Immunology Essay 1

***Question**: A 68-year-old patient with chronic lymphocytic leukaemia receives the anti-CD20 antibody Obinutuzumab as part of their treatment. Discuss the role that natural killer cells may have in this scenario and design a novel alternative therapeutic strategy that could lead to enhanced NK cell responses in this setting.*

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Introduction: 221

Chronic lymphocytic leukaemia (CLL) is a $CD5^+$ B-cell malignancy arising from the clonal expansion of mature B lymphocytes. It presents with the accumulation of abnormal, mature B-lymphocytes in the blood, bone marrow and lymphatic tissue [1][2]. This accumulation of cells occurs through defective apoptosis due to overexpression of BCL-2, survival signals from the microenvironment and ongoing proliferation in the lymph nodes [3].

Although defined as a $CD5^+$ B-cell malignancy, CLL cells still retain CD20 at reduced levels on their surface, making it a crucial target for anti-tumour antibodies such as rituximab and Obinutuzumab. Rituximab, a chimeric CD20 monoclonal antibody, was initially used to treat patients with CLL [4]. However, Obinutuzumab was developed to enhance antibody-dependent cellular cytotoxicity (ADCC) through glycoengineering, thereby increasing direct B-cell death and eliciting a stronger clinical response in CLL. A comparison between rituximab and Obinutuzumab is shown in Table 1. Essentially, Obinutuzumab represents a next-generation, engineered evolution of rituximab with more potent anti-tumour activity.

Table 1; Comparison of anti-CD20 monoclonal antibodies Rituximab and Obinutuzumab for the treatment of Chronic Lymphocytic Leukaemia.

Feature	Rituximab	Obinutuzumab
Antibody type	Type 1	Type 2
Fc glycosylation	Fucosylated	Afucosylated
ADCC	Moderate	High
CDC	Strong	Weak
Direct cell death	Low	High
NK activation	Moderate	Strong

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

CLL cells rely on survival signals received in lymphoid tissues from neighbouring non-neoplastic cells within the microenvironment [2]. This is critical for the proliferation and survival of malignant B-cells, with key pathways involving low-level BCR signalling and nurse-like cells secreting chemokines such as CXCL12 and CXCL13. These interactions trigger pro-

tumour pathways, such as PI3K/Akt/mTOR and BTK pathways, preventing apoptosis of malignant CLL cells.

Immune system in response to CLL: 221

CLL cells disrupt both innate and adaptive immunity, creating an immunosuppressive microenvironment that, in turn, increases the incidence of secondary malignancies and other infections.

Innate immunity exhaustion occurs through impaired NK cells, dysfunctional macrophages, neutrophils, and dendritic cells, all of which lose cytotoxic and antigen-presenting functions.

The macrophage population in CLL patients have a pro-tumoral (M2-like) phenotype, termed nurse-like cells. The tolerance of these cells is driven by CLL cells through the secretion of IL-10, adenosine, nicotinamide, and nicotinamide phosphoribosyl transferase [5].

Dendritic cells manifest their dysfunction through an altered cytokine profile, the lack of the maturation antigen CD83, and the co-stimulatory molecule CD80. They cannot activate proper type 1 T cell responses, thus rendering the patient more susceptible to infections, cancers, or autoimmune disorders. This occurs through the upregulation of SOCS5, a negative regulator that prevents STAT6 activation in response to IL-4R signalling, thereby hindering the differentiation of functional, mature dendritic cells and reducing the secretion of pro-inflammatory cytokines [5].

Adaptive immune system abnormalities occur when T cells undergo oligoclonal proliferation due to chronic exposure to malignant B cells. In the early stages, $CD4^+$ and $CD8^+$ T cell counts increase, although the expansion of $CD8^+$ cells soon overtake, resulting in a reduced $CD4^+$: $CD8^+$ T cell ratio [6][7]. B-cell dysfunction occurs through impaired antibody production (hypogammaglobulinemia), increasing vulnerability to infections.

NK cell response to CLL (300): 441

Natural killer cells originate from hematopoietic stem cells in the bone marrow, then mature in peripheral tissues, which instruct NK cells to adopt tissue-specific phenotypes. NK cells adjust based on both their intrinsic ability to adapt and signals from the surrounding microenvironment, leading to highly flexible development to meet the body's shifting demands. Therefore, diseases such as CLL, NK cell development can be altered to suppress cytolytic activity and promote tumour growth [8]. In the setting of CLL, NK cell function is impaired through several mechanisms, summarised in Table 2.

Table 2; Summary of the mechanisms by which NK cell dysfunction is seen in CLL.

Mechanism	Effect on NK function
Decreased CD16 expression	Reduces ADCC
IL-10/ TGF-β	Suppress NK signalling
Increased HLA-E	Activate NKG2A inhibition
Defective synapse	Poor granule delivery
NK cell exhaustion	Reduced cytotoxic capacity
Loss of KIR+ cells	Increased immature NK cells

NK cells in CLL display elevated CD27 expression on the cytolytic CD56dim subset, a characteristic associated with the decline of mature cells and the expansion of immature subsets. This is accompanied by defective expression of the NKG2D coreceptor, an activating receptor involved in anti-tumour immunosurveillance [9]. A loss of KIR+ cells, specifically KIR2DL1 and KIR3DL1, also results in a reduction of mature NK cells that express KIR receptors. KIR receptors interact with HLA class 1 ligands and are essential for NK cells to recognize and eliminate leukemic cells. With these characteristics, CLL cells can evade the immune system, thereby promoting tumour progression [9].

In usual settings, once NK cells recognise their target cell, they can lyse it by releasing lytic granules containing perforin and granzyme B, and by engaging death receptors such as FASL and TRAIL [10]. In addition, NK cells produce cytokines and chemokines which modulate and coordinate other immune mediators of cytotoxicity.

Despite the NK cells' role in controlling and killing tumour cells, CLL impairs NK cell function, thereby hindering their activity. This can be due to elevated levels of immunosuppressive cytokines such as IL-10 and TGF-β. TGF-β lowers the expression of activating receptors NKG2D and NKp30, which decreases NK cell recognition of target cells. As well as this, it blocks the production of IFN-γ and TNF-α, which are essential in NK cell anti-tumour responses via SMAD3-mediated signalling.

CLL also increases inhibitory signals by upregulating ligands, such as HLA-E, which block NKG2A on NK cells. Following this, immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in NKG2A are phosphorylated, recruiting SHP-1 tyrosine phosphatase, which dampens immune cell (NK cell) activation [11].

CLL cells respond to NK cell attack by rapidly polarising actin filaments at the synapse, creating a shield which protects the malignant B cells by reducing intracellular levels of granzyme B delivered by NK cells [12].

Although studies show that peripheral blood NK cell levels increase in patients with CLL, decreased cytotoxicity and increased exhaustion among these cells are ongoing patterns. This provides an area of interest for novel therapeutics to regain NK cells' practical nature as an anti-tumour surveillance mechanism.

Mechanism of action of Obinutuzumab and how it interacts with NK cells in this scenario.
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Obinutuzumab is a type 2 antibody that binds at a different angle and epitope, resulting in a more rigid and stable interaction than rituximab. Obinutuzumab promotes B cell death through direct, non-apoptotic pathways involving actin remodelling, lysosomal membrane permeabilization and caspase-independent cell death. This provides a complement-independent mechanism, unlike rituximab, which is effective in patients with CLL whose complement activity is usually compromised.

As shown in Table 1 above, Obinutuzumab has a glycoengineered Fc region that lacks fructose. This dramatically boosts binding to FcγRIIIa receptors on immune cells such as NK cells and macrophages, in turn activating these cells to release cytotoxic granules such as perforin and granzymes, which trigger death receptor pathways (like FasL). These characteristics define the ADCC mechanism and lead to the direct killing of CD20-expressing cancer cells [13]. This can be quantitatively demonstrated by studies showing that afucosylated antibodies increase IgG binding to CD16a by up to 50-fold compared to fucosylated antibodies [14].

Once Obinutuzumab-coated CLL cells interact with NK cells, CD16 transmits activating signals into the NK cell. This occurs through ITAMs on the associated FcεRIγ and CD3ζ chains becoming phosphorylated, activating Syk/ZAP70, PLCγ, calcium flux, and granule trafficking pathways, resulting in complete NK cell activation [15].

The activation cascade of NK cells continues as they release perforin, creating pores in the target cell membrane. Granzyme B then enters through these perforin, inducing apoptosis via caspase activation and mitochondrial damage. This results in the rapid apoptotic death of $CD20^{+}$ CLL cells.

As show in figure 1, Obinutuzumab binds to CD20 receptors on malignant CLL B cells leading to the activation of NK cell mediated ADCC, through the release of perforin and granzymes. In addition to NK cell engagement, Fc receptors on macrophages are activated, promoting ADCP. Due to the nature of NK cells and their ability to mediate ADCC, Obinutuzumab targets antigens expressed by tumour cells and recruits NK cells through FcγR-Fc interactions, leading to NK cell activation and target cell lysis [10].

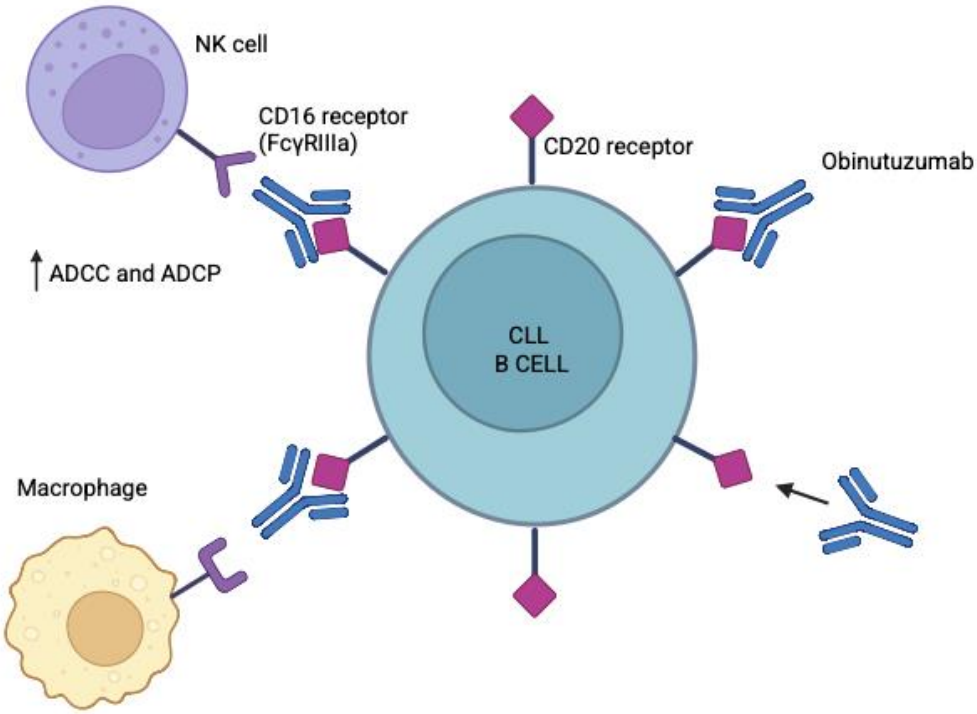

Figure 1; Mechanism of CD20 monoclonal antibody Obinutuzumab-mediated immune effector activation in CLL. Created using BioRender software (Created in https://BioRender.com). CD20 receptor, shown by pink squares, attach to malignant CLL B cell targeted by the Obinutuzumab antibodies. NK cell expresses the CD16 (FcγRIIIa) receptor. ADCC, antibody-dependent cellular cytotoxicity ; ADCP, antibody-dependent cellular phagocytosis.

Alternative therapeutic strategies. 926

In this case study, the patient being assessed is 68 and may therefore struggle to tolerate intensive treatments due to increased side effects. Before administering any therapy, a geriatric assessment should be conducted to confirm the best treatment strategy, with other health factors, such as nutrition and cardiovascular health, considered.

Exploiting NK cells to reactivate their innate killing functions (missing-self recognition, ADCC via CD16, secretion of IFN-γ, perforin, and granzymes) using therapeutic strategies is helpful because they can deliver direct, targeted cytotoxicity against CLL cells. NK-based strategies are also deemed safer than T-cell approaches and can be combined with other current CLL treatments, such as Obinutuzumab. Table 3 presents a comprehensive comparison of treatment strategies that could be administered to a patient with CLL alongside Obinutuzumab to increase tumour cell death.

CAR-NK cells are a promising strategy to enhance NK cell responses in CLL [16]. Clinical trials have studied cord blood-derived CAR-NK cells in patients with relapsed CLL, with the outcome showing satisfactory responses [17]. Results from this study show that the CAR-NK cells expanded and persisted at low levels for at least 12 months without the development of many toxic side effects. Figure 2, taken from the study, illustrates the persistence of CAR-NK cells after the initial infusion, indicating sustained anti-cancer activity and a durable response. The data shows that higher doses lead to stronger and more sustained expansion, and that better persistence correlates with clinical response (P = 0.02), indicating that survival and expansion of CAR-NK cells are important for determining therapeutic effectiveness. A drawback is that inter-patient variability is high, and while some patients show substantial expansion, others show fewer detectable copies.

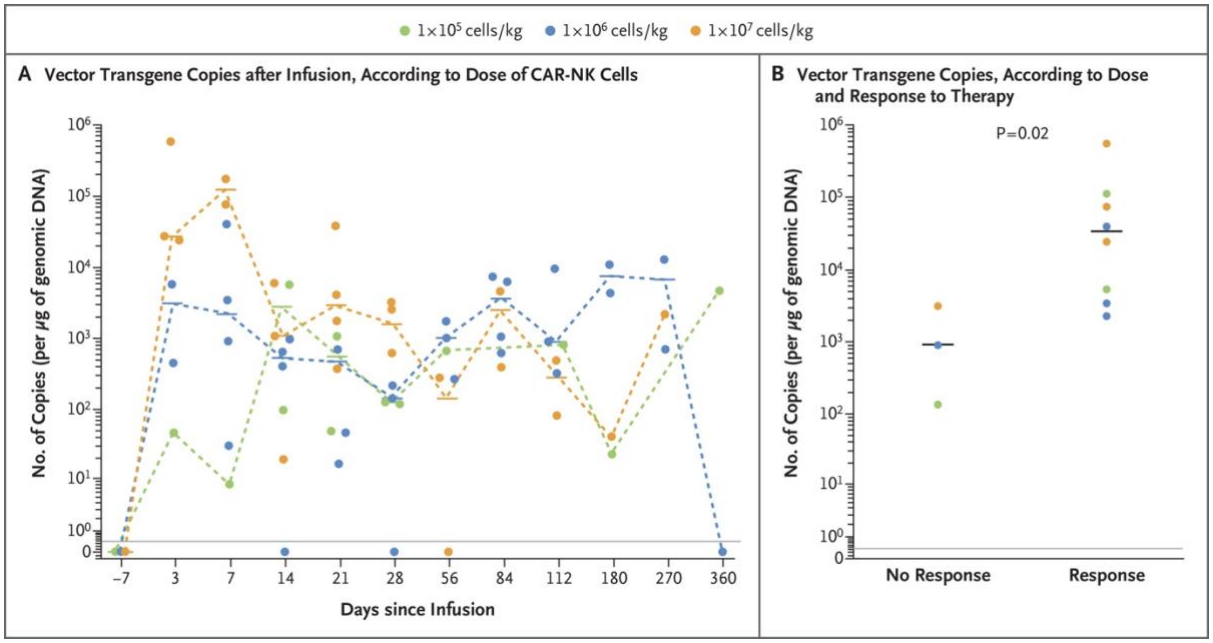

Figure2; Persistence of CAR-NK cells following infusion. Reproduced from Liu et al., 2020. Panel A shows quantitative measurements of CAR-NK cells in peripheral blood samples. A horizontal grey line depicts the lower limit of quantification for this assay, and solid horizontal bars indicate the median copy numbers at various time points for each dose. Panel B shows the peak number of copies of CAR-NK cells in the first 28 days after infusion [17].

The mechanism of CAR-NK function is illustrated in Figure 3. This diagram shows engineered CAR-NK cell binding to the CD19 antigen on malignant B-CLL cells, bypassing the need for natural NK activation signals and resulting in increased release of perforin (PFN), granzyme B (GzmB), and TNFα. These initiate cytotoxic pathways by pro-inflammatory and pro-apoptotic signalling, thus driving the depletion of malignant B cells in CLL.

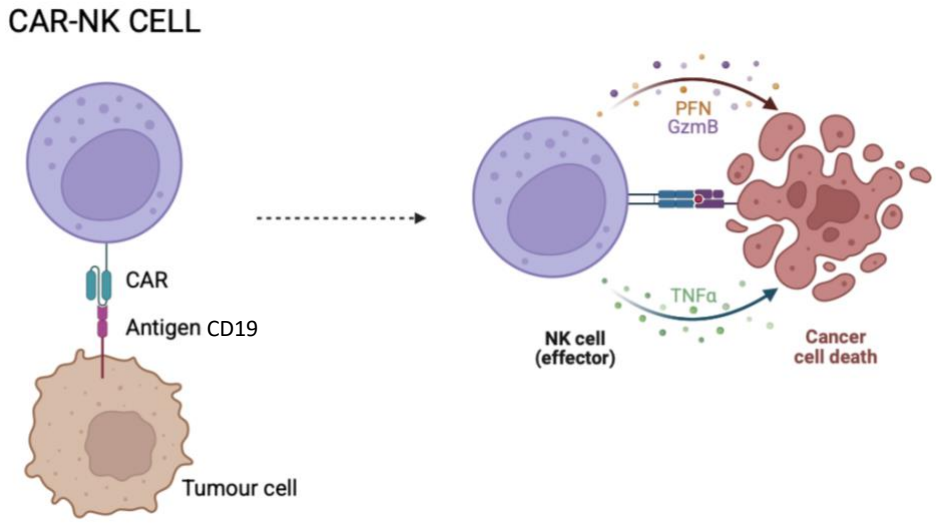

Fig3; Diagram to show the mechanism of CAR-NK cells. Created using BioRender software (Created in https://BioRender.com).

The limitations of this therapy could include the CLL microenvironment suppressing NK function through cytokines and inhibitory ligands. To combat this, dual CARs targeting specific antigens are being explored, for example CD19/ROR1, so that CLL survival pathways can be blocked while inducing cell death to prevent cancer cells from evading elimination [18]. Wnt5a-induced ROR1 signalling is a crucial pathway that reduces CLL apoptosis and enhances cell survival against NK cells by increasing resistance. Targeting both CD19 and ROR1 on CLL cell surfaces overcomes this resistance by countering CLL's survival signals, hence leading to more direct cell death and broader immunity.

Another method to further improve this therapeutic strategy would be tp engineer CAR-NK cells that lack NKG2A, resulting in a more potent anti-tumour response. The interaction between HLA-E and the inhibitory receptor NKG2A usually impairs NK function. Therefore, removing this will mean that when CAR binds to the antigen, the NK cell will receive a stronger activating signal, leading to an increase in degranulation and cytokine release [19]. IFN- γ secreted by the CLL microenvironment promotes HLA-E expression on the tumour cell surface, which then binds to its receptor NKG2A encoded by the KLRC1 gene. A study shows that to develop NK cells that are resistant to HLA-E mediated inhibition, KLRC1 KO NK cells were generated using CRISPR technology [20].

Aside from CAR-NK therapy, Tri-specific killer engagers (TriKEs) could also serve as a novel therapeutic strategy to enhance NK cell responses in Obinutuzumab treated patients with CLL. They work by simultaneously engaging and activating NK cells through CD16 and IL-15 and work alongside antibodies to target cancers. An advantage of TriKEs in comparison to other immunotherapy options, such as CAR-T cell therapy, is that they target cancer cells without hyperactivating T cells, thereby posing as a safer treatment option [21]. NK-cell engagers, BiKEs, similarly work by linking NK cells via CD16a to tumour cells. BiKEs from the basis for more complex TriKEs.

The final strategy explored is IL-15 superagonists which could also enhance NK effects and lead to anti-tumour responses. It does this by mimicking natural IL-15 signalling with improved stability and potency (exhibiting longer serum half-life and increased in vivo activity). This promotes proliferation, survival, and activation of NK cells leading to an increase in cytotoxic function, ADCC, and better production of immune signals (such as chemokines), which in turn lead to a broader anti-tumour response [22]. Clinical trials have confirmed the efficacy of using IL-15 superagonists alongside monoclonal antibodies; in this study, rituximab is used [23].

When proposing these novel strategies, it is important to consider that much of the testing is based on in vitro assays using NK92 cell lines. NK92 cells are immortalised NK cells with greater cytotoxicity compared to human NK cells. They have higher NK:CLL ratios than those observed in patient blood samples, which makes results from Obinutuzumab appear stronger in vitro than they would in real patients [24][25]. Therefore, this disparity could highlight a key challenge in translating in vivo results directly to clinical outcomes.
Using primary human NK cells alongside NK-92 cells, or testing in patient-derived CLL cells would help address this challenge when designing preclinical experiments.

Overall, the best proposed strategy to enhance cytotoxic NK cell function would be to engineer a CAR-NK cell with dual targets, such as CD19/ROR1, or a CAR-NK cell that lacks NKG2A. CAR-NK cells represent a promising therapeutic option for cellular therapy, as they do not require HLA compatibility and can be produced at large scale, potentially translating into “off-the-shelf” treatments [10][26].

Table 3; Comparison of different therapeutic strategies used to enhance NK cell function for patients with CLL treated with Obinutuzumab.
Abbreviations: TriKEs, Tri-specific Killer Engagers; CAR-NK cells, Chimeric Antigen Receptor NK cells.

Therapeutic Strategy	**Features and Mechanism**	**Impact on CLL**
CAR-NK cells	Express chimeric antigen receptor CAR Antigen specific recognition	“Off the shelf” potential Direct cytotoxic killing
TriKEs	NK-cell engaging domain (anti-CD16) Tumour antigen binder IL-15 moiety	Simultaneous target of CLL cells, activation of NK cytotoxicity, NK expansion.
IL-15 superagonist	Promotes NK and CD8 T cell proliferation. Longer half-life than native IL-15	NK cell expansion, increase immune-mediated control of CLL cells. Augment other NK associated therapies.
NK-cell engagers: BiKEs	Bridge NK cells to tumour cells by binding NK activating receptor and tumour antigen on CLL cells.	Redirecting endogenous NK cells. NK degranulation and target killing.

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