states. Most of them may exhibit an effector memory phenotype ($CD45RA^-$ $CD27^-$), followed by a central memory phenotype ($CD45RA^-$ $CD27^+$) (174). In addition, positive regulation of CD56, CD80 and CD86 is also provided (174), suggesting that, in addition to exerting strong antileukemic activity, these cells may also act as APCs and improve the antitumor responses.

Vγ9Vδ2 cell expansion using IL-2 may not even promote satisfactory proliferative rates; however, it is clear that the synergism between IL-2 and IL-15 confers a substantial increase in an inflammatory profile (174, 181), as these cytokines promote a higher transcription factor T-bet expression (181), which in turn, is related to greater cytotoxicity. In addition, the advantage of γδ T cells expanded with IL-2 + IL-15 can be maintained under one of TME's hallmarks *in vivo*, namely hypoxia (181). In fact, a striking feature of the leukemic microenvironment is the low partial pressure of oxygen that favors the tumor-associated immunosuppressive pathways, while at the same time promoting expansion of LCs (185). In this context, the persistence of γδ T cells in hypoxia further demonstrates their clinical importance.

Alternatively, the combination of ZOL, IL-2 + IL-18 also promotes the proliferation of effector cells (186, 187) since IL-18 is an important inducer of IFN-γ secretion (188). Given this, it has been reported that this cytokine indirectly induces the expansion of γδ T cells. Tsuda et al. (186) showed that Vγ9Vδ2 cells are efficiently expanded in response to ZOL, IL-2 + IL-18, but in a $CD56^{bright}$ $CD11c^+$ NK-like cell dependent fashion (187). Many studies have reported that the involvement of NK-like cells in the proliferation of γδ T cells implies greater expansion efficiency when compared to methods using dendritic cells (DC) or monocytes (187, 189–191). These findings suggest an approach targeted at feeder cells that may be responsible for γδ T cell clonal proliferation in different methods *in vitro* and, perhaps, *in vivo*.

IL-18 can also directly support γδ T cell expansion, even in the absence of feeder cells (192). When Vγ9Vδ2 cells are treated only with ZOL, there is a delay in their *in vitro* expansion, as prolonged exposure subjects these cells to acute ZOL toxicity (193). However, when IL-18 combined with geranylgeranyl pyrophosphate (GGPP) is added, the proliferative capacity is restored by inhibiting the toxic effects of ZOL, which allows a substantial expansion of viable γδ T cells to occur. IL-18 + GGPP also were able to activate γδ T cells, exhibiting a central memory or effector memory phenotype and with higher IFN-γ production and CD56 expression (192).

In a subsequent study, treatment with ZOL + IL-2 and culture with Mo-DCs stimulated an activated phenotype in γδ T cells. In this context, immature Mo-DCs have been shown to have a particularly higher capacity to intensify γδ T cell cytotoxicity against LCs, whether in autologous or allogeneic condition (194). Furthermore, IL-15 producing DCs isolated from healthy patients and patients with leukemia (in remission) can potentiate γδ T cell cytotoxicity *in vitro* (182). These DCs induced NKp30, CD16, CD80 and CD86 expression in γδ T cells in an IL-15 dependent manner. This methodology was able to produce γδ T cells with higher expression of co-stimulatory molecules and low expression of inhibitory proteins. In addition, stimulation with DCs + IPP + allogeneic LCs led to high IFN-γ secretion and strong antitumor activity (182).

Deniger et al. (162) demonstrate a new strategy that involves the use of artificial APCs (aAPCs) derived from the K562 leukemic lineage. These feeder cells were modified to express molecules, such as CD19, CD64, CD86, 4-1BBL and IL-15, on their membrane surface. When γδ T cells are cultured with aAPCs + IL-2 + IL-21, there is a remarkably robust 4900 ± 1700-fold polyclonal expansion (162). Most of these cells expressed different γδ TCR domains. Resultant γδ T cells also were able to kill LCs *via* TCR, NKG2D and DNAM-1 (162).

In the same vein, Cho et al. (175) used $CD80^+$, $CD83L^+$ and 4-$1BBL^+$ aAPCs. At low IL-2 concentrations, these co-stimulatory molecules promoted a remarkable Vγ9Vδ2 cell expansion that secreted higher levels of IFN-γ and TNF (175). Notwithstanding, there was no significant proliferation rate (106-fold increase) when compared to the hefty increase observed in the previous study (162). Triple co-stimulation with these molecules induced not only the high IFN-γ and TNF production, but also the positive regulation of a range of other molecules such as IL-2, IL-6, perforins, Gzm A and Fas ligand (FasL) (175). Most importantly, the expanded cells exhibited a terminal effector phenotype ($CD27^{low}$ $CD45RA^{high}$), followed by an effector memory phenotype (175).

Unlike most of the investigations discussed above, other studies have focused on γδ T cells that express the Vδ1 TCR chain. Substantial evidence has demonstrated the ability of this subtype to kill LCs, as already reviewed. Unlike the Vγ9Vδ2 subtype, these cells do not show susceptibility to activation-induced cell death (AICD), which has been reported in several experimental trials (125, 195, 196). These cells can also exercise immune surveillance for long periods, favoring the longevity of cancer immunity (197–199). Several unique attributes have been discovered that particularly place Vδ1 cells as attractive targets in antileukemic therapies. So far, a few studies have emerged that have sought to translate the functional role of these lymphocytes and their applicability, as we will highlight below.

Siegers et al. (30) developed an *in vitro* expansion protocol that enabled the proliferation of γδ T cells isolated from PB after treatment with lectin-based compounds named Concanavalin-A (Con-A). Thus, it was possible to expand Vδ1 cells in a greater proportion than the Vγ9Vδ2 subtype when Con-A was combined with IL-2 + IL-4. The low Vγ9Vδ2 cell proportion was motivated by the period of exposure to Con-A, which induced AICD in these lymphocytes (30). Noteworthy, the resulting Vδ1 cells exerted an efficient cytotoxic activity against LCs through TCR, NKG2D, CD56 and FasL (30).

Subsequently, proof-of-concept studies were performed on leukemia xenograft models using a newly established cell generation protocol called Delta One T (DOT), which was designed by Almeida et al. (31). Specifically, this clinical-grade protocol consists of two steps. First, γδ T cells are isolated from PB of healthy donors or patients with leukemia using magnetic beads and are cultured *in vitro* for 14 days. During this time, these lymphocytes are expanded using a combination of molecules, such as IFN-γ, IL-1β, IL-4 + IL-21, in association with anti-CD3 antibodies (Abs). Then, the expanded cells are transferred to a new culture medium, where they are restimulated by anti-CD3 combined with IL-15 and IFN-γ for another 7 days (31). Overall, this is a 3-week protocol that involves γδ TCR and cytokine stimulation that can accomplish its goals efficiently.

When γδ T cells were submitted to the DOT protocol, expansion was obtained with rates greater than 1000-fold, thus allowing the viable and efficient proliferation of highly cytotoxic cells. It is noteworthy that, with this cell proportion rate, Vδ1 cells, which are generally less frequent in the blood (55, 56), expand from less than 0.5% of all circulating T cells to more than 70% (25, 31). Notably, Vδ1 cells with high expression of NKp30, NKp44, DNAM-1 and 2B4 are also provided, all well established as key-receptors in antileukemic responses (66, 74). These lymphocytes do not regulate inhibitory proteins on their membrane surface, even after 3 weeks of continuous stimulation. In addition, many cell adhesion molecules and chemokine receptors are positively regulated, while these lymphocytes can kill autologous and allogeneic LCs *in vivo*, and ignoring normal cells (31).

Finally, the same protocol was tested by Lorenzo et al. (200), in which γδ T cells from PB were reinforced using a range of stimulatory molecules (31). While the previous study sought to mobilize Vδ1 cells against a CLL xenograft model (31), the latter work applied the DOT protocol to an AML xenograft model (200). It is important to highlight that in both cases there was an efficient regression of tumors, and this increased mice survival (31, 200). In addition, γδ T cells avoided systemic metastasis of LCs (31). They exerted their antileukemic activity against AML blasts in a partially TCR-dependent manner, while they depended on the B7-H6 expression (200), which binds to NKp30 (67).

Blocking Immune Checkpoints in γδ T Cells and Leukemic Cells

Although they are potent, γδ T cell antitumor responses can be regulated by immune checkpoints (IC). Many inhibitory proteins, such as PD1, CTLA4, LAG3, BTLA, T cell immunoreceptor with Ig and ITIM domains (TIGIT) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), are key mediators in inflammatory regression and cell suppression, in the context of the TME (93, 201, 202). Generally, these molecular interactions can act synergistically with the infiltration of suppressive cells that support tumor evasion through the establishment of a strongly tolerogenic environment (26, 76). However, recent advances in cancer immunotherapy using monoclonal Abs (mAbs) targeting ICs, the immune checkpoint inhibitors (ICI), have shown that combinatorial blocking of proteins, such as PD1 and PD-L1, can restore cellular functions and reestablish antitumor activity (203, 204).

The mechanisms of γδ T cell regulation mediated by ICs are diverse and poorly understood, but seemingly unified by the fact that these receptors functionally complement each other and ensure the adjustment of the immune response. PD1 and BTLA are the most potent ICs shown to suppress γδ T cell cytotoxicity in cancer (205, 206). Although CTLA4 expression has not been consistently assessed, it is known that this molecule is rarely expressed in activated γδ T cells (207, 208). Importantly, the expression of these ICs may vary between γδ T cell subtypes, where, for example, Vδ1 cells exhibit higher PD1 expression than their Vγ9Vδ2 counterpart (209).

Early after activation, when the γδ TCRs find their cognate ligands, γδ T cells begin to rapidly display many of these ICs on the cell surface (205, 207). Collectively, the expression of these proteins is low or stable, but temporary, and is sufficient to reduce cytokine production, proliferation and survival of γδ T cells (205, 206, 208, 210, 211). These changes can also be observed in leukemia, as γδ T cells increase the expression of PD1, CTLA4 and BTLA, while LCs strongly regulate the expression of their ligands, such as PD-L1, CD80 and/or CD86, and herpesvirus-entry mediator (HVEM), respectively (212). This represents an important barrier, as these molecules can prevent the efficient activation of γδ T cells and the associated antitumor response. Blocking the expression of these inhibitory receptors through the use of ICIs may be an interesting alternative to reverse the state of anergy and/or cell exhaustion.

The influence of ICIs on γδ T cells and their potential impact on the associated cytotoxic activity, in the context of the leukemic microenvironment, has not yet been characterized and is, therefore, an open question. Despite this, PD1 has been shown to negatively regulate Vγ9Vδ2 cell responses, while the addition of ZOL + anti–PD-L1 was able to bypass the inhibitory signals and promote γδ T cell reactivation against LCs in a TCR-dependent fashion (205). Therefore, this discovery allows us to suggest that γδ TCR-mediated activation is capable of overcoming the inhibitory effects of the PD1/PD-L1 pathway, since the application of ICIs plus ZOL, which is a strong Vγ9Vδ2 TCR stimulator, apparently synergizes the activation of γδ T cells and restores their tumor reactivity (205).

Notably, Hoeres et al. (213) demonstrated that although PD1 signaling can modulate the production of IFN-γ in leukemia-reactive γδ T cells, its additional blockage and stimulation with ZOL can increase the production of this cytokine. Although it did not show a significant effect on the destruction of LCs by γδ T cells, the action of anti-PD1 + ZOL in these lymphocytes was able to induce high IFN-γ secretion, which is a potent inflammatory and antitumor factor (213). As noted, cytokine secretion, such as IFN-γ, can be negatively regulated, and we can infer from this study that the application of ICIs potentially reverses this suppressive condition and is able to stimulate the triggering of an antitumor response.

In addition to PD1/PD-L1, other inhibitory proteins are highly regulated in LCs (i.e., CTLA4, BTLA, TIGIT, TIM3 and LAG3) and their effects on γδ T cells have not yet been fully investigated (26, 159, 214–216). However, previous studies have shown that some of these receptors have great potential for deregulating their antitumor activity, reflecting in cytokine production (213, 217). Nonetheless, evaluating these components before proceeding to a clinical application is important, since these molecules most likely prevent the efficient killing of LCs. This is one of several mechanisms of tumor escape that are commonly observed in recent and innovative treatment modalities, and which also include the chimeric antigen receptor (CAR) T cell therapy (218).

Focusing on γδ T Cell-Engager Molecules in the Leukemic Microenvironment

Antibodies Direct γδ T Cells Against LCs

As we have shown herein, data from *in vitro* experiments and mouse models unequivocally demonstrate the potential of γδ T cells against leukemia. Knowledge obtained regarding the many signals that regulate their activation and the tumor resistance underlying γδ T cells offers additional approaches that, in addition to inducing an activated status, a (poly)clonal expansion or a more pronounced Th1 phenotype, may also allow more specific targeting against the tumor. Improving γδ T cell efficiency against LCs, however, requires strategies based on their cytotoxic nature, which include, for example, antibody-dependent cell cytotoxicity (ADCC) (75, 219). Therefore, this implies a role for CD16, mAbs and bispecific antibodies (bsAbs) that bind to their respective target antigens.

CD16-mediated ADCC plays an important role in tumor destruction. For this to occur, CD16 must bind to the constant fraction of Abs IgG, thus constituting an optional axis in target cell killing. γδ T cells constitute the major blood T cell population that expresses CD16 (220, 221), although this expression is variable (222). Given this, the potential engagement of therapeutic Abs with the product of Vγ9Vδ2 cells can provide an efficient alternative against LCs (223). Several studies have shown that γδ T cells mediate leukemic regression *via* a CD16-dependent pathway (136, 223–226), in particular the Vγ9Vδ2 subtype, which positively regulates CD16 and TNF expression when stimulated with pAgs (227).

mAbs-coated LCs are efficiently destroyed by $CD16^+$ γδ T cells *via* ADCC and these lymphocytes subsequently exhibit APC functions and activate αβ T cells, apparently through the tumor Ags presentation by MHC class II (228). It has been shown that the application of therapeutic CD20-targeting Abs, such as Rituximab (RTX), improves the antileukemic effect of these lymphocytes through tumor destruction by ADCC *in vitro*. This leads γδ T cells to secrete high levels of IFN-γ, perforins and CCL5 (219). In addition, BrHPP implementation potentiates the RTX bioactivity and consequently also increases γδ T cell cytotoxicity against $CD20^+$ LCs *in vitro* and *in vivo* (75).

When peripheral blood mononuclear cells (PBMC) are stimulated with ZOL + IL-2 *ex vivo* and then cultured with LCs and Obinutuzumab (anti-CD20), it is observed that γδ T cells perform ADCC more efficiently than NK cells (223). Most importantly, the cytotoxicity of these lymphocytes cultured with Obinutuzumab is more potent compared to other tested mAbs, such as RTX. This view was reinforced when LCs treated with Obinutuzumab were substantially lysed in a CD16-dependent manner (223).

Benyamine et al. (136) demonstrated that BTN3A-targeting mAbs (anti-BTN3A 20.1) sensitize LCs and act indirectly in tumor destruction. This is due to the anti-BTN3A Abs binding in three different target molecules: BTN3A1, BTN3A2 and BTN3A3. The combination of these mAbs with γδ T cells and the subsequent infusion in a leukemia murine model was able to decrease the leukemic load in the PB and BM, increasing survival in these mice (136). Taken together, these data create the expectation that targeting mAbs to BTN proteins can be potentially useful in new therapeutic approaches.

Like most other surface molecules expressed in LCs, CD19 is also a potential target to be considered. When LCs are incubated with γδ T cells and modified anti-CD19 Abs (Ab 4G7SDIE), a significant increase in the degranulation marker CD107a is observed, as well as the strong IFN-γ and TNF production (224). In addition, the adoption of bsAbs targeting CD19/CD16 (bsAbs N19-C16) is also able to increase the expression of these inflammatory molecules (224). Interestingly, bsAbs targeting CD19/CD3 (bsAbs N19-CU) also strongly activated γδ T cells and, unlike the other previously tested Abs, mediated the lysis of LCs (224). It should be noted that the use of Abs modified to have a triple specificity to CD16 and CD19 (triplebody SPM-1) was also able to activate these lymphocytes against $CD19^+$ target cells, which was evidenced by the expression of antitumor mediators (225).

The projection of a bsAbs targeting the Vγ9 TCR chain and CD123 (anti-TRGV9/CD123 engager) was also able to recruit γδ T cells against AML blasts (229). This engagement induced its activation and cytotoxicity against endogenous LCs, as evidenced by CD69, CD25 and Gzm B positive regulation. Interestingly, these activated γδ T cells exhibited a low secretion of IL-6 and IL-10, which are cytokines that are highly related to cytokine release syndrome (CRS) in patients undergoing αβ T cell-based therapies (229–231). The efficacy of this approach is evidenced when anti-Vγ9/CD123 directed γδ T cells were infused into a leukemia mouse model and controlled the leukemic proliferation in different compartments in these mice (229).

Finally, it has been shown that CD1d is also an attractive target. A recent study showed that CD1d specific single domain Abs can guide γδ T cells (226). These engagers were able to mobilize and activate these lymphocytes against autologous LCs from patients with CLL. This allowed γδ T cells to produce many inflammatory molecules and maintain their pAgs reactivity (226). Taken together, the many studies reviewed here allow us to suggest that the therapeutic application of Abs can be improved with the use of N-BPs that enhance γδ T cell activation. However, their therapeutic application against leukemia still needs more detailed investigation.

γδ T Cells Expressing CARs

While the application of therapeutic Abs has significantly increased the effectiveness of leukemia treatments, other approaches are also emerging with promising healing potential. Current advances in genetic engineering enable CAR transduction in NK cells, macrophages and T cells, thus offering new horizons for cell therapy, although this has been primarily focused on conventional αβ T cells (232, 233). In this context, γδ T cells are also undergoing a number of improvements in order to enhance their antitumor capacities.

The fact is that γδ T cells can be redirected with CARs against surface molecules expressed by LCs (234). Their unique innate properties and their high capacity for tumor sensing and killing place them in an interesting position in potential approaches against leukemia. CAR γδ T cells can offer a triple activity because, for example, they can recognize LCs (i) through the direct engagement of γδ TCR to their cognate ligand, (ii) through NKRs and their associated ligands, or (iii) through CAR specificity to the target antigen **(Figure 3)** (234, 235). Besides this, their APC functions (211) may allow the prolongation of immune response in the TME (228), since the CAR acquisition preserves the ability of γδ T cells to present tumor Ags (235).

The applicability of these genetically modified T cells has been established by some of the previous studies that evaluated the viability of viral transduction (236, 237) or electroporation (238) of the CAR. Rischer et al. (236) demonstrated for the first time that Vγ9Vδ2 cells can be efficiently transduced with CAR genes. Their study also showed that γδ T cells expressing anti-CD19 CARs destroy $CD19^+$ LCs and produce high levels of IFN-γ in a target-dependent fashion (236). Subsequently, Deniger et al. (238) showed that the introduction of CAR by electroporation in PB-derived γδ T cells is able to produce polyclonal CAR T cells that express Vδ1, Vδ2 and Vδ3 TCR chains (238). For this to happen, approaches already reviewed here were used (162).

Noteworthy, one study demonstrated that CAR γδ T cells adopt a highly activated, but not exhausted, phenotype, as highlighted by the low regulation of CD57 (238). In addition, these lymphocytes tend to assume distinct phenotypic states of effector memory, while positively regulating homing molecules. Specifically, these homing receptors included CXCR4, a molecule associated with migration to BM, as well as CD62L and CCR7, which are linked to migration to lymph nodes (238). This is encouraging since BM and lymph nodes are sites of high tumor growth in acute and chronic leukemias (1, 4, 26, 78, 239–241).

Surprisingly, it has also been confirmed that CAR γδ T cells recognize and kill LCs in BM regardless of the CD19 target. Rozenbaum et al. (242) recently showed that these modified lymphocytes have high IFN-γ production and reactivity to $CD19^{+/-}$ LCs *in vitro*, which was even enhanced with the addition of ZOL. To investigate *in vivo* efficacy, the authors injected CAR γδ T cells in a leukemia mouse model. Although it did not induce a complete remission, the infusion of these cells led to a drastic reduction in the leukemic burden in the BM of these mice, which was even more pronounced when ZOL was administered (242).

These studies demonstrate that the production of CAR γδ T cells is viable and supports the high effectiveness of these lymphocytes against many malignancies, especially in leukemias. In contrast to conventional CAR T cell therapy, approaches based on γδ T cells can overcome several currently reported limitations, such as modulation of tumor antigen expression (242, 243) and CRS (229–231).

How About Molecular Switching of TCRs?

One interesting strategy for targeting lymphocytes against the tumor is to design γδ T cells with αβ TCRs or to design αβ T cells with γδ TCRs (244). This therapeutic concept has great potential for combining some unique γδ T cell properties, such as the rapid responsiveness to the tumor, the expression of individual molecules, and the absence of alloreactivity, with the high proliferative capacity and specific reactivity of conventional αβ T cells. Combining these unique aspects through TCR transduction leads us to expect that the resulting antileukemic responses will be long-lasting and based on immunological memory.

This new concept of modified T cells, named T cells engineered with defined γδ TCRs (TEG), was adopted in some studies that showed that TEGs kill LCs *in vitro* and *in vivo* models (245). TEGs tend to deregulate the intrinsic αβ TCR expression in their membrane surface, avoiding the graft-*vs*-host disease (GvHD) (245, 246). In addition, $CD4^+$ TEGs retain their ability to induce a complete maturation of DCs, and stimulation with PAM can potentiate the cytotoxicity of $CD8^+$ or $CD4^+$ TEGs since it promotes higher production of inflammatory molecules, such as IFN-γ, TNF, and IL- 2, *in vivo* (245).

Similar results were obtained when TEGs cultured with LCs reduced the tumor *in vitro* (247). In addition, the infusion of TEGs plus IL-2 + PAM in an AML murine model enabled reactivity directed to LCs without affecting the healthy hematopoietic compartment and without being influenced by the TME, when inserted into mice that expressed IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and stem cell factor (SCF) (247), which are molecules that support tumor growth *in vivo* (248). Therefore, TEGs demonstrated efficiency in reducing the tumor in xenograft models with minimal alloreactivity, which stimulated the projection of a robust manufacturing procedure of TEGs that were validated under good manufacturing practice (GMP) conditions (244, 249).

Finally, γδ T cells transduced with αβ TCR plus CD4 and CD8 co-receptors showed high antitumor activity against LCs (250). As similarly observed in TEGs, transduction of αβ TCR induced a low expression of endogenous γδ TCR. In addition, modified $CD8^+$ or $CD4^+$ γδ T cells expressed high levels of IFN-γ and IL-4, although IFN-γ production was more pronounced in $CD8^+$ cells. Most importantly, these transduced cells were able to kill LCs *in vitro*, although $CD8^+$ γδ T cells have shown more efficiency than $CD4^+$ cells (250, 251). This evidence supports the important role of γδ T cells in TCR gene transfer-based approaches while suggesting an improved antileukemic capacity when TCR transduction is combined with co-receptors, in particular, with the CD8 protein.

Converting γδ T Cells Into Living Drugs

Source, Isolation and Pre-Activation

γδ T cells and their subtypes are present in several tissues, but the ideal source for obtaining all these lymphocytes is still being determined. Despite this, therapeutic γδ T cells for infusion can be obtained from peripheral blood (252, 253) or umbilical cord blood (UCB) (254, 255). It is important to note that the frequency of γδ T cells varies between 5-10% of peripheral blood T cells (51, 52), while they constitute <1% of T cells in UCB (254). The functional differences between γδ T cell subtypes in these sources are not yet clear, but it is already established that while the subtype expressing Vγ9Vδ2 TCR predominates in PB (51, 55, 56), polyclonal γδ T cells expressing the Vδ1 TCR domain predominate in UCB (52, 256, 257).

γδ T cell expansion from PB is a well-established method and is usually adopted in clinical and experimental trials. For isolation of these lymphocytes, the starting material is the product of leukapheresis, which can be initially enriched through stimuli with several soluble factors (e.g., cytokines and N-BPs) and later undergoes removal of αβ T cells and $CD19^+$ B cells through the use of magnetic beads, depletion or separation kits (optionally maintaining NK cells) (169, 252, 253). Since increasing the γδ T cell product from leukapheresis can further improve its therapeutic handling, adopting the use of molecules as the granulocyte colony-stimulating factor (G-CSF) may mobilize a large amount of antileukemic γδ T cells for peripheral blood, as shown in several studies (258–262).

Alternatively, physical exercise and the consequent systemic activation of β-adrenergic receptors (β-AR), immediately before PBMC isolation, has been shown to substantially increase mobilization for PB, *ex vivo* expansion and antitumor capacity. In their study, Baker et al. (263) showed that the practice of physical exercises can predict the expansion potential of γδ T cells, which is mobilized in a β-AR type 2 dependent fashion. Therefore, patients with high levels of physical activity mobilized γδ T cells that expanded *ex vivo* in much higher percentages compared to blood at rest when stimulated with IL-2 + ZOL for 14 days (263). These cells had higher expression of CD56 and NKG2D and showed high cytotoxicity against LCs *in vitro*.

On the other hand, γδ T cell isolation from UCB is still poorly investigated and so far, it has not been the target of cell expansion protocols in clinical trials. Berglund et al. (264) showed that it is possible to expand γδ T cells derived from UCB *in vitro*. The authors developed an expansion protocol based on the application of ZOL + IL-2 in culture for 14 days. This promotes the growth of Vγ9Vδ2 cells that mostly adopt a central memory phenotype and secrete higher levels of IL-1β, IL-2 and IL-8 (264). In general, the acquisition and handling of UCB-derived γδ T cells still need to be investigated more fully. Some factors, such as the low frequency of Vγ9Vδ2 cells (more easily expanded *in vitro*) in UCB and the poorly defined phenotypic diversity in this environment, make handling more limited (254). The approaches discussed here are viable targets for adoptive cell therapy because they also serve as adequate and economical adjuvants for hematopoietic stem cell transplantation (HSCT) (263, 264).

It is not clear whether pre-activation with ZOL + IL-2 can trigger the total antitumor capacity of γδ T cells. However, many *in vitro* approaches that use other molecules, such as IL-15, have demonstrated greater potential in stimulating the activation of these lymphocytes. As already reviewed, IL-15 associated with pAgs promotes high cytotoxicity in γδ T cells, which is evidenced by the high T-bet expression (181). In addition, the combined use of IL-2 + IL-15 can provide γδ T cells with antileukemic properties (174, 181, 182) even in hypoxia (181).

A mix of cytokines combined with Abs can also promote a pre-activated state in γδ T cells, as evidenced in studies using the DOT protocol. Notably, the use of IFN-γ, IL-1β, IL-4, IL-15, and IL-21 with anti-CD3 Abs positively regulates many NKRs, while ICs, such as PD1, CTLA4 and CD94/NK group 2 member A (NKG2A), are negatively regulated on the cell surface (31, 200). In addition, many homing receptors, such as signal-regulatory protein alpha (SIRPα), integrin-β7, CD31, CD56, CD96 and intercellular adhesion molecule 1 (ICAM-1), are expressed, as well as chemokine receptors, such as CXCR3, CCR6 and CX3C chemokine receptor 1 (CX3CR1) (31). Noteworthy, the junction of these cytokines promotes γδ T cells with APC functions and a higher potential to migrate and recirculate between blood and tissues (31, 174). Therefore, pre-activation using these approaches may lead to better crosstalk with other cytotoxic cells (e.g., NK) or LCs in different compartments (265).

The HSCT Questions

The functional importance of γδ T cells in HSCT has received enormous attention after many years of research. The fact is that the frequency of these lymphocytes may fluctuate between treated and untreated individuals, either during chemotherapy (27, 266) or after HSCT (267–273), implying relevant roles for γδ T cells in the patient's recovery (274). Several initial reports have shown that αβ TCR depleted allogeneic HSTC (allo-HSCT) was able to increase disease-free survival (2-5 years) after transplantation (267, 268, 273). Notably, this was correlated with a high γδ T cell frequency circulating in the PB and mediating the graft-*vs*-leukemia (GvL) effect (267). The Vδ1 subtype represented the highest proportion of these cells in the blood of patients (267, 273, 275).

Given that γδ TCRs are not restricted to HLA expression, the triggering of the GvHD effect is less likely, since tumor detection depends on more ubiquitous targets (273, 276). Therefore, the high frequency of these cells contributes to the restoration of the hematopoietic niche and is related to antileukemic responses (273); although this is not their only contribution to the success of HSCT. Higher γδ T cell percentages and a lower incidence of infection was been observed in many patients after HSCT, indicating protective roles in fungal, bacterial and viral infections (268, 273, 276). This made it possible to increase survival in patients with a high frequency of these cells when compared to patients with low or normal counts (277).

Cytomegalovirus (CMV) infection and its reactivation is a major concern after HSCT and, notably, γδ T cells can be essential effectors in controlling viral expansion. Knight et al. (278) reported for the first time that Vδ1 and Vδ3 cells expand as

a result of an active response against CMV in patients after allo-HSCT; although there were previous data that showed that these subtypes expand in CMV infection in immunocompetent individuals (275, 276, 279). Interestingly, CMV reactivation after allo-HSCT mobilized these non-Vδ2 subtypes against infected cells and against LCs *in vivo* (280). This is intriguing and leads us to infer that the reactivation of CMV after HSCT can benefit patients with leukemia, as it impacts the incidence of disease recurrence (281).

Epstein-Barr virus (EBV) infection is also a problem. Farnaut et al. (282) showed that EBV infection resulted in a significant Vδ1 cell expansion in a patient with ALL transplanted with UCB, which represented more than 80% of the total circulating γδ T cells. One year after transplantation, these cells were highly differentiated and exhibit CD57 and CD8 expression while minimally expressing the BTLA protein (282). These data suggest a strongly adaptive response from Vδ1 and Vδ3 cells that possibly improves the efficacy of allografts (269).

Overall, the graft enriched with γδ T cells provides a lower relapse incidence during immune reconstitution after HSCT (274). This is evidenced when patients with low frequencies of these lymphocytes have a high rate of death from relapse (283). In addition, γδ T cell innate and adaptive responses can also prevent the occurrence of infections after HSCT (269, 284, 285). Finally, their functional plasticity can assist in immunological tolerance to the graft and avoid GvHD, as evidenced in many studies (258, 260). Therefore, the data highlighted here position γδ T cells as potential targets in applications aimed at improving clinical results after HSCT, since they induce a potent GvL effect in the absence of GvHD.

THE STATE-OF-THE-ART FOR CLINICAL TRIALS

Although promising, γδ T cells have not yet been fully translated into clinical research that targets leukemia. Although clinical studies carried out over two decades have shown that γδ T cells have low toxicity and reactivity against the host (274), the clinical efficacy of adoptive therapy with γδ T cells has not been consistently reported (**Table 2**). *In vivo* stimulation, that is, the activation of autologous γδ T cells using N-BPs + IL-2, induced few measurable responses in patients with leukemia. Wilhelm et al. (286) included 4 patients with CLL in a clinical study based on PAM + IL-2 *in vivo* infusion. None of the 4 patients were able to obtain objective or complete responses, which was also evidenced by the low expansion of endogenous γδ T cells *in vitro* when isolated from these patients.

Kunzmann et al. (287) evaluated stimulation with ZOL + IL-2 in several tumors. In this clinical trial, 8 patients with AML were included. Only 2 of them had an objective response, and they achieved a partial remission. Notably, ZOL infusion in pediatric patients with acute leukemia after HSCT depleted for αβ TCR and $CD19^+$ B cells prolonged the disease-free survival in these patients, since it was associated with high numbers of circulating γδ T cells (271). This was also reported in a subsequent clinical trial that evaluated 46 pediatric patients with acute leukemia and reported that 3 or more repeated ZOL infusions offer a lower rate of transplant-related death, lower occurrence of relapses and absence of GvHD. Global disease-free survival is also improved (272).

The efficiency degree of donor γδ T cell *ex vivo* expansion is evidenced when the graft is depleted for αβ TCR, as this was able to induce a remarkable clinical recovery in 74 patients with acute and chronic leukemia, in which 43 achieved an objective response and 25 achieved complete remission, with no risk of recurrence and with improved survival after allo-HSCT (267). The subsequent follow-up of 153 patients with acute leukemia after allo-HSCT showed that γδ T cell-enriched graft, even inducing few complete remissions (36 patients), was able to confer a long-term survival advantage in patients who exhibited high γδ T cell frequency in the blood (268). Finally, ZOL + IL-2 *in vivo* stimulation after infusion of PBMC depleted for αβ T cells in 2 patients resulted in a higher *in vivo* expansion of donor γδ T cells and NK cells that induced complete remission in these patients (288).

It is important to highlight that many Phase I clinical trials are emerging to investigate γδ T cells as alternative axes in several established therapies since the available clinical and preclinical data suggest that γδ T cell-based strategies be combined with agents that better target these cells against the tumor. Therefore,

TABLE 2 | Executed clinical trials with γδ T cell-based strategies.

Leukemia subtype	*N* included	Interventions	Objective response	Complete response	Ref.
***In vivo* stimulation (autologous)**					
CLL	4	PAM and IL-2	0/4	0/4	(276)
AML	8	ZOL and IL-2	2/8	0/8	(277)
ALL and AML	43	ZOL	ND	ND	(261)
ALL, AML and MPAL	46	ZOL after allo-HSCT depleted for αβ T cells/$CD19^+$ B cells	ND	ND	(262)
***Ex vivo* expansion (donor γδ T cells)**					
ALL, AML and CLL	74	Allo-HSCT depleted for αβ T cells	43/74	25/43	(257)
ALL and AML	153	Allo-HSCT depleted for αβ T cells	100/153	36/153	(258)
AML and SPL	2	ZOL and IL-2 after CD4/CD8 depleted haplo-PBMC	2/2	2/2	(278)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; CLL, chronic lymphocytic leukemia; haplo, haploidentical; IL, interleukin; MPAL, mixed phenotype acute leukemia; ND, not determined; PAM, pamidronate; PBMC, peripheral blood mononuclear cell; SPL, secondary plasma cell leukemia; ZOL, zoledronate.

TABLE 3 | Currently registered γδ T cell-based clinical trials.

Disease or clinical condition	*N* intended inclusion	Interventions	Phase	Start	Status	Study identifier
***In vivo* stimulation (autologous)**						
ALL and AML	22	ZOL after haplo-HSCT depleted for αβ T cells/CD19$^+$ B cells	I	January, 2016	Recruiting	NCT02508038
Eligible patients for HSCT	20	ZOL and IL-2	I	March, 2019	Recruiting	NCT03862833
***Ex vivo* expansion (autologous)**						
AML	20	PB collection and BM aspirate (OS)	NA	August, 2018	Recruiting	NCT03885076
Relapsed or refractory AML	38	γδ T cell infusion	I	September, 2019	Recruiting	NCT04008381
Relapsed or refractory CLL	6	γδ T cell infusion	I	October, 2019	Recruiting	NCT04028440
ALL, AML and CML	38	EAGD T cell infusion after HSCT	I	January, 2020	Recruiting	NCT03533816
Genetic engineering						
AML	18	TEG001	I	June, 2017	Recruiting	NTR6541
ALL and CLL	48	anti-CD19 CAR γδ T cells infusion	I	October, 2017	Not yet recruiting	NCT02656147

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; EAGD T cell, expanded/activated γδ T cells; haplo, haploidentical; HSCT, hematopoietic stem cell transplantation; IL, interleukin; NA, not applicable; OS, observational study; PB, peripheral blood; TEG, T cells engineered to express a defined γδ TCR; ZOL, zoledronate.

several studies aiming at the optimization of γδ T cell antitumor reactivity through genetic engineering approaches are currently registered **(Table 3)**. The use of these lymphocytes as platforms for CAR (NCT02656147) and TEG (NTR6541) engineering can overcome many obstacles observed in conventional adoptive therapy with αβ T cells and NK cells, although they also have their limitations (215, 228). Finally, *in vivo* stimulation and *ex vivo* expansion are also being insistently evaluated in the context of allo-HSCT (NCT02508038, NCT03862833) and the γδ T cell product infusion (NCT03885076, NCT04008381, NCT04028440, NCT03533816) in the expectation that a safe, effective and tolerable method for the treatment of patients will be discovered.

CONCLUDING REMARKS AND OUTLOOKS FOR THE FUTURE

Through this review, we hope to shed light on a relatively unexplored unconventional T cell. Nonetheless, it is one that has proven to be an important component in the leukemic microenvironment, since it responds effectively against the tumor and is able to affect the clinical outcome in patients with leukemia, as we recently reviewed (289). γδ T cells have unique immunological properties that allow the development of an off-the-shelf immunotherapy with universal applicability, that is, independent of histocompatibility related factors since γδ T cells respond regardless of MHC/HLA expression and recognize Ags presented by ubiquitous monomorphic molecules in many tumors in humans.

Furthermore, the clinical responses reported in clinical and pre-clinical trials, already reviewed here, highlight the importance of further increasing γδ T cell reactivity, either by raising intracellular pAg concentrations to "sensitize" LCs or by projecting γδ T cells with higher expression of receptors associated with cytotoxicity, adhesion and homing, as this allows recirculation and immune surveillance in different tumor compartments, even under hypoxia. The fact that these cells predominate in the blood and healthy or malignant tissues provides a migratory advantage over αβ T cells or NK cells and a greater ability to infiltrate and respond in the leukemic microenvironment; in particular the Vδ1 subtype, which has improved cytotoxicity and resistance to exhaustion or AICD.

The difficulty that still needs to be overcome for the therapeutic use of these cells is, in fact, is that of how to obtain a clinically significant cell proportion. As such, new techniques for cell expansion (or improvement) are necessary. In addition, ensuring that γδ T cell antileukemic phenotype is not diverted by TME stimuli also represents another challenge to be faced. Therefore, the modulation and effective targeting of these cells need to be achieved. Finally, improving and maintaining their *in vivo* persistence and invigorating exhausted γδ T cells also represent additional barriers that can be reversed using molecular factors that support their cytotoxicity in TME *in vivo*. The fact is that the innate and adaptive γδ T cell properties will lead to advances in better antileukemic approaches and potentially establish which of these will provide a real and applicable translational perspective.

AUTHOR CONTRIBUTIONS

NDA and MSB established the initial conception, projected, and wrote this manuscript. NDA, MSB and TLPR collected, analyzed, and reviewed the data. MSB designed the illustrations and tables. NDA, FM-G, FSHA, AMT, AM and AGC supervised the project development, interpreted the data, and reviewed this manuscript. All authors contributed to the article and approved the submitted version.

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CAPÍTULO III

Artigo com dados experimentais para publicação

Análise de células T não convencionais e mediadores imunológicos solúveis em pacientes com Leucemia Linfoblástica Aguda de Células B

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RESUMO

As células T não convencionais existem como subconjuntos de linfócitos que apresentam padrões simplificados de expressão do receptor de antígeno de célula T (TCR). Apesar dessas células apresentarem uma baixa frequência, eles desempenham um papel crucial em várias doenças imunomediadas, inclusive no câncer. Neste estudo, usamos um painel de citometria de fluxo para identificar células T γδ, NKT e células MAIT em amostras de medula óssea [MO (n=25)] e sangue periférico (n=25) de pacientes diagnosticados com LLA-B, e amostras de pacientes sem diagnóstico de doenças onco-hematológicas para o grupo controle [GC (n=25)]. Os resultados revelaram que as células T γδ no sangue periférico (SP) em pacientes com Leucemia Linfoblástica Aguda de Células B (LLA-B) no dia do diagnóstico (D0) apresentaram-se aumentadas com diferença estatística

significativa quando comparado ao grupo controle de pacientes sem a doença. Curiosamente também foi observado um aumento nessas células entre o grupo com LLA-B entre os dias D0 e D35 tanto nas amostras de sangue periférico quanto de medula óssea. As células NKT apresentaram um comportamento semelhante, aumentando sua frequência no sangue periférico em pacientes com LLA-B no dia do diagnóstico (D0). Foi observado um aumento nessas células entre o grupo com LLA-B entre os dias D0 e D35 nas amostras de SP, mas não foi observado o mesmo comportamento quando comparado entre os grupos de amostra de MO nos tempos D0 e D35. Em relação as células MAIT houve um aumento significativo quando comparado o D0 de pacientes com LLA-B com o GC, e quando comparado o grupo de pacientes com LLA-B na MO nos tempos D0 e D35. Em paralelo, foi demonstrado que a maioria dos mediadores imunológicos solúveis, tais como citocinas, quimiocinas e fatores de crescimento que tiveram um aumento significativo em diferentes compartimentos (SP e MO) em diferentes tempos de indução ao tratamento da doença em relação ao grupo controle, com exceção de IFN-γ, IL-17 e PDGF que foi observado aumento apenas no D35. O papel desempenhado pelas células T não convencionais encontradas no sangue periférico e no microambiente medular neoplásico e a produção de citocinas podem estar relacionadas com a resposta imune antitumoral de pacientes com leucemia linfoblástica aguda de células B comum e supostamente estão participando para manutenção ou não da patogênese da doença após o tratamento quimioterápico.

Palavras-chaves: Células T não convencionais; leucemia linfoblástica aguda; microambiente tumoral; citocinas.

INTRODUÇÃO

As leucemias compreendem um grupo heterogêneo de neoplasias hematológicas, que podem ser classificadas em leucemias linfoblásticas ou mieloblásticas e divididas em tipos agudos e crônicos, dependendo do tipo de célula afetada, estágio de maturação e contagem de blastos, respectivamente[1]. Enquanto as leucemias agudas são caracterizadas por um bloqueio profundo na diferenciação hematopoiética e resultam em uma superprodução de blastos imaturos, as leucemias crônicas são caracterizadas pela produção excessiva de células diferenciadas parcialmente maduras[2,3].

Os mecanismos imunológicos em pacientes com leucemia são pouco conhecidos. No entanto, com o aumento dos avanços nas pesquisas sobre o microambiente tumoral na leucemia, houve grande progresso no campo da imunoterapia. Estudos demonstraram que as LCs secretam fatores que perturbam os nichos saudáveis da MO, reprogramando-os e transformando-os em "nichos leucêmicos", além de induzir uma interrupção na produção equilibrada de citocinas e favorecer a persistência leucêmica e o potencial metastático[4,5]. No entanto, apesar do microambiente pró-tumoral criado pelas células leucêmicas (CL), estudos relatam que uma resposta imune específica pode ser desencadeada e, portanto,

contribuir para a defesa contra o tumor, embora não seja suficiente para controlar a neoplasia[6].

Vários estudos têm descrito o potencial imunoterapêutico de células T CD8 e CD4 que reconhecem antígenos peptídicos (Ag) apresentados por moléculas polimórficas do complexo principal de histocompatibilidade (MHC) classe I e MHC classe II, respectivamente[7]. Algumas populações de células T são consideradas "não convencionais", que também estão implicadas na imunidade tumoral, embora seu papel nelas não seja bem compreendido. Coletivamente, essas populações de células T diferem de suas contrapartes convencionais principalmente na maneira como reconhecem e respondem a moléculas estranhas[8].

Ao contrário das células T reativas ao MHC, as células T não convencionais geralmente apresentam padrões simplificados de expressão do receptor de antígeno de célula T (TCR) e geralmente têm como alvo moléculas monomórficas apresentadoras de Ag e outros ligantes, onde, após sua ativação, promovem rápida e forte respostas efetivas[9]. Essas populações de células T incluem células T γδ, células NKT e células MAIT[10].

Dessa maneira, no contexto da LLA-B, a contribuição do nosso trabalho com o objetivo de estimar a frequência dessas células T não convencionais, e de seus produtos (citocinas, quimiocinas e fatores de crescimento) para a conversão do microambiente normal em nichos leucêmicos é um tópico ainda distante de ser totalmente elucidado, mas tornou-se, um alvo atraente em termos para futuras pesquisa translacionais para melhorar o manejo clínico da doença ([11-13]).

MATERIAL E MÉTODOS

Aspectos éticos

Este estudo faz parte de um projeto maior intitulado "*Biomarcadores celulares e moleculares envolvidos na resposta imunológica de pacientes com leucemia linfoblástica aguda: novas abordagens aplicadas ao diagnóstico, prognóstico e terapêutica*" aprovado pelo Comitê de Ética em Pesquisa (CEP) da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), sob número de parecer 4.982.395/2021 e CAAE 51257921.2.0000.0009.

População do estudo

A população do estudo foi constituída de pacientes atendidos no serviço de hematologia pediátrica da Fundação HEMOAM com diagnóstico de LLA-B, obtidas por demanda espontânea no período de março de 2021 a março de 2023.

Além dos pacientes, foram recrutadas crianças (sem leucemia) atendidas na triagem clínica da Fundação HEMOAM, que realizarem o hemograma de rotina e cujo resultado não apresentaram alterações em nenhum dos constituintes sanguíneos, nem infecções detectáveis pelos testes realizados de sorologia.

Critério de exclusão

Como critérios de exclusão, foram adotados os seguintes parâmetros: pacientes diagnosticados com LLA que não sejam classificados em LLA-B comum, material biológico insuficiente ou coagulado.

Obtenção das Amostras

Para este estudo, foram utilizadas amostras de SP e MO de pacientes pediátricos com suspeita de Leucemia Aguda, obtidas através de punção aspirativa da crista ilíaca, esterno ou tíbia e através de punção venosa, respectivamente. A obtenção destas amostras ocorreu paralelamente aos procedimentos médicos habituais, de modo que foram aproveitadas da rotina médica para a pesquisa. As coletas ocorreram em dois *timepoints* da quimioterapia de remissão, sendo estes: dia do diagnóstico (Dia 0, D0) e no 35º dia da terapia de indução da remissão (Dia 35, D35). Foram coletados 2ml de MO e 3ml de SP em tubos com sistema a vácuo, contendo anticoagulante EDTA (ácido etilenodiamino tetra-acético) pela equipe médica da Fundação HEMOAM.

Diagnóstico de LLA-B

Para o diagnóstico e a caracterização imunofenotípica em LLA-B, foram utilizadas amostras de aspirado medular que apresentaram 20% ou mais de blastos na leitura da lâmina de Mielograma. Essas células, na imunofenotipagem (IF), apresentaram-se de tamanho pequeno e baixa complexidade interna, com intensidade de fluorescência fraca a moderada para CD45. Em seguida, foi realizada a IF para especificar a linhagem celular transformada e a subclassificação em LLA-B comum, através da identificação de antígenos de membrana, citoplasmáticos ou nucleares, também conhecidos como clusters

de diferenciação (CD), presentes na superfície das CLs ou em seu compartimento intracelular **(Tabela 3).**

Imunofenotipagem de LLA-B comum e células T não convencionais

Foi realizado a imunofenotipagem (IF) por citometria de fluxo para classificação das Leucemias Linfoblásticas de Célula B comum, e também IF das subpopulações de células T gamma/delta (γδ) (Anti-CD3, ANTI-γδ, anti-CD45, Anti-CD95), células NKT (anti-CD3, Anti-CD16, anti-CD45, Anti-CD56 e anti-CD161) e células MAIT (anti-CD3, anti-Vα7.2, anti-CD45, e anti-CD161), tanto de amostras de SP quanto de MO, pela técnica de Citometria de Fluxo. As subpopulações celulares propostas neste estudo foram marcadas utilizando um painel de anticorpos monoclonais conjugados com fluorocromos específicos para os marcadores de cada população celular (FITC, PE, PERCP, PE-Cy7, APC e APC-Cy7 ou APG-H7). Os resultados obtidos na imunofenotipagem foram analisados através do software FlowJo (v10).

Dosagem de citocinas, quimiocinas e fatores de crescimento para a quantificação de biomarcadores solúveis sistêmicos

O plasma obtido das amostras de SP e MO nos tempos D0 e D35 da quimioterapia de remissão foram armazenados em biorrepositório para a dosagem de mediadores solúveis. Para as quantificações dos biomarcadores solúveis sistêmicos utilizou-se a plataforma Luminex Bio-Plex Pro™ human cytokines que permitiu a pesquisa dos respectivos analitos: CXCL8, CCL11, CCL3, CCL4, CCL2, CCL5, CXCL10, IL-1β, IL-6, TNF-α, IL-12(p70), IFN-γ, IL-15, CXCL10, CXCL11, IL-1β, IL-1Ra, IL-4, IL-6, IL-7, IL-10, IL-13, IL-17, IL-1Rα, IL-4, IL-5, IL-9, IL-10, il-13, FGF-Básico, PDGF, VEGF, G-CSF e GM-CSF, através do software *Luminex xPONENT* versão 3.1.

Análise de dados

Os dados demográficos (idade e sexo), epidemiológicos e características clínicas dos pacientes foram apresentados em formas de tabelas e gráficos, elaboradas com o programa Excel 2013 (*Microsoft Corporation, Redmond, WA, USA*). As análises estatísticas foram realizadas com os softwares *Graphpad Prism* (v8) e *Stata* (v15). Inicialmente, foi realizado teste para verificar normalidade dos dados através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com teste Mann-Whitney, enquanto para as comparações das variáveis com três ou mais grupos, com o teste Kruskal-Wallis, seguido de pós-teste de Dunn's, para múltiplas

comparações entre os grupos. Para a construção, das redes biológicas para demonstração das interações complexas entre as subpopulações de células T não convencionais e os mediadores imunológicos solúveis avaliados, foi realizada com o teste de correlação de Spearman com elaboração das redes com o software Cytoscape® (v3.0.3).

RESULTADOS E DISCUSSÃO

A população do estudo consistiu de amostras de Sangue Periférico (SP) de crianças sem leucemia, que foram atendidas no serviço de hematologia pediátrica na Fundação HEMOAM, cujo resultado do hemograma não apresentou alterações, para o GC (n=25), e pacientes que receberam o diagnóstico de LLA-B comum (n=25), sendo para este último utilizadas amostras de SP (LLA-B SP) e amostras de MO (LLA-B MO). Os pacientes do GC apresentaram idade mediana de 10 (IQR 6-18), enquanto com LLA-B a mediana de idade foi de 7 (IQR 4-9) anos. O sexo masculino foi predominante tanto nos pacientes do GC (13/12), quanto nos pacientes com LLA-B (18/7) conforme observado na **Tabela 1.**

Tabela 1. Distribuição dos pacientes dos grupos Controle e LLA-B.

Variáveis	**GC** ***n=25***	**LLA-B** ***n=25***	***Valor - p***
Gênero ***(Masculino/Feminino)***	13/12	18/7	0,243
Idade ***(anos, mediana [IQR])***	10 [6-18]	7 [4-9]	**0,0016**

GC, grupo controle; LLA-B, pacientes com Leucemia Linfoblástica Aguda de Células B; IQR, intervalo interquartil. Diferença significativa em p<0,05 está representada em negrito.

Os dados hematológicos de cada grupo, juntamente com as medianas e os resultados da análise estatística estão descritos na **Tabela 2**. Pacientes com LLA-B no D0, tanto no Sangue Periférico (SP) quanto de Medula Óssea (MO) apresentaram uma diminuição significativa de neutrófilos, hemoglobina e plaquetas em comparação ao GC, refletindo a usual tríade da leucemia, caracterizada por anemia, neutropenia e trombocitopenia[14].

Não foi observado um aumento de leucócitos totais no grupo LLA-B (SP-D0) em relação ao GC. No entanto, houve uma diferença significativa entre o grupo LLA-B (SP-

D35) quando comparado aos grupos (SP D0) e CG. Além disso, o grupo LLA-B (MO-D0) apresentou um aumento significativo no número de leucócitos totais quando comparado ao grupo LLA-B (MO-D35), corroborando com a ideia de que em alguns casos nem sempre pode-se observar uma leucometria alta no SP, com a presença rara de blastos naqueles pacientes leucopênicos, mas uma presença numerosa naqueles com leucocitose na MO [12-14].

Na LLA-B, os marcadores mais importantes incluem CD10, CD19 e CD79a (196, 197). O pool menos diferenciado da linhagem B expressa marcadores mais precoces, que são CD19, CD22 e CD79a, definindo, por tanto, a LLA Pró-B. A positividade para CD10 (antígeno *Calla*) define o subtipo LLA-B comum, e a expressão da cadeia mμ pesada citoplasmática de imunoglobulina (Ig) define o subtipo Pré-B; enquanto a expressão adicional de Ig de superfície, com expressão fraca ou ausente de CD10, define a LLA-B madura[15].

Tabela 2. Dados do Hemograma de pacientes do grupo controle e pacientes do grupo LLA-B, sendo estes últimos estratificados nos tempos D0 e D35.

Parâmetros	**GC (n=25)**	**LLA-B (n=25)**			
		SP		**MO**	
		D0	**D35**	**D0**	**D35**
Hemácias	4,9 (4,5 - 5,2)	2,7 (2,4 – 3,3)[a]	3,0 (2,8 – 3,3)[b]	2,5 (2,0 – 3,0)[d]	2,9 (2,6 – 3,3)
Hemoglobina	14,0 (12,7 - 14,4)	7,7 (6,6 – 9,1)[a,c]	8,6 (8,1 – 9,6)[b]	7,0 (5,6 – 8,2)[d]	8,8 (7,6 – 9,6)
Plaquetas	285 (180 - 364)	34 (20 – 87)[a,c]	155 (68 – 256)[b]	32 (23 – 65)[d]	98 (49 – 201)
Leucócitos Totais	7,4 (6,1 - 9,1)	6,5 (2,8 – 18,3)[c]	2,4 (1,8– 5,2)[b]	33,3 (7,0 – 120)[d]	6,6 (3,3 – 17)
Neutrófilos	3,7 (3,0 – 4,5)	0,4 (0,1 – 2,1)[a]	1,0 (0,3 – 1,7)[b]	0,6 (0,1 – 3,3)[d]	3,0 (0,7 – 6,3)

SP, sangue periférico; MO, medula óssea; D0, dia do diagnostico; D35, trinta e cinco dias após o diagnóstico; IQR, intervalo interquartil. A análise descritiva está expressa em Mediana (IQR).

Valor de referência: Hemácias: 4,5 - 6,0 x10^6/μL; Hemoglobina: 12 - 18 g/dL; Plaquetas: 130 - 400 x10^3/μL; Leucócitos Totais: 5,2 - 12,4 x10^3/μL; Neutrófilos: 1,9 - 8,0 x10^3/μL; Eosinófilos: 0,1 - 5,0 x10^3/μL; Basófilos: 0,0 - 2,0 x10^3/μL.

[a]Diferença significativa de SP-D0 para o GC; [b]Diferença significativa de SP-D35 para o GC; [c]Diferença significativa de SP-D0 para SP-D35; [d]Diferença significativa de MO-D0 para MO-D35; Fonte em negrito indica significância estatística em $p<0,05$.

Tabela 03. Perfil imunofenotípico dos Linfoblastos encontrados na Medula Óssea (MO) de pacientes diagnosticados com Leucemia Linfoblástica Aguda de Células B comum.

[1] ID dos Pacientes	[2] Tamanho	[3] Complexidade	[4] Expressão de marcador Pan-Leucocitário	[5] IF dos marcadores para linhagem Linfoblástica de células B						[6] IF de marcadores de imaturidade		[7] IF de marcador anômalo-mieloide
	(FSC)	*(SSC)*	CD45	CD10	CD19	CD20	cCD79a	CD81	cIgM	CD34	CD38	CD66c
001	Pequeno	Baixa	Fraca	+	+/++	-/+	+	+/++	(-)	-/+	-/+	-/+
002	Pequeno	Baixa	Fraca	-/+	++	-/+	+/++	++	(-)	-/+	+/-	(-)
003	Pequeno	Baixa	Fraca	+/-	+/++	(-)	+	+/++	(-)	+/-	-/+	+/-
004	Pequeno	Baixa	Moderada	-/+	+/++	-/+	+/-	+/++	(-)	+/++	(-)	+/-
005	Pequeno	Baixa	Fraca	+	+/++	(-)	+	+	(-)	+	-/+	+/-
006	Médio	Baixa	Fraca	++	+/++	(-)	+	+	(-)	-/+	(-)	+/-
007	Pequeno	Baixa	Moderada	+/++	+/-	(-)	+/-	+	(-)	(-)	-/+	-/+
008	Médio	Baixa	Fraca	++	+/++	(-)	+/-	+	(-)	+	-/+	+/-
009	Médio	Baixa	Fraca	++	+	(-)	+	+	(-)	+/-	(-)	+
010	Pequeno	Baixa	Fraca	+	+/++	-/+	+	+/++	(-)	(-)	+	(-)
011	Pequeno	Baixa	Moderada	-/+	+	(-)	+	+/++	(-)	+	+/-	+/++
012	Pequeno	Baixa	Moderada	+	++	(-)	++	+	(-)	(-)	(-)	(-)
013	Pequeno	Baixa	Moderada	+/-	+/++	(-)	+/-	+/++	(-)	+	(-)	+/++
014	Pequeno	Baixa	Fraca	+/++	+/++	(-)	+	+	(-)	-/+	-/+	+/-
015	Pequeno	Baixa	Moderada	+/-	++	(-)	+	++	(-)	(-)	+/-	+/-
016	Pequeno	Baixa	Moderada	++	++	(-)	+	++	(-)	+/-	-/+	+/-
017	Pequeno	Baixa	Moderada	+/++	+	(-)	+/-	++	(-)	(-)	+/-	(-)
018	Pequeno	Baixa	Fraca	+/++	+/++	(-)	+	+	(-)	-/+	+/-	(-)
019	Pequeno	Baixa	Fraca	+/++	+/++	(-)	+/-	+	(-)	-/+	-/+	(-)
020	Grande	Media	Ausente	++	++	+	++	++	(-)	+	-/+	(-)
021	Pequeno	Baixa	Fraca	++	+/++	(-)	+	+/-	(-)	+/-	-/+	+/++
022	Pequeno	Baixa	Moderada	+	++	(-)	+	+/++	(-)	-/+	+	++
023	Pequeno	Baixa	Fraca	+/++	+	-/+	+/-	++	(-)	-/+	+/-	-/+
024	Pequeno	Baixa	Fraca	+/++	+/++	(-)	+/-+	+	(-)	+	-/+	(-)
025	Pequeno	Baixa	Fraca	++	+/++	-/+	+	+/++	(-)	-/+	+/-	+

[1] ID: Identificação dos pacientes. [2] Tamanho (*FFC: Forward Scatter* – Classificação do tamanho em pequeno, médio ou grande). [3] Complexidade (*SSC: Side Scatter* – Classificação da complexidade/granulosidade em baixa, média ou alta). [4] Expressão Do Marcados Pan-Leucocitário CD45: Ausente, Fraca ou Moderada. [5] IF: Intensidade da Fluorescência dos Marcadores de Linfoblastos de células B comum. [6] IF: Intensidade da Fluorescência dos Marcadores de imaturidade. [7] IF: Intensidade da Fluorescência de Marcador de Anômalo (mieloide). Intensidade de Fluorescência: +: Intensidade Moderada. ++: Intensidade Forte. +/-: >50% das células positivas, com Moderada Intensidade. -/+: <50% das células positivas, com Moderada Intensidade.

Nossos dados apontam que 17/25 pacientes chegaram a fase de Consolidação de Remissão da doença com classificação em medula M1 (< 5% na contagem de blastos), 6/25 M2 (intervalo entre > 5% e < 25% na contagem de blastos) e apenas 2/25 com classificação M3 (> 25% na contagem de blastos) (Tabela 4). A análise desses parâmetros pode indicar a necessidade de mudanças na abordagem terapêutica e no grupo de risco, de modo a melhorar o suporte clínico e aumentar a sobrevida dos pacientes com a doença [16].

A análise de DRM é um importante indicador clínico de resposta à terapia, pois consiste na avaliação de CLs residuais sobreviventes à quimioterapia e que são capazes de repovoar a MO, resultando em episódios de recidiva da doença, caso não tratada. Dessa forma, quando um paciente exibe níveis de DRM ≥0.01% (10^{-4}) de CLs na MO e no SP o risco de recidiva é significativamente maior do que aqueles pacientes que exibem níveis inferiores a 0.01%. Por tanto, quanto maior o valor de DRM ao final da fase de indução, maior é o risco de recaída e menor é a taxa de sobrevida, de acordo com diferentes relatórios[17].

A Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) atualmente adota os critérios estabelecidos pelo GBTLI-2009 na rotina clínica para o tratamento da LLA-B comum. Este protocolo é organizado em 6 fases, sendo elas as terapias de Pré-fase, Indução da Remissão, Consolidação da Remissão, Intensificação, Consolidação Tardia, e, por último, Manutenção. Neste protocolo, a estratificação de grupos de risco baseia-se na resposta à terapia, avaliada no 8° dia da fase de indução (D8) pela contagem de blastos no SP, no 15° dia da fase de indução (D15) pela avaliação morfológica da MO e DRM por citometria de fluxo, e no 35° dia da terapia de indução (D35) através da análise morfológica da MO[18].

Tabela 04. Leucometria e quantitativo de blastos na Medula Óssea (MO) dos pacientes com LLA-B nos tempos D0 (dia do diagnóstico do paciente), D15 (quinze dias após o início da Terapia de Indução de Remissão da Doença, de acordo com o protocolo estabelecido), para pesquisa de Doença Residual Mensurável (DRM) e D35 (trinta e cinco dias após o início do tratamento**),** para avaliação medular.

	*DIAGNOSTICO		**DRM		***REMISSAO MORFOLOGICA		
TEMPOS	**D0**		**D15**		**D35**		
ID. DOS PACIENTES	**LEUCOMETRIA (/mm3)**	***BLASTOS (%)**	**LEUCOMETRIA (mm3)**	****BLASTOS (%)**	**LEUCOMETRIA (mm3)**	*****BLASTOS (%)**	**CLASSIFICACAO MEDULAR**
001	5.760	58 %	1.010	0,5%	2.320	<5 %	M1
002	521.400	93 %	7.260	15%	15.240	>25%	M3
003	111.900	90 %	12.090	0,3 %	34.050	5 - 25%	M2
004	231.500	88 %	2.590	10,8 %	28.200	5 - 25%	M2
005	12.630	75 %	3.070	9,10 %	3.120	<5 %	M1
006	6.350	82 %	940	0,27 %	4.860	<5 %	M1
007	5.600	65 %	1.910	0,1 %	6.680	<5 %	M1
008	270.400	95 %	2.590	21,2 %	3.270	5 - 25%	M2
009	5.420	73 %	6.430	6,4 %	4.510	<5 %	M1
010	13.990	63 %	3.410	0	10.020	<5 %	M1
011	114.400	79%	5.190	16,0 %	25.880	5 - 25%	M2
012	3.640	32 %	5.680	1,3 %	15.59	<5 %	M1
013	31.930	67 %	1.140	26,2 %	49.280	<5 %	M1
014	8.790	60 %	2.950	25,9 %	1.200	<5 %	M1
015	33.350	61 %	76.440	38,1 %	3.420	5 - 25%	M2
016	7.670	21 %	14.290	3,3 %	20.850	<5 %	M1
017	92.570	84 %	3.750	0,11 %	4.970	<5 %	M1
018	9.960	78 %	3.620	11,4 %	1.940	<5 %	M1
019	109.900	95 %	2.160	0,11 %	4.110	5 - 25%	M1
020	5.740	20 %	520	0,7 %	2.150	<5 %	M1
021	594.100	88 %	3.860	35,8 %	12.670	>25%	M3
022	8.140	22 %	480	0,04 %	10.300	<5 %	M1
023	212.600	88%	5.640	0,001 %	14.510	5 - 25%	M2
024	125.800	85%	1.260	0	4.830	<5 %	M1
025	74.090	95%	9.670	2 %	19.710	<5 %	M1

ID: Identificação dos pacientes. D0: Dia do diagnóstico do paciente. D15: 15 dias após o início do tratamento. D35: 35 dias após o início do tratamento. Doença Residual Mensurável (DRM). M1: < 5% na contagem de blastos. M2: intervalo entre > 5% e < 25% na contagem de blastos. M3: > 25% na contagem de blastos. (%): percentual. (/mm^3): por milímetros cúbicos* Imunofenotipagem por Citometria de Fluxo. ** Imunofenotipagem por Citometria de Fluxo. ***Leitura do Mielograma por Microscopia Óptica.

Em relação a frequência das células T não convencionais, em nossos dados, observou-se que os pacientes com LLA-B apresentaram uma frequência aumentada de células T γδ no momento do diagnóstico (D0) (**Figura 6**). Houve uma diminuição no D0 e D35 no SP de pacientes com LLA-B em relação ao GC, porém houve um aumento estatisticamente significativo quanto a essas células nos tempos D0 e D35 na MO.

Alguns estudos *in vitro* mostraram que células T não convencionais reconhecem e destroem os blastos de leucemia, mas os mecanismos adjacentes ainda são pouco estudados. A exemplo, análises revelaram uma abundância de células T γδ infiltrantes de tumor em coortes de pacientes com leucemia[19]. Essa alta frequência foi positivamente correlacionada com a sobrevida desses pacientes. Da mesma forma, foi relatado que as células Tγδ têm porcentagens aumentadas em pacientes com LLC e que esse subtipo se expande no sangue periférico, exibindo alta liberação de grânulos citotóxicos contendo granzimas B Tomados em conjunto, esses dados sugerem que a leucemia afeta a frequência de células T não convencionais e que essas células têm alguma influência durante a regressão ou progressão da doença[20-21].

Um estudo de Weinkove e colaboradores mostrou que a frequência de células NKT foi menor em pacientes com leucemia crônica em comparação com o grupo controle saudável e que a terapia por 14 dias com IFN-γ e 1 μM IM (mesilato de imatinibe) em combinação com 100 ng/ml de α- GalCer nesses pacientes resultou em um aumento significativo nas células NKT. Da mesma forma, Weinkove et al. demonstraram que LCs purificadas e estimuladas in vitro com 200 ng/ml de α-GalCer por 5 dias incitaram a proliferação de células NKT autólogas e alogênicas, mas não em quantidade significativa também foi demonstrado que houve estimulação de produção de IFN-γ por células NKT em pacientes leucêmicos. No entanto, observou-se que a cultura prolongada dessas células resultou na polarização das células NKT para um perfil Th2 e resultou em altos níveis de citocinas associadas à tolerância ao tumor[22].

As células MAIT, como sua alta frequência em humanos e sua capacidade de secretar rapidamente um repertório de mediadores que induzem ativação e regulação de outras células do sistema imunológico, além da capacidade de reconhecer antígenos restritos a MR1, a pesquisa com células MAIT, bem como outras populações de células T restritas a MR1, tem despertado grande interesse na área da oncologia. Atualmente, o papel funcional desencadeado por essas células T inatas no câncer não é claro e tem sido objeto de vários estudos[23].

As células T não convencionais podem promover a rejeição do tumor e oferecer vantagens que as indicam como alvos potenciais para a imunoterapia baseada em células T. Embora eficazes, é importante notar que as células T não convencionais não estão isentas da influência dos receptores de checkpoint, uma vez que essas células regulam positivamente o receptor PD1 inibitório em sua superfície celular após a ativação [185-187]. No entanto, a terapia de bloqueio de checkpoint usando drogas baseadas em anti-PD1 e anti-CTLA-4 está provando ser uma abordagem poderosa para impedir que as células efetoras entrem em um estado de anergia causado por células cancerígenas, proporcionando assim uma resposta imune persistente [188-24].

O fato é que o papel das células T não convencionais durante o processo neoplásico costumam estar relacionado a uma melhor vigilância imunológica ou resposta antitumoral em pacientes com leucemia, e que, até o momento, as células T não convencionais ainda são pouco exploradas. Fatores, como a ausência de barreiras relacionadas à histocompatibilidade, já que as moléculas que apresentam Ag para células T não convencionais são monomórficas; ativação por mecanismos dependentes e independentes de TCR; capacidade de reunir respostas rápidas e poderosas; além da alta frequência em tecidos específicos em humanos, todos demonstram a necessidade de mais estudos sobre o potencial imunoterapêutico dessas células e, principalmente, a tradução desses estudos em ensaios clínicos.

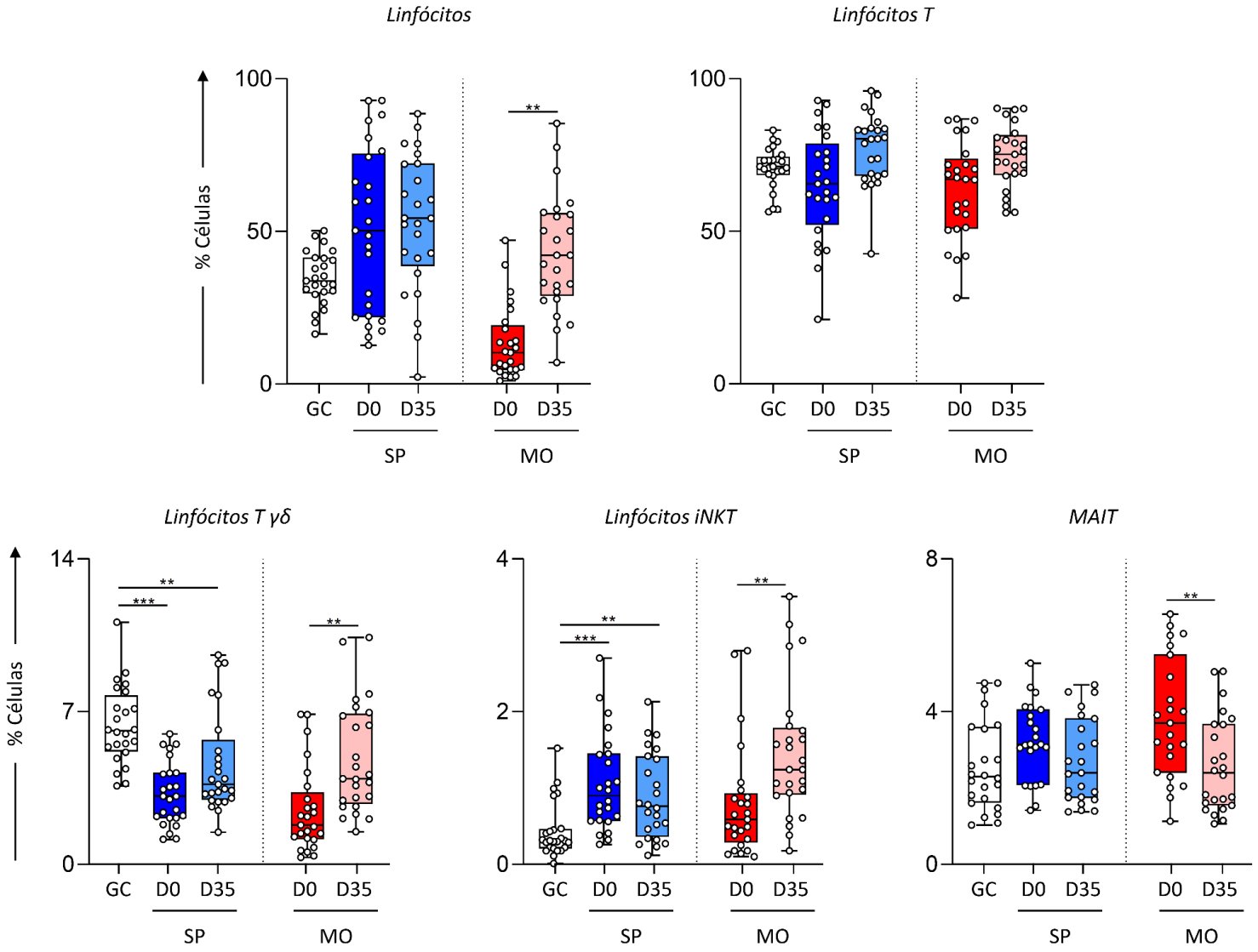


Figura 05. Frequência de células Tγδ, NKT e MAIT no sangue periférico (SP) e medula óssea (MO) de pacientes com LLA-B nos tempos D0 e D35. O Grupo Controle (GC) foi composto apenas por amostras de Sangue Periférico de crianças atendidas na Fundação HEMOAM. Para verificar a normalidade dos dados, foram realizadas análises através do teste Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com Mann-Whitney. Enquanto para a comparação das variáveis com três ou mais grupos, foi realizada o teste de Kruskal-Wallis. Diferenças significativas são indicadas por linhas de conexão asteriscos para p <0,01 (*), p <0,05 (**) ou p <0,001 (***).

Dados sobre a complexa rede de mediadores imunológicos solúveis na circulação e, especialmente na medula óssea de pacientes com LLA-B ainda são limitados, representando um vasto campo para investigações. Nesse sentido, com objetivo de fornecer um panorama sobre a complexa rede imunológica na LLA, bem como sua correlação com as células T não convencionais, foi realizado a dosagem de 26 analitos, incluindo quimiocinas, citocinas pró-inflamatórias, citocinas regulatórias e fatores de crescimento celular (**Figuras 7A, 7B, 7C e 7D, respectivamente**).

De maneira geral, os dados demonstram que os pacientes pediátricos com LLA exibiram um aumento significativo nos níveis de mediadores imunológicos solúveis, ao D0 e D35, em comparação aos níveis observados em amostras de sangue periférico do GC, indicativo de uma intensa e complexa resposta imunológica. Diferentemente do perfil da maioria dos mediadores solúveis, foi observado uma diminuição significativa da quimiocina CXCL10 e da citocina regulatória IL-10, tanto na circulação, quanto no compartimento medular. Esses achados corroboram com estudos anteriores realizados pelo grupo de pesquisa, que demonstraram o declínio de CXCL10 e IL-10 ao D35, no compartimento medular. Além disso, através de redes biológicas, o estudo demonstrou uma forte correlação estabelecida entre os dois mediadores[19].

A quimiocina CXCL10, juntamente com CXCL9 e CXCL11, correspondem a ligantes seletivos para CXCR3. Em condições normais, o eixo CXCL9-10-11/CXCR3 regula a migração e ativação de células imunes e exibe um efeito antitumoral[20]. No entanto, investigações mostraram que, no contexto do câncer, o eixo CXCL9-10-11/CXCR3 é capaz de contribuir para a proliferação e metástase de células cancerígenas [21]. Estudo envolvendo pacientes pediátricos com LLA, demonstrou que a CXCL10 induzia quimiotaxia de células leucêmicas que expressavam CXCR3, e diminuiu a apoptose induzida por quimioterapia nas células leucêmicas CXCR3^{+} [22,24,27,28].

De maneira similar, a IL-10 também tem demonstrado exibir um importante papel negativo, relacionado a diminuição de respostas citotóxicas especificas e a estimulação da imunossupressão celular apresentada por estes pacientes no momento do diagnostico, [25]. Altos níveis de IL-10 na circulação e no microambiente tumoral têm sido usualmente correlacionados com um pior prognóstico em neoplasias sólidas e malignidades hematológicas, incluindo LLA, onde estudos relataram um aumento nas porcentagens de células T regulatórias (Treg) junto a níveis elevados de IL-10 em amostras de sangue

periférico, indicando a importância do perfil Treg no processo de imunossupressão destes pacientes[26].

Surpreendentemente, os dados aqui observados indicam que o tratamento quimioterápico pode reverter a imunossupressão celular apresentada por estes pacientes ao D0, visto que é responsável pela eliminação das células neoplásicas. Em concordância, foi observado que os pacientes com LLA-B apresentaram um aumento significativo de IFN-γ ao D35, o que é consistente com achados de estudos anteriores[27]. Além disso, estudos realizados em pacientes com LMA, relatam que as células leucêmicas segregam fatores solúveis que impedem as células T de secretar as citocinas relacionadas ao perfil Th1[28]. O que apoia nosso achado, uma vez que se espera que ao D35 haja a eliminação ou redução da carga leucêmica a células residuais. Por fim, ao D35 também foi observado aumento significativo nos níveis dos fatores de crescimento PDGF, VEGF e GM-CSF, indicando um aumento na atividade hematopoiética [29,30.]

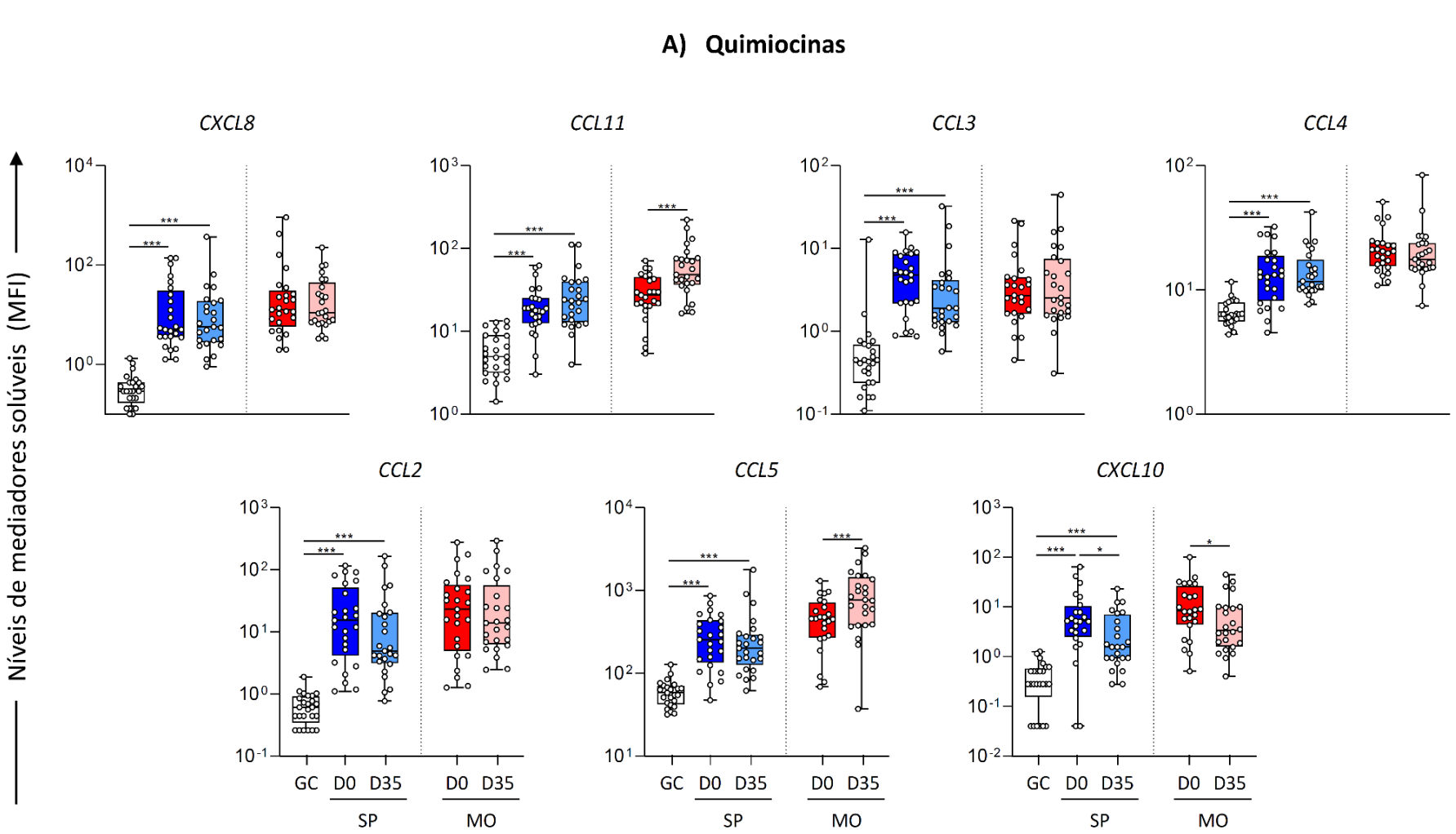


Figura 6a. Dosagem de Quimiocinas no sangue periférico (SP) e medula óssea (MO) de pacientes com LLA-B nos tempos D0 e D35. O Grupo Controle (GC) foi composto apenas por amostras de Sangue Periférico de crianças atendidas na Fundação HEMOAM. Para verificar a normalidade dos dados, foram realizadas análises através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com Teste *t* (Student) ou Mann-Whitney. Enquanto para a comparação das variáveis com três ou mais grupos, foi realizada o teste de Kruskal-Wallis. Diferenças significativas são indicadas por linhas de conexão asteriscos para p <0,01 (*), p <0,05 (**) ou p <0,001 (***).

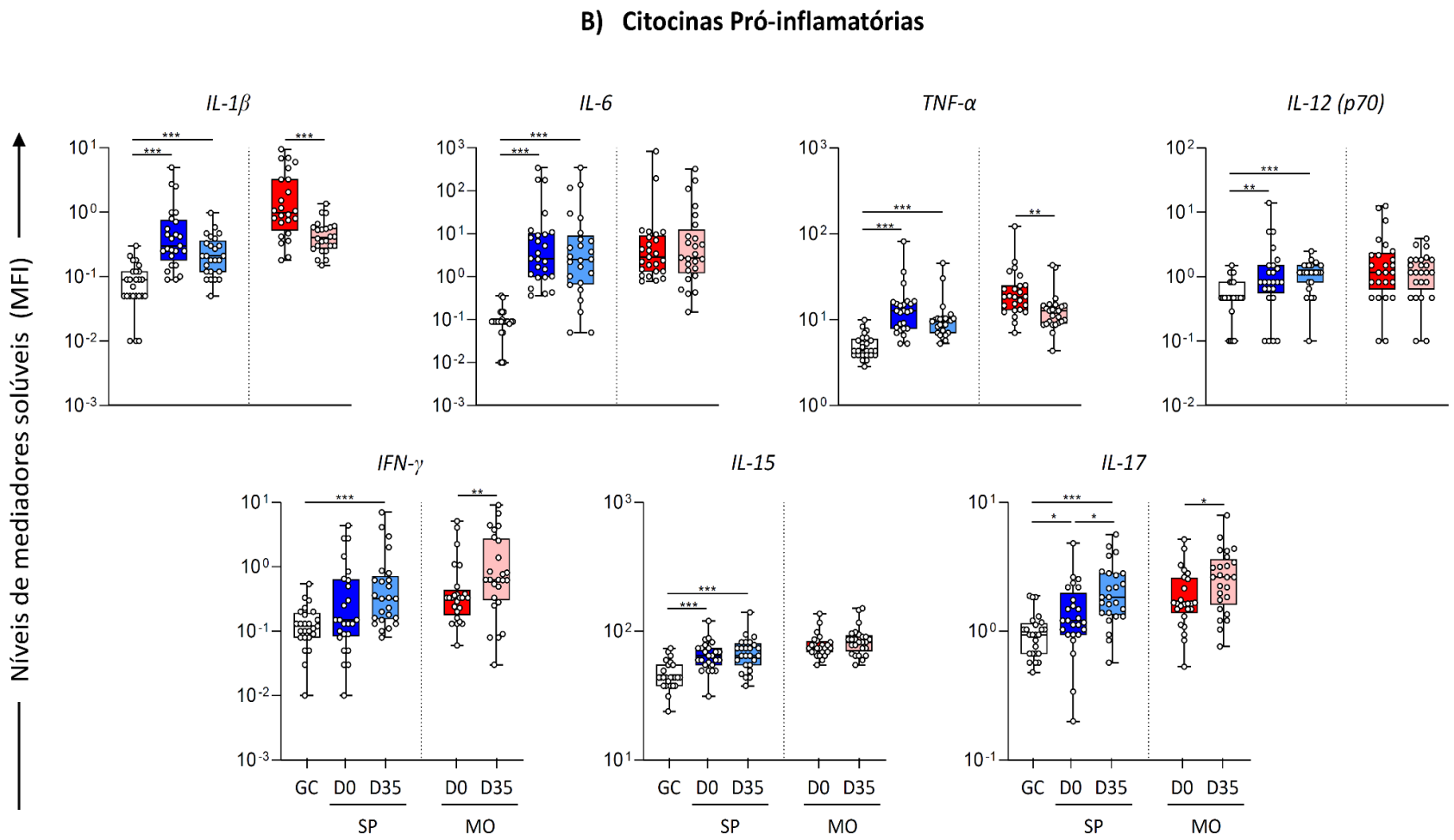


Figura 6b. Dosagem de Citocinas pró-Inflamatórias no sangue periférico (SP) e medula óssea (MO) de pacientes com LLA-B nos tempos D0 e D35. O Grupo Controle (GC) foi composto apenas por amostras de Sangue Periférico de crianças atendidas na Fundação HEMOAM. Para verificar a normalidade dos dados, foram realizadas análises através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com Teste *t* (Student) ou Mann-Whitney. Enquanto para a comparação das variáveis com três ou mais grupos, foi realizada o teste de Kruskal-Wallis. Diferenças significativas são indicadas por linhas de conexão asteriscos para p <0,01 (*), p <0,05 (**) ou p <0,001 (***).

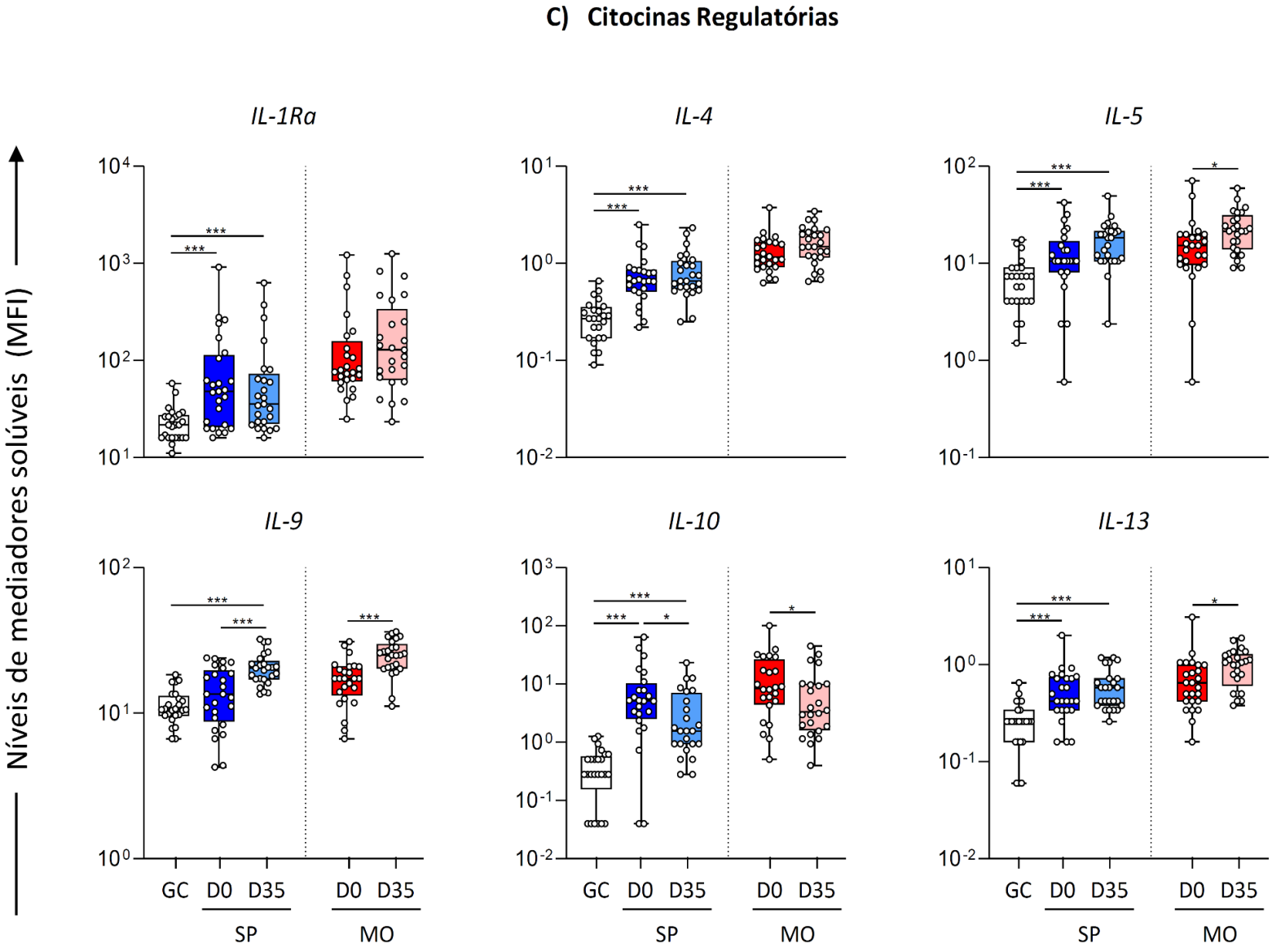


Figura 6c. Dosagem de Citocinas Regulatórias no sangue periférico (SP) e medula óssea (MO) de pacientes com LLA-B nos tempos D0 e D35. O Grupo Controle (GC) foi composto apenas por amostras de Sangue Periférico de crianças atendidas na Fundação HEMOAM. Para verificar a normalidade dos dados, foram realizadas análises através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com Teste *t* (Student) ou Mann-Whitney. Enquanto para a comparação das variáveis com três ou mais grupos, foi realizada o teste de Kruskal-Wallis. Diferenças significativas são indicadas por linhas de conexão asteriscos para p <0,01 (*), p <0,05 (**) ou p <0,001 (***).

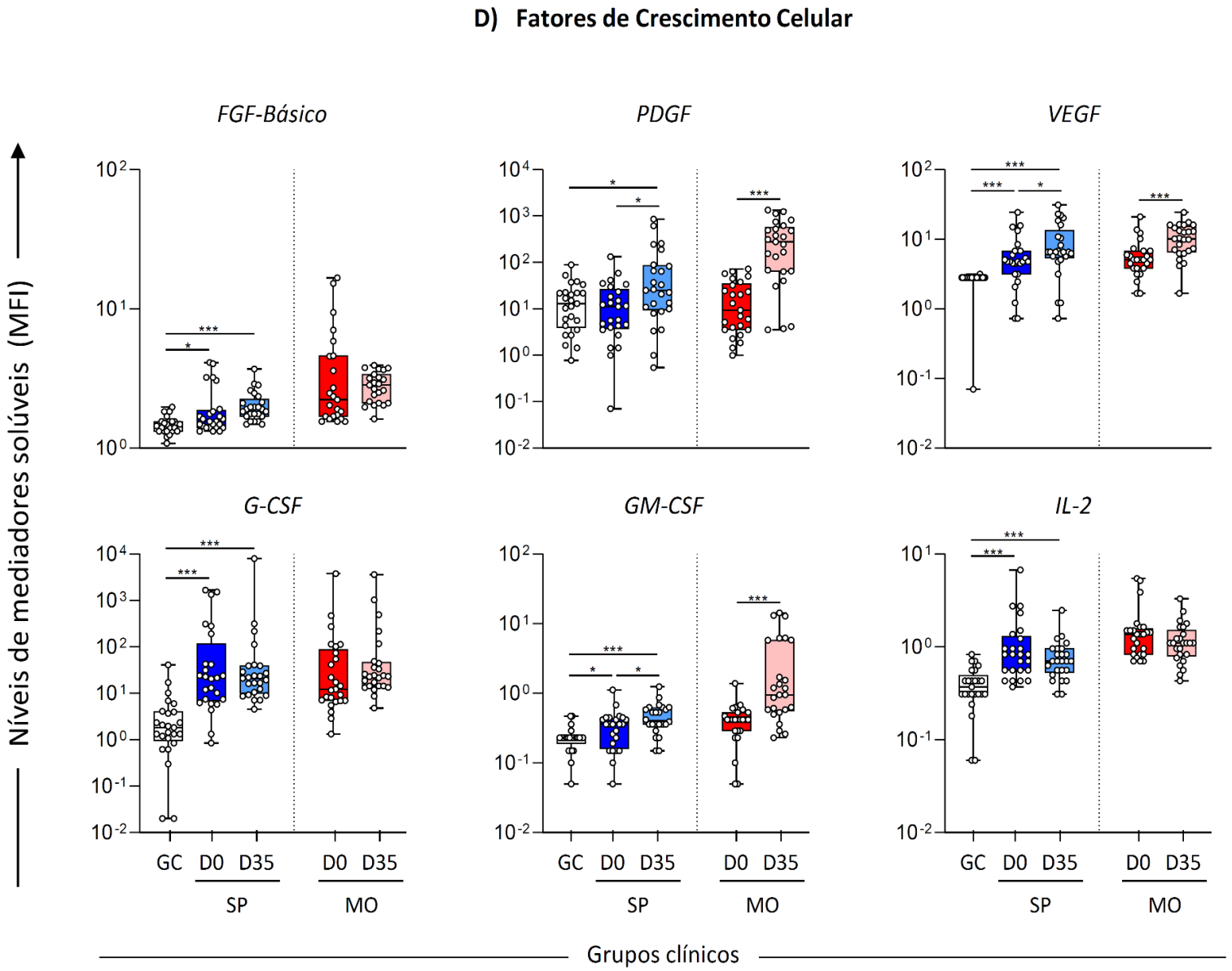


Figura 6d. Dosagem de Fatores de Crescimento no sangue periférico (SP) e medula óssea (MO) de pacientes com LLA-B nos tempos D0 e D35. O Grupo Controle (GC) foi composto apenas por amostras de Sangue Periférico de crianças atendidas na Fundação HEMOAM. Para verificar a normalidade dos dados, foram realizadas análises através do teste de Shapiro-Wilk. As comparações de valores entre dois grupos de dados foram realizadas com Teste *t* (Student) ou Mann-Whitney. Enquanto para a comparação das variáveis com três ou mais grupos, foi realizada o teste de Kruskal-Wallis. Diferenças significativas são indicadas por linhas de conexão asteriscos para p <0,01 (*), p <0,05 (**) ou p <0,001 (***).

Foram construídas redes biológicas integrativas entre as moléculas imunológicas solúveis e as células de interesse ao estudo **(Figura 3).** A análise dos dados demonstrou que pacientes com diagnóstico de LLA-B possuem uma rede de interações imunológicas distintas do Grupo Controle e também entre os compartimentos analisados (Sangue Periférico e Medula Óssea) nos diferentes tempos observados (D0 e D35). Ao D0, no Sangue Periférico vemos a evidente participação das células NKT e Tγδ. Ao comparar no mesmo compartimento ao D35 observamos uma mudança no perfil de participação, com as células NKT mais evidentes, com correlações negativas entre células e moléculas durante o seguimento clínico.

Em comparação com a Medula óssea, ao D0 observa-se a participação massiva e evidente de Linfócitos, com correlações moderadas e positivas entre quimiocinas, citocinas regulatórias e fatores de crescimento. Ao D35 observamos um menor número de correlações entre as células e moléculas, com a ausência da participação dos linfócitos. É observado que o Sangue Periférico (LLA-B SP) tende a refletir o perfil da MO (LLA-B MO) durante a terapia de indução, porém com um número menor de interações que podemos observar entre os compartimentos ao D0. Este fato pode ser explicado pela maior complexidade do microambiente medular, portanto, um maior número de correlações.

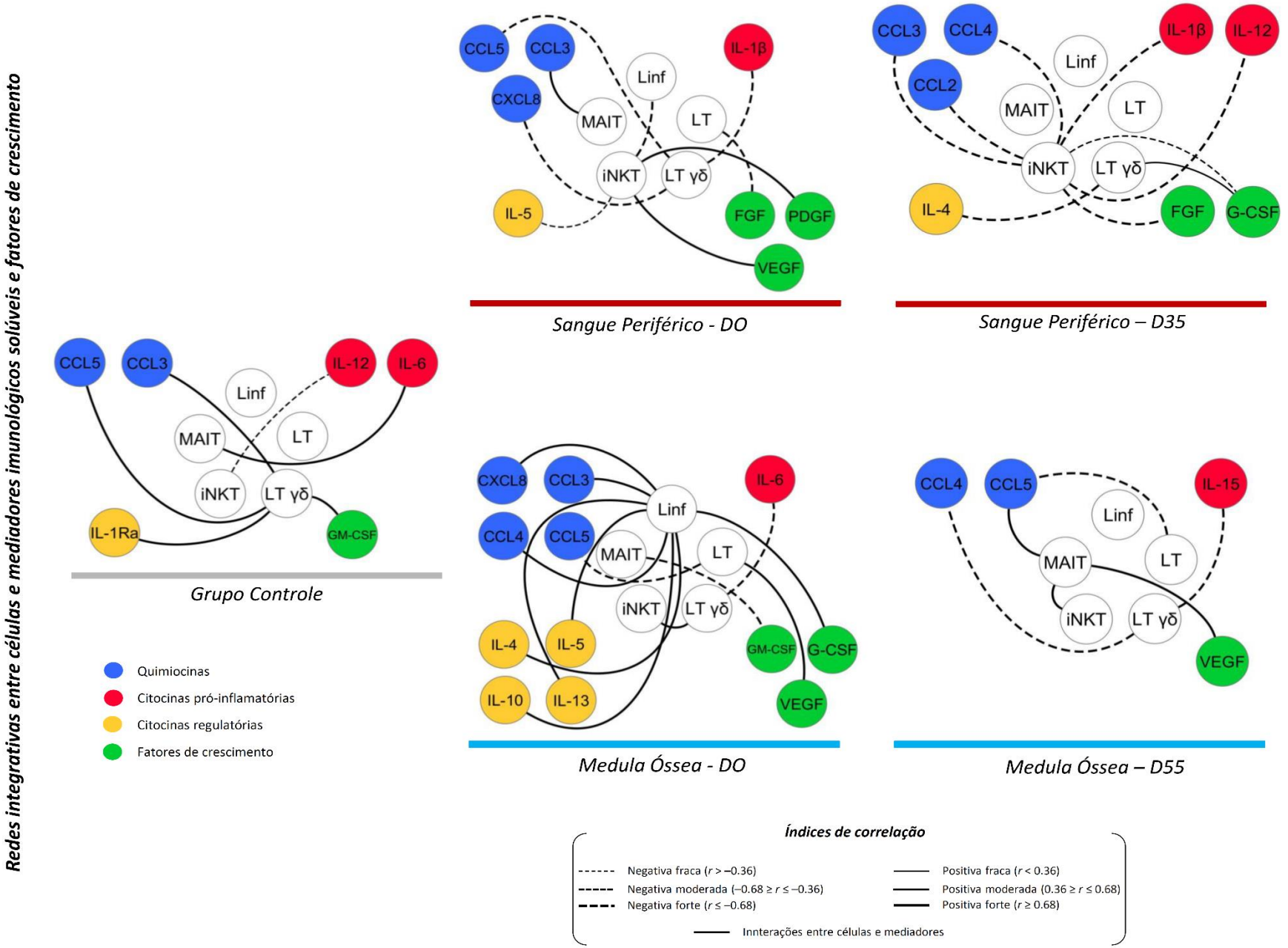


Figura 7. Rede de correlações demostra as interações entre os mediadores inflamatórios e células durante o seguimento de estudo. Cada grupo de cores é usado para identificar as moléculas e suas interações, sendo estas as quimiocinas (■), citocinas pró-inflamatórias (■), citocinas regulatórias (■) e fatores de crescimento (■) nos diferentes compartimentos, Sangue Periférico e Medula Óssea no grupo LLA-B. As linhas tracejadas entre as moléculas indicam uma correlação negativa, enquanto as linhas sólidas indicam uma correlação positiva. A espessura destes indica a força da correlação. As linhas cinzas indicam a correlação entre os parâmetros laboratoriais e moléculas solúveis. O índice de correlação (r) foi utilizado para categorizar a força da correlação em fraca (r≤0,35), moderada (r≥0,36 a r≤0,67) ou forte (r≥0,68).

Esse estudo apresentou algumas limitações, tais como o número de pacientes (n), o painel de marcadores disponíveis para avaliar imunofenotipicamente cada população de células T não convencionais, o número de óbitos.

No entanto, nossos dados demonstraram que a frequência de células T não convencionais na MO e SP podem estar relacionados à forte imunossupressão que os pacientes com LLA-B exibem no momento do diagnóstico. Isto pode ser explicado, em parte, pela alta carga leucêmica, que contribui criticamente para um desequilíbrio imunológico. Além disso, uma tendência de aumento de células T não convencionais na MO sugere que uma resposta imune local esteja ocorrendo neste compartimento; no entanto, é importante que estudos adicionais sejam realizados. Um número maior de amostras e um GC para MO poderia revelar com mais precisão o real significado destas perturbações induzidas pelo microambiente leucêmico e o seu impacto prognóstico.

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CONCLUSÃO

Em conclusão, nosso estudo indica que:

(i) As populações de células T não convencionais têm sido associadas a um repertório de respostas anti- ou pró-tumorais e desempenham papéis importantes na dinâmica de tumores sólidos e malignidades hematológicas, incentivando assim o desenvolvimento de novas investigações na área;

(ii) A revisão 1 que realizamos deu enfoque aos conhecimentos atuais sobre o papel de populações de células T não convencionais na resposta imune antitumoral na leucemia e discutiu porque mais estudos sobre o potencial imunoterapêutico dessas células são necessários;

(iii) Além disso, os avanços na biotecnologia e a compreensão atual da engenharia de células T levaram a novas abordagens nessa luta, melhorando assim a resposta imune mediada por células contra o câncer, principalmente células Tγδ, como demonstrado na revisão 2;

(iv) Nesse sentido, destacamos várias características das células Tγδ e suas interações na leucemia, explorando estratégias para maximizar suas funções antitumorais, visando ilustrar os achados destinados a uma melhor mobilização de células Tγδ contra o tumor.

(v) Finalmente, delineamos nossas perspectivas sobre sua aplicabilidade terapêutica e indicamos questões pendentes para futuras pesquisas básicas e clínicas na leucemia, na esperança de contribuir para o avanço dos estudos sobre células Tγδ na imunoterapia do câncer.

(vi) Por fim, demonstramos com nossos dados originais que pacientes com LLA-B apresentaram uma alta frequência para as células T não convencionais analisadas: Tγδ, NKT e células MAIT, quando comparado com o GC no grupo do SP e também quanto comparado entre o compartimento medular nos tempos D0 e D35.

(vii) Até onde sabemos, este é o primeiro estudo que descreve os percentuais da frequência de células T não convencionais em compartimento medular em pacientes com LLA-B comum.

(viii) A produção de citocinas está diretamente relacionada com a resposta imune antitumoral de pacientes com leucemia linfoblástica aguda de células B comum.

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ANEXO - Carta de aprovação no Comitê de Ética em Pesquisa (CEP) da Fundação HEMOAM.

FUNDAÇÃO DE HEMATOLOGIA E HEMOTERAPIA DO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Biomarcadores celulares e moleculares envolvidos na resposta imunológica de pacientes com Leucemia Linfoblástica Aguda: Novas abordagens aplicadas ao diagnóstico, prognóstico e terapêutica

Pesquisador: Allyson Guimarães da Costa

Área Temática:

Versão: 1

CAAE: 51257921.2.0000.0009

Instituição Proponente: Fundação de Hematologia e Hemoterapia do Amazonas - HEMOAM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.982.395

Apresentação do Projeto:

Trata-se de um estudo observacional, do tipo longitudinal, retrospectivo e prospectivo para avaliação dos componentes celulares e moleculares envolvidos na resposta imunológica de pacientes com LLA, durante a terapia de indução da remissão e terapia de consolidação da remissão dos protocolos de tratamento. As amostras serão coletadas na Fundação HEMOAM, durante os procedimentos de rotina dos pacientes. As coletas ocorrerão em 4 momentos da quimioterapia de remissão, referidos como: diagnóstico (D0), dia 15 da terapia de indução da remissão (D15), final da terapia de indução da remissão (D35) e no dia 84 da terapia de consolidação da remissão (D84) dos protocolos de tratamento, sendo coletados 2 mL de MO e 3mL de SP em tubos com sistema a vácuo, contendo anticoagulante EDTA (Ethylenediaminetetraacetic) pela Equipe Médica da Fundação HEMOAM. Serão incluídas no estudo 158 amostras de DNA e plasma de pacientes com LLA criopreservadas e 203 amostras de DNA e plasma de doadores de sangue. A identificação de biomarcadores imunológicos como instrumento preditivo de prognóstico e resposta terapêutica fornecerá meios para identificar os pacientes que apresentarão uma boa resposta à quimioterapia ou a ausência dela, indicando neste último caso, a necessidade de intervenções terapêuticas alternativas ou mais assertivas de maneira antecipada, diminuindo a duração da exposição a terapias ineficazes e potencialmente toxicas, o que proporcionará melhores resultados e maior qualidade de vida aos pacientes.

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