Faculty of Graduate Studies

Targeting triple-negative breast cancer with developed nanoparticles-based T cell engagers carrying dual antibodies

استهداف سرطان الثدي ثلاثي السلبية من خلال مشغلات الخلايا التائية المستندة إلى الجسيمات النانوية والتي تحمل أجسامًا مضادة

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Clinical Laboratory Science from the Faculty of Graduate Studies at Birzeit University, Palestine.

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18/7/2023

Targeting triple-negative breast cancer with developed nanoparticles-based T cell engagers carrying dual antibodies

By

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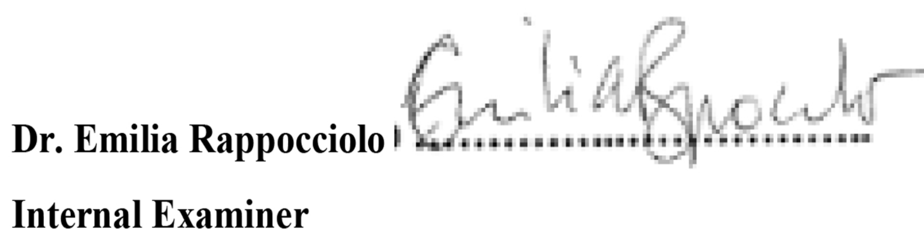

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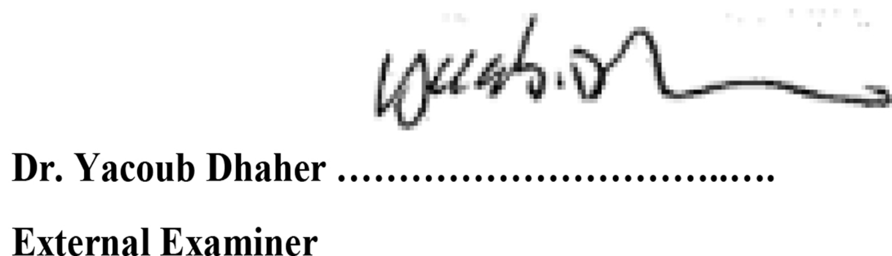

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Declaration

Targeting triple-negative breast cancer with developed nanoparticles-based T cell engagers carrying dual antibodies

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The work provided with this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Dedication

To the cherished mothers, beloved daughters, precious sisters, and revered aunts, and to all the remarkable women across the world, your unwavering strength in the face of breast cancer fills my heart with profound gratitude. Your resilience and tenacity provide me with the unwavering determination to press forward, and no words can adequately convey the depth of my appreciation.

To mom…

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I humbly dedicate my thesis to a chosen few people who have had a significant impact on my life and have left a lasting impression on my heart. My mentor, Dr. Mahmoud Watad, is foremost among them. He has been my mentor throughout this academic path, has never wavered in his confidence in my skills, and never ceases to inspire me. I will always be appreciative of his priceless advice.

To my soul mate, my mother, who has been with me every step of the way, offering support and assistance when I needed it most. Her presence has been a beacon of strength and comfort, reminding me that I am never alone in this academic pursuit. I also extend my heartfelt appreciation to my father and my special sisters, Laialy, Nana, Layla, and Bissan, whose love and encouragement have been rocks on which I stood during moments of intense study and dedication. To my precious sister Layla, who resides in my heart, and Laialy, a second mother to my kids, I am deeply grateful.

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To every woman in this world who may be fighting or surviving breast cancer, you are my eternal wellspring of strength. Your resilience and determination inspire me to push forward, dedicating my efforts to finding a brighter future for all.

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List of Abbreviations

Abbreviation	Full Name
TNBC	Triple-negative breast cancer
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
DCIS	Ductal carcinoma in situ
IDC	Invasive ductal carcinoma
IBC	Invasive breast cancer
ILC	Invasive lobular carcinomas
AXL	AXL Receptor Tyrosine Kinase
HIF	Hypoxia-inducible factor
CAR-T	Chimeric Antigen Receptor T cell therapy
BTCEs	Bispecific T cell engagers
nanoTCEs	Nano-sized T cell engagers
nanoBTCEs	Bispecific Nanoparticles T cell engagers
MM	Multiple myeloma
3DTEBM	3D tissue-engineered bone marrow
scFvs	single chain variable fragments

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Targeting Triple-Negative Breast Cancer with Developed Nanoparticles-Based T cell Engagers Carrying Dual Antibodies

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Abstract

Triple-negative breast cancer (TNBC) is a particularly challenging type of breast cancer, known for its difficult diagnosis and the poorest prognosis compared to other subtypes. It is characterized by the absence of three key receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) - as confirmed by negative immunohistochemistry staining. Over the past decade, efforts have been made to develop immunotherapies for TNBC. However, therapies often require advanced laboratory facilities and intricate techniques, incurring significant costs and demanding continuous changing with high toxicities. In this research, we addressed the issue of targeting TNBC cells with nano-sized T cell engagers (nanoTCEs) which are liposomes with two monoclonal antibodies, one targeting cytotoxic T cells and the other targeting TNBC cells. This innovative approach allows for specific targeting of T cells to TNBC cells in vitro to induce the killing of cancer cells. Hereby we demonstrate the presence of a target protein termed AXL on murine 4T1 TNBC cell lines. Moreover, we prepared liposomes containing antibodies against AXL and CD3 (T cell protein), and demonstrated an effective and specific binding of AXL on 4T1 by the AXL/CD3-nanoTCEs, compared to the control isotype. Furthermore, we have observed that AXL/CD3-nanoTCEs activate T cells and promote their ability to induce the killing of 4T1 cells, indicating success in the treatment of solid tumors using this method. These findings present this nanoTCE technology as a novel and promising immunotherapeutic approach for the treatment of TNBC. Our research serves as a foundation for future investigations, including the validation of TCEs in animal models and eventually in human subjects.

1. Literature Review

1.1. Types of Breast Cancer

Breast cancer can be categorized based on its histological grade and histological type. Histological grade refers to the assessment of the tumor's differentiation, including tubule formation and nuclear pleomorphism, as well as its proliferation pattern, which is determined by the mitotic index. This grading system helps determine the tumor's aggressiveness [1]. In contrast, histological type pertains to the growth pattern of tumors. For instance, breast cancer's diverse adenocarcinomas exhibit specific morphological and cytological patterns, which consistently correlate with particular clinical presentations and outcomes. These specific patterns are referred to as types [1]. There are currently 17 distinct histological special types identified (Table 1 and Fig. 1) according to the World Health Organization [2].

Table 1: Prevalence of Histological Types in Breast Cancer Cases

Histological type	**Prevalence (%) [3]**	**Prevalence (%) [4]**	**Prevalence (%) [5, 6]**	**Prevalence (%) [7]**
Invasive ductal carcinoma (IDC)	65–80	50–80	56.4	78
Carcinoma with osteoclastic giant cells	0.5–1.2			
Invasive lobular carcinoma (ILC):	5	5-15	8.2	11.1
Classical			7.4	
Alveolar			0.1	
Solid			0.3	
Tubulo- lobular			0.4	
Pure tubular carcinoma	<2	<2	4.4	2.2
Invasive cribriform carcinoma	<4	0.8–3.5	0.6	0.3
Medullary carcinoma	<5–7	1–7	2.6	1.1

Typical			0.3	
Atypical			2.3	
Mucinous carcinoma	<2	2	1.4	2.2
Neuroendocrine carcinoma		2–5		0.0
Invasive papillary carcinoma	1–2	1–2	0.4	0.7
Invasive micropapillary carcinoma	<2.7	<2		<2.7
Apocrine carcinoma	<1–4	<4		
Metaplastic carcinoma	<5	<1		0.2
Lipid- rich carcinoma	<1	<1–6		
Secretory carcinoma	Few cases	<0.15		
Oncocytic carcinoma		Few cases		
Adenoid cystic carcinoma	Few cases	0.1		0.1
Acinic- cell carcinoma		Few cases		
Glycogen- rich clear cell carcinoma	<1–3	1–3		
Sebaceous carcinoma		Few cases		
Mixed types			25.3	
NST and invasive lobular carcinoma	3.3	4.0		
NST and special type	2.1			
Invasive lobular mixed	3.1			
Tubular carcinoma mixed	16.8			
Miscellaneous			0.6 (Metaplastic and adenoid cystic carcinoma)	0.0 (Signet ring cell carcinoma)

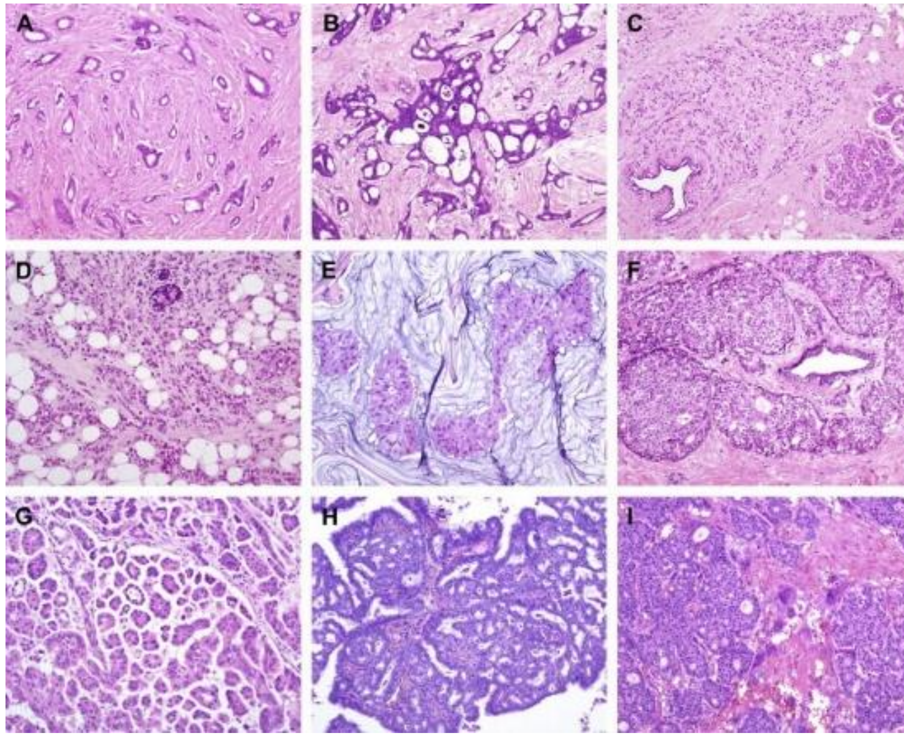

Figure 1. Breast cancers with histological special types tend to exhibit a preference for being positive for the ER. These special types include: (A) Tubular carcinoma, (B) cribriform carcinoma, (C) classic ILC, (D) pleomorphic ILC, (E) mucinous carcinoma, (F) neuroendocrine carcinoma, (G) micropapillary carcinoma, (H) papillary carcinoma, (I) low-grade IDC with osteoclast- like giant cells.

In the past decade, novel molecular techniques have been employed to investigate breast cancer, including high-throughput microarray-based gene expression profiling. This technique enables the evaluation of metastatic potential, histological grade, prognosis signatures (response to therapy), and transcriptomes of breast cancers, along with cDNA microarray analysis and immunophenotyping [2]. Breast cancer has been categorized into five types using global gene expression profiling and hierarchical clustering techniques. These types are known as Luminal A, Luminal B, human epidermal receptor 2 (HER2)-overexpressing, basal-like breast cancers, and normal-like tumors [8]. Immunophenotyping classifications have also revealed that breast cancers can be grouped into two distinct clusters based on the expression of the estrogen receptor (ER): ER+ (in Luminal A and B) and ER- (in HER2-overexpression, basal-like breast cancers and normal-like tumors) [8].

1.1.1. Ductal Carcinoma in situ Breast Cancer

Ductal carcinoma in situ (DCIS) is a type of noninvasive breast cancer characterized by abnormal epithelial cell growth within the mammary ductal system, without infiltration of the basement membrane [9]. DCIS typically does not exhibit noticeable symptoms and often remains asymptomatic. The primary method for detecting DCIS is through mammographic screening programs, although it can also be identified through pre-screening techniques like palpable lumps or nipple discharge. Among core biopsies of breast tissue, approximately 8% of cases are attributed to DCIS, and around 74% of these cases are confirmed as DCIS following further excision [9].

DCIS is further classified into three degrees of histological cellular atypia, which are described in Table 2 below [9].

Table 2: The Degrees of Histological Atypia in DCIS

Low degree	Intermediate degree	High degree
- Monotonous nuclei - Small nucleic size - Occasional nucleoli and mitoses - Akin to luminal epithelial cell size	- The classification of the observed cases falls neither into the low-grade nor high-grade category. - Poor inter-observer reproducibility in assessing these cases.	- Marked nuclear pleomorphism, large nucleic size, conspicuous mitosis, and irregular chromatin (observed in 42-53% of cases) - These features indicate a high risk of recurrence and suggest a higher likelihood of the presence of invasive disease

Allred categorized DCIS into distinct subtypes: the Comedo group, large cell subtype, which represents a more aggressive form of DCIS with ducts exhibiting a cheesy-like or comedo-like appearance, and the non-comedo group, small cell subtype, which is considered less aggressive and can be further classified into cribriform, micropapillary, and solid forms. Non-comedo DCIS generally carries a lower risk of progressing to invasive cancer compared to the comedo-type DCIS [10].

The incidence of DCIS has increased with the introduction of mammography. Alongside mammography exposure, the risk factors for DCIS are comparable to those for invasive breast cancer (IBC) [11]. However, despite these shared risk factors, mortality

rates from DCIS have decreased, primarily due to early detection and a shift in tumor types, leading to a reduction in the proportion of DCIS cases with poor prognoses [11].

Notably, the non-comedo subtypes of DCIS, which are typically not associated with subsequent invasive cancer, have shown the greatest increase in incidence [11]. Between 1983 and 2003, the overall incidence of DCIS has risen by 500%. However, during this period, there has been a decline in DCIS cases among women aged 50 and older, while the incidence of DCIS among women under the age of 50 continues to rise (Fig. 2) [11].

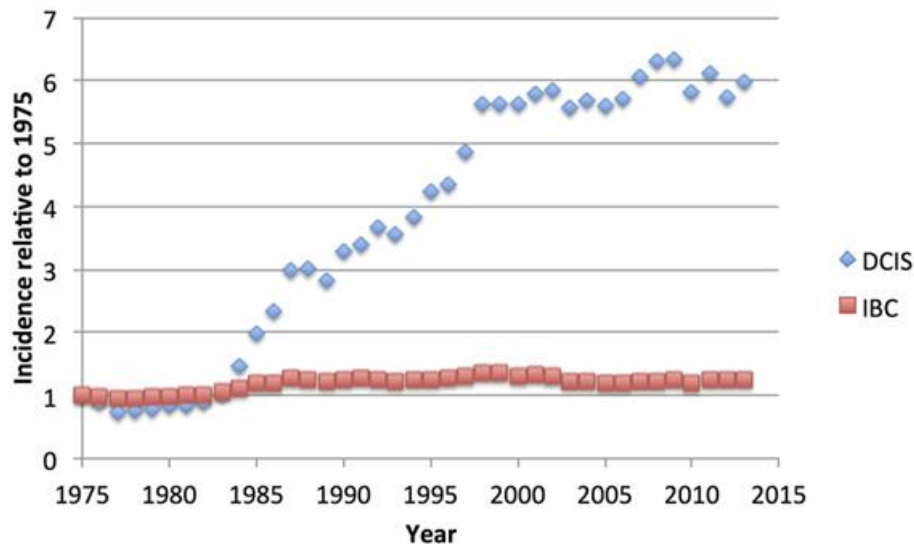

Figure 2. The age-adjusted incidence rates of DCIS and IBC in the United States, as reported by the National Cancer Institute Surveillance, Epidemiology, and End Results, have significantly changed since 1975. DCIS cases have exhibited a dramatic increase over time, while IBC cases have not shown a noticeable decrease [12].

The management of DCIS often involves excision and radiotherapy to prevent invasive disease, even though most DCIS cases never progress to an invasive state nor lead to morbidity [13]. Approximately 3-5% of DCIS patients, whether treated or untreated with radiation, may experience an ipsilateral local recurrence, with about half of these recurrences being invasive [13].

Recent studies have highlighted the potential use of targeted treatments, such as trastuzumab, for DCIS patients exhibiting HER2 overexpression [14]. This finding underscores the importance of considering personalized treatment decisions that effectively control the disease while balancing patient preferences with factors such as treatment toxicity, cosmesis, financial implications, and overall quality of life [14].

1.1.2. Invasive Breast Cancer

Breast cancers that start spreading into surrounding tissues from where they started (ductal or tubular) are called IBCs. Most breast cancers are invasive, but there are different types of IBCs; the most common are: invasive lobular carcinomas (ILC) which consist of about 10% of IBCs, and ductal carcinoma (IDC) which consists of about 70-80% [15].

The classical form of ILC morphology is small, non- cohesive cells that infiltrate the stroma in a single file pattern, about 90% of ILCs lack the adhesion protein E-cadherin making this feature a hallmark of ILC [23]. The E-cadherin deregulation is due to genetic alterations in the gene CDH1 (located at chromosome 16q22.1) [15].

Specific genetic features unique to ILC, such as mutations in PTEN, TBX3, and FOXA1, in addition to the well-known loss of E-cadherin. PTEN loss led to increased AKT phosphorylation, which was most prominent in ILC compared to other breast cancer subtypes. The study also revealed spatially clustered mutations in FOXA1, which correlated with higher expression and activity of FOXA1. On the other hand, GATA3 mutations and high expression were characteristic of luminal A IDC, indicating different regulation of ER activity between ILC and IDC. By analyzing proliferation and immune-related gene signatures, the researchers identified three distinct ILC transcriptional subtypes associated with differences in survival outcomes. Furthermore, the study explored cases with mixed histology of IDC and ILC and molecularly classified them as either ILC-like or IDC-like, suggesting no true hybrid features between the two subtypes. Overall, this comprehensive molecular atlas of ILC provides valuable insights into its genetic basis and potential clinical options for managing this subtype of breast cancer.

Other related mutations were identified and as ILC features are PTEN, a tumor suppressor gene that controls cell growth, that found to be absent in ILC, PTEN loss led to increased AKT phosphorylation, which was most prominent in ILC compared to other breast cancer subtypes [16]. TBX3, also known as T-box 3, is another oncogene whose specific function in ILC is not yet fully understood, but it is known as a transcription factor that plays an essential role in promoting proliferation and metastasis and found to be overexpressed in ILC [16]. FOXA1 plays a crucial role as a modulator of ER transcriptional activity, it functions within a large protein complex, where it alters chromatin accessibility and facilitates long-range DNA interactions [16]. Loss of the cell-cell adhesion molecule E-cadherin (CDH1) is a key hallmark of ILC, the second most prevalent subtype of IBC. CDH1 loss is believed to be responsible for the characteristic highly disorganized morphology of ILC and is often associated with tumor invasion and metastasis in other cancer types as well. Ciriello and Gatza identified mutations in the CDH1 gene in approximately 63% of patients with ILC, and these mutations were often accompanied by loss of chromosome 16q, where CDH1 is located, leading to complete loss of the protein. The downregulation of CDH1 transcript and protein levels was observed in ILC cases with CDH1 mutations. Contrary to previous reports, Ciriello's study did not find significant DNA hyper-methylation at the CDH1 promoter in ILC, suggesting that epigenetic silencing is not a major mechanism for CDH1 downregulation in IBC. However, the discrepancy with prior literature might require further investigation, as the methods used in previous studies could be a factor contributing to the difference in results. Overall, the study confirms that E-cadherin loss is a defining molecular feature of ILC, shedding new light on its genetic basis and providing insights for potential clinical implications (Fig. 3) [16].

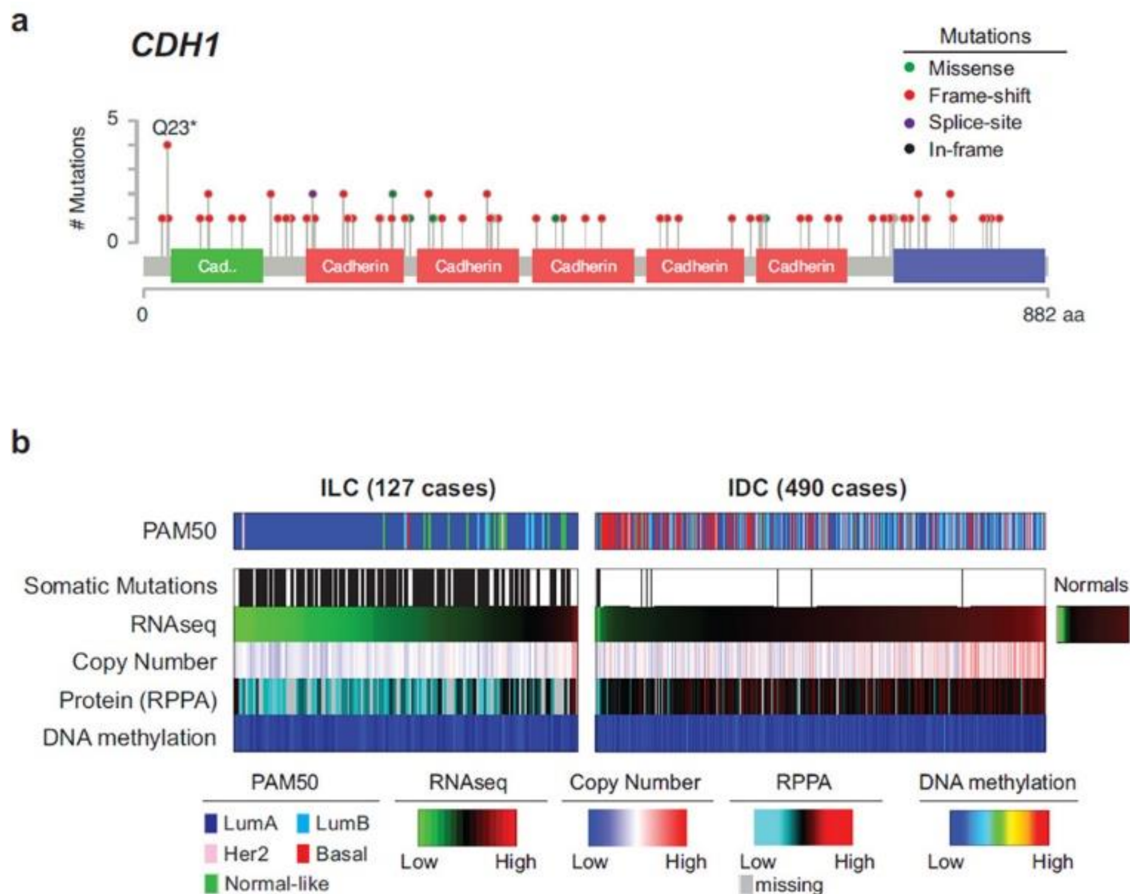

Figure 3. Loss of E-cadherin in ILC. (A) Mutations that affect the CDH1 gene in ILC are found throughout the entire sequence and are predominantly predicted to cause truncation (indicated in red). (B) A comparison of E-cadherin status between ILC and invasive IDC reveals frequent hemizygous copy-number losses at the CDH1 gene locus, resulting in the downregulation of both mRNA and protein levels. Analysis of DNA methylation at the CDH1 promoter using six probes indicates no significant changes in DNA methylation in both ILC and IDC samples [16].

IDC and ILC start in the terminal duct lobular unit of the breast. These cells show common gene expression profiles, and the different morphological appearances of these tumors can be explained by different mechanisms of their carcinogenesis [17]. Over half of ILCs differ from IDCs in global transcription programs, the remaining ILCs closely resemble IDCs [17]. 52% of ILCs clustered together and show different gene expression profiles from IDCs, most of the different genes in both types are genes encoding proteins involved in cell adhesion/motility, fatty acid transport and metabolism, immune/defense response, and electron transport; CDH1 found to be downregulated in lobular carcinomas,

and in some other tumors this protein is absent [17]. Both tumors show upregulation of genes involved in tumor-extra cellular matrix interactions, cell adhesion, and migration pathways including metastasis [17].

ILC is a type of breast cancer that may be less responsive to neoadjuvant chemotherapy compared to other breast cancer types. ILC's low proliferation rate and high ER expression make it less sensitive to chemotherapy, resulting in lower pathological complete response rates [18]. Neoadjuvant endocrine treatment with aromatase inhibitors or selective ER modulators may be considered for ILC patients due to their specific characteristics [18]. Mastectomy is more common in ILC due to larger tumor size, multifocality, and difficulties estimating tumor size pre-operatively [18]. Adjuvant anti-HER2 therapy appears to be equally beneficial for ILC compared to other types of breast cancer [18]. Gene expression-based prognostic tests, although not specifically developed for ILC, can provide valuable prognostic information and may aid in determining the need for adjuvant chemotherapy [18]. Endocrine-based treatment with the addition of CDK4/6 inhibitors is the standard of care for metastatic ER-positive ILC [18]. Although specific data on some therapies in ILC are limited, further research is needed to explore the efficacy of targeted therapies in ILC, particularly HER2-targeting antibody-drug conjugates and HER2-targeted therapies (Fig. 4) shows detailed therapeutics [18].

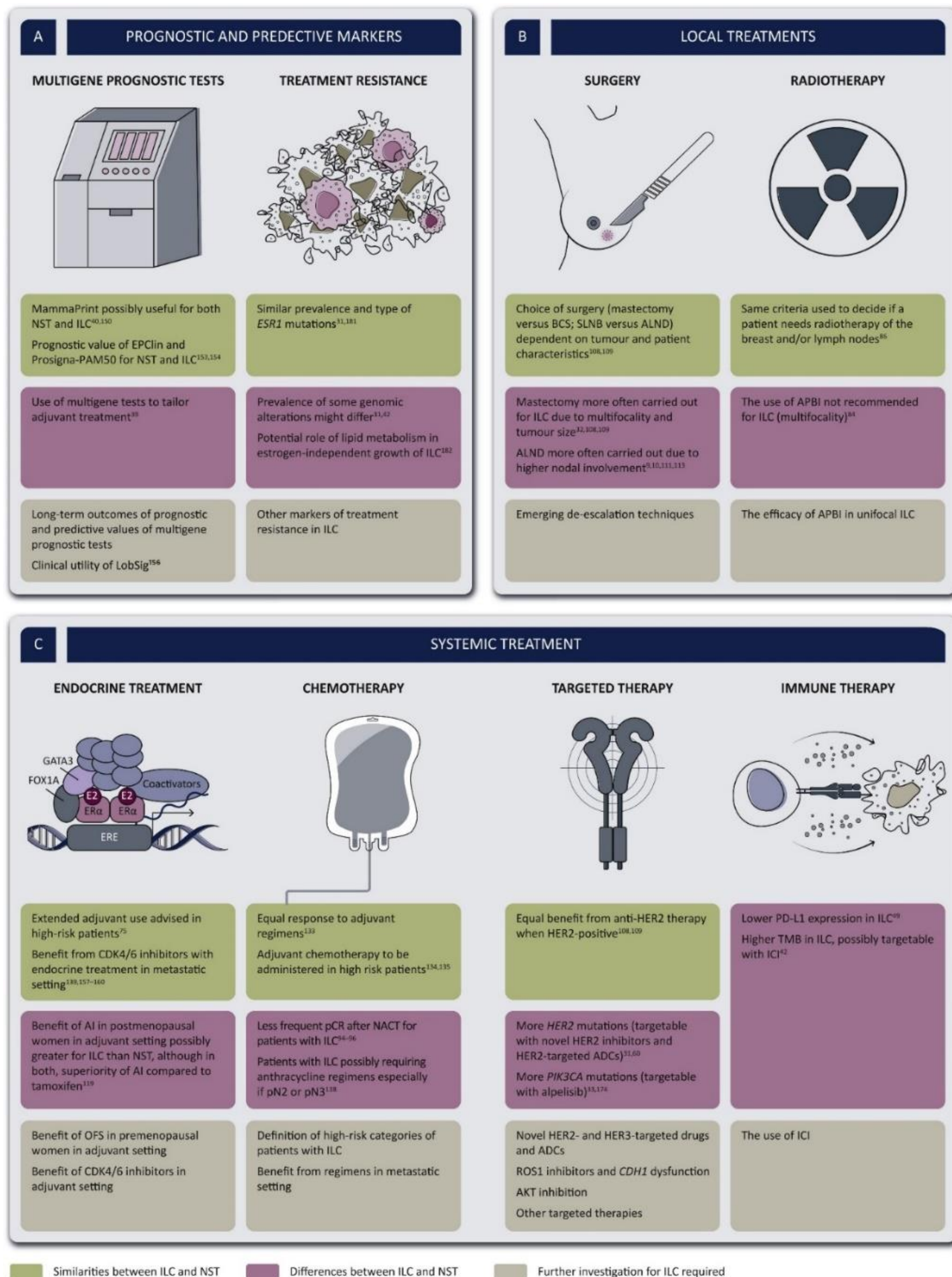

Figure 4. This analysis explores the treatment and treatment markers in ILC and NST patients, highlighting similarities, differences, and areas requiring further investigation. It covers prognostic and predictive markers, local treatment approaches, and systemic treatment strategies [18].

1.1.2.1. Inflammatory Breast Cancer

Inflammatory breast cancer is an aggressive and lethal form of breast cancer characterized by inflammation-like symptoms such as breast edema and redness [19]. However, this inflammation is not a physiological response but rather caused by the blockage of lymphatic vessels in the breast by tumor cells [19]. The term "inflammatory breast cancer" was coined in 1924, but before that, it had various names like von Volkmann's syndrome, lactation cancer, acute mammary carcinoma, etc. [19]. The clinical signs of IBC can vary, ranging from localized edema and redness to affecting the entire breast [19]. The breast affected by inflammatory breast cancer presents a well-modeled, diffuse swelling with no flattening or puckering, and associated the presence of symmetrical hypertrophy and orange skin as characteristic of inflammatory breast cancer [19].

Mainstay treatment for inflammatory is pre-operative chemotherapy or neoadjuvant chemotherapy; Locoregional treatments, including radiotherapy and surgery, play a significant role following systemic treatment [20]. The sequence of locoregional treatment depends on the response to induction chemotherapy [20]. Surgical-modified radical mastectomy is recommended after optimal remission, followed by radiotherapy [20]. Anthracyclines and taxanes are the most effective cytotoxic drugs for inflammatory breast cancer treatment, and their sequential use has shown higher rates of objective remission [20]. The optimal schedule for administering paclitaxel, a taxane, is still under investigation, and patients who have extensive residual disease after optimal preoperative chemotherapy have a poor prognosis, and the role of additional adjuvant chemotherapy is uncertain [20]. Radiotherapy alone or followed by surgical resection is considered appropriate for patients with suboptimal debulking response [20]. Potential therapeutic targets for inflammatory breast cancer have been developed, such as angiogenic modulators and farnesyltransferase inhibitors; restoring p53 function could also have significant benefits [20]. Despite all, further studies are still needed to improve the prognosis of women with inflammatory breast cancer.

1.1.2.2. Triple-Negative Breast Cancer

Breast cancer is the primary form of cancer affecting women in the United States, ranking as the second leading cause of cancer-related deaths [21]. It particularly poses a significant threat to women aged 45 to 55 years, becoming the leading cause of mortality within this age group [21]. In 2009, around 192,370 American women received a breast cancer diagnosis, tragically resulting in an estimated 40,170 deaths caused by breast cancer [21]. Among breast cancer cases, approximately 15% are classified as triple-negative breast cancer (TNBC) [21]. TNBC refers to the immunophenotype of breast cancer that lacks expression of the progesterone receptor (PR), ER, and HER2 [21]. The majority of TNBCs tend to be grouped within the basal-like subtype [21].

Variable responses observed in breast cancer treatments and highlight the benefits of hormonal therapy for receptor-positive subtypes compared to TNBCs, which typically rely on 5-Fluorouracil, Epirubicin, and Cyclophosphamide (FEC) chemotherapy. Despite hormonal therapy being effective in receptor-positive breast cancers, some patients with TNBCs experience disease recurrence and metastasis, indicating resistance to FEC chemotherapy. The text suggests that epigenetic silencing, involving DNA methylation, histone methylation, acetylation, and sumoylation, may play a key role in FEC chemoresistance.

Molecular and epigenetic profiling has successfully classified breast cancer subtypes and identified potential driver mechanisms for TNBC progression. However, the functional mechanisms behind chemoresistance related to these molecular markers remain unclear. While epigenetic inhibitors have been used in cancer management, they have shown more significant benefits in hematopoietic cancers than in solid tumors [22]. Administering epigenetic drugs may lead to the recovery of tumor suppressor genes, but it may also activate global metastatic genes, potentially promoting cancer spread. The Polycomb repressive complex, a conserved regulatory structure capable of suppressing genes through various physiological roles and types of epigenetic patterning found in humans and higher organisms, including EZH2, SETD1A, and DNMT, is known to repress gene regulation and inhibit cell proliferation and invasion in breast cancers.

Targeting these individual epigenetic regulators could be a potential option to enhance chemo-drug delivery in TNBCs and reprogram cellular and biological responses for chemotherapy when compared to chemotherapy outcomes in receptor-positive subtypes [22] (Fig. 5).

The standard FEC chemotherapy regimen is commonly used but faces resistance in some TNBC patients due to the absence of the three receptors. Epigenetic mechanisms such as DNA methylation, histone methylation, acetylation, and sumoylation contribute to FEC chemoresistance [22]. While breast cancer subtypes have been classified successfully using molecular and epigenetic profiling, the functional mechanisms underlying chemoresistance remain unclear [22]. Epigenetic inhibitors have been explored in cancer management, but their effectiveness is primarily observed in hematopoietic cancers rather than solid tumors. Epigenetic drugs may lead to the reactivation of tumor suppressor genes but can also activate global metastatic genes [22]. Certain epigenetic regulators, including EZH2, SETD1A, and DNMT, have shown inhibitory effects on cell proliferation and invasion in breast cancers, targeting those epigenetic regulators may enhance drug delivery in cancer treatment [22].

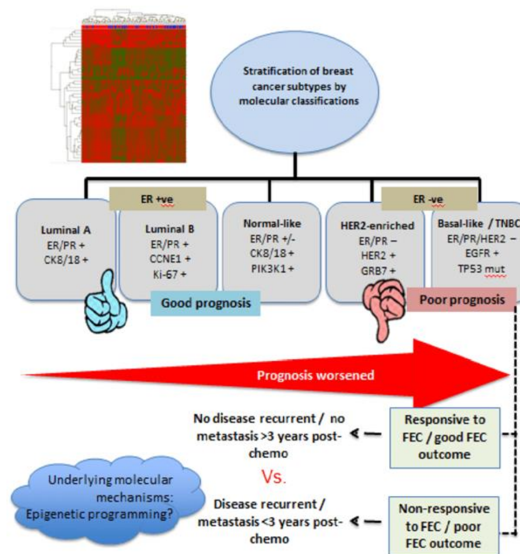

Figure 5. Molecular classification of breast cancer reveals two distinct groups: the ER-positive and ER-negative groups. Within the ER-positive group, further subdivisions were identified, namely Luminal A, Luminal B, and Normal-like subtypes. The ER-negative group, on the other hand, includes HER2-positive and TNBC/basal-like breast cancers (BLBC). It is worth noting that the prognosis tends to deteriorate in the ER-negative group [22].

Patients with TNBC show relapse earlier than in other subtypes; in addition, metastases tend to be more aggressive emphasizing the need for finding novel treatments [23]. In TNBC metastasis occurs more in the viscera-brain and lungs and is less likely in bones [23]. Gene expression profile classification of TNBC immunohistochemistry reveals six subtypes identified with unique gene expression patterns (Table 3) [23]. The TNBC tumor clusters identified four main gene expression clusters associated with specific signatures: stromal genes (fibroblast activation protein), luminal genes (ESR1, FOXA1), immune genes (CD8A), and basal epithelial genes (keratin 5 and 14) [23]. The stromal and immune gene clusters play a crucial role in identifying Lehmann's M, MSL, and IM subtypes, representing gene expression patterns from the tumor microenvironment, including invading fibroblasts and immune cells [23].

PAM50 is a 50-gene signature that classifies breast cancer into five molecular intrinsic subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like, and Normal-like. Each of the five molecular subtypes varies by their biological properties and prognoses. Luminal A generally has the best prognosis; HER2-enriched and Basal-like are considered more aggressive diseases. Less common subtypes, such as Claudin-low, Interferon-rich, and Molecular Apocrine, have also been identified using other gene expression profiling assays [24]. Molecular subtyping using the PAM50 gene signature can be performed using gene expression derived from microarrays, RNASeq, or qRT-PCR. Until the recent development of Prosigna™, a rapid PAM50-based molecular subtype classifier using the NanoString nCounter Dx Analysis System, the complexities of using PAM50 and other gene signature assays for molecular subtyping have limited their use in clinical practice and led to the development of immunohistochemical surrogate definitions to classify tumors into molecular subtypes [24]. The PAM50 subtypes of TNBC were identified, with HER2E and luminal tumors showing high expression of the LAR cluster, and true normal and normal-like tumor samples highly expressing the stromal/fibroblast cluster. The basal-like tumors, defined by PAM50, were further divided into three groups based on the expression of immune-related genes,

stromal-related genes, and basal genes [23]. Additionally, claudin-low tumors were scattered within the larger basal-like group, characterized by their expression of immune and/or stromal gene clusters. Unfortunately, according to a study on Taiwanese women, TNBC tends to display a worse clinical course (Fig. 6) [25].

Table 3: Classification of TNBC Subtypes with Unique Gene Expression Patterns

Subtype and name	Genes	Features	Reference
BL1-Basal-like	Elevated expression of: Basal cytokeratins: CK5/6, CK4/17, and epidermal growth gene EGFR	High proliferation Aggressive Poor prognosis	[23]
BL2-Basal-like	Elevated cell cycle genes expression And EGFR	High proliferation Aggressive Poor prognosis	[23]
M-Mesenchymal	Elevated mesenchymal markers: Vimentin⁺, Downregulation of: E-cadherin and claudin	High motility and invasiveness	[23]
MSL-Mesenchymal stem-like	TβRIII exhibited the highest differential expression among the genes involved in TGF-β signaling. TβRIII regulates the expression of integrin-α2, influencing MSL cell migration, invasion, and tumorigenicity.	associated with younger ages showing metaplastic and mesenchymal features	[26]
LAR-Luminal androgen receptor	High expression of estrogen related genes: ESR1, PGR, MUC1, and GATA3, and low expression of cell cycle- related genes ($p < .0001$) such as KI67 and aurora kinase B	Apocrine features	[26]
IM-Immunomodulatory	Upregulation of genes encoding human leukocyte antigens (HLAs), such as HLA-DRB1, HLA-DQB1, and HLA-DQA1. Elevation of genes encoding cytokines or cytokine receptors, such as CXCL13, IL-17A, and IL-10 interferon signaling pathway, such as IFITM1, IFITM2, and STAT1	Younger age Immune-related gene expression: regulation, immune cell infiltration, and immune response pathways and Immune checkpoint expression: Immunomodulatory markers, such as PD-L1	[27]

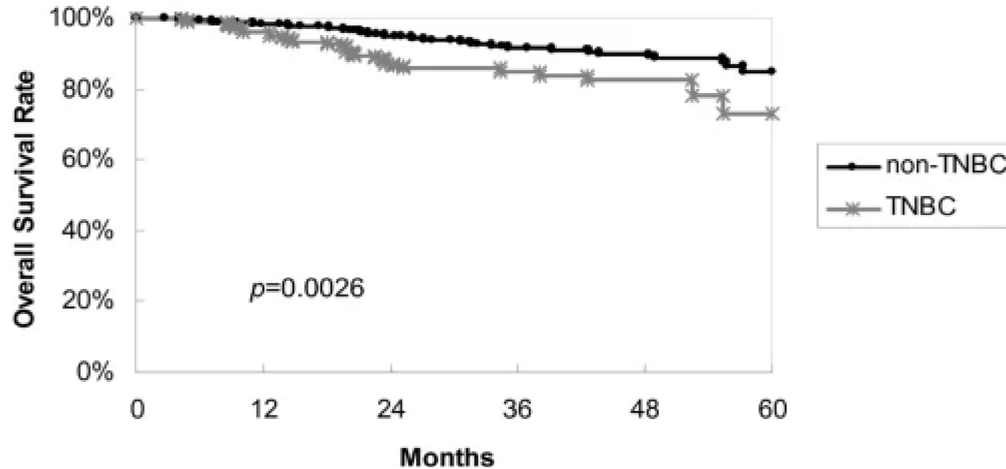

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Figure 6. Using log-rank analysis, the overall survival curves for tumor subgroups were compared. TNBC showed a worse 5-year overall survival ($p = 0.0026$) compared to non-TNBC [25].

1.1.2.2.1. Tumor Hypoxia

Hypoxia, which is a major feature of solid tumors, is defined as a decreased availability of oxygen that causes treatment resistance and favors tumor progression [28]. The massive proliferation of cells in the tumor microenvironment leads to a further decrease in oxygen levels [28]. Oxygen levels in the expanding rapidly proliferating tumor tissues are lower on average than in normal tissues (oxygen tension of 0-20 mmHg compared to 40 mmHg or higher in normal tissues) [29]. Hypoxia induces a plethora of signaling pathways in tumors (Fig. 7) that culminate in the activation of hypoxia-inducible factor (HIF); HIF expression is induced directly by oxidative stress and indirectly through the PI3K/AKT/mTOR, MAPK, and NF-κB signaling pathways [29]. HIF was shown to be a negative prognostic factor in TNBC, and it was correlated with more aggressive disease and shorter survival [29].

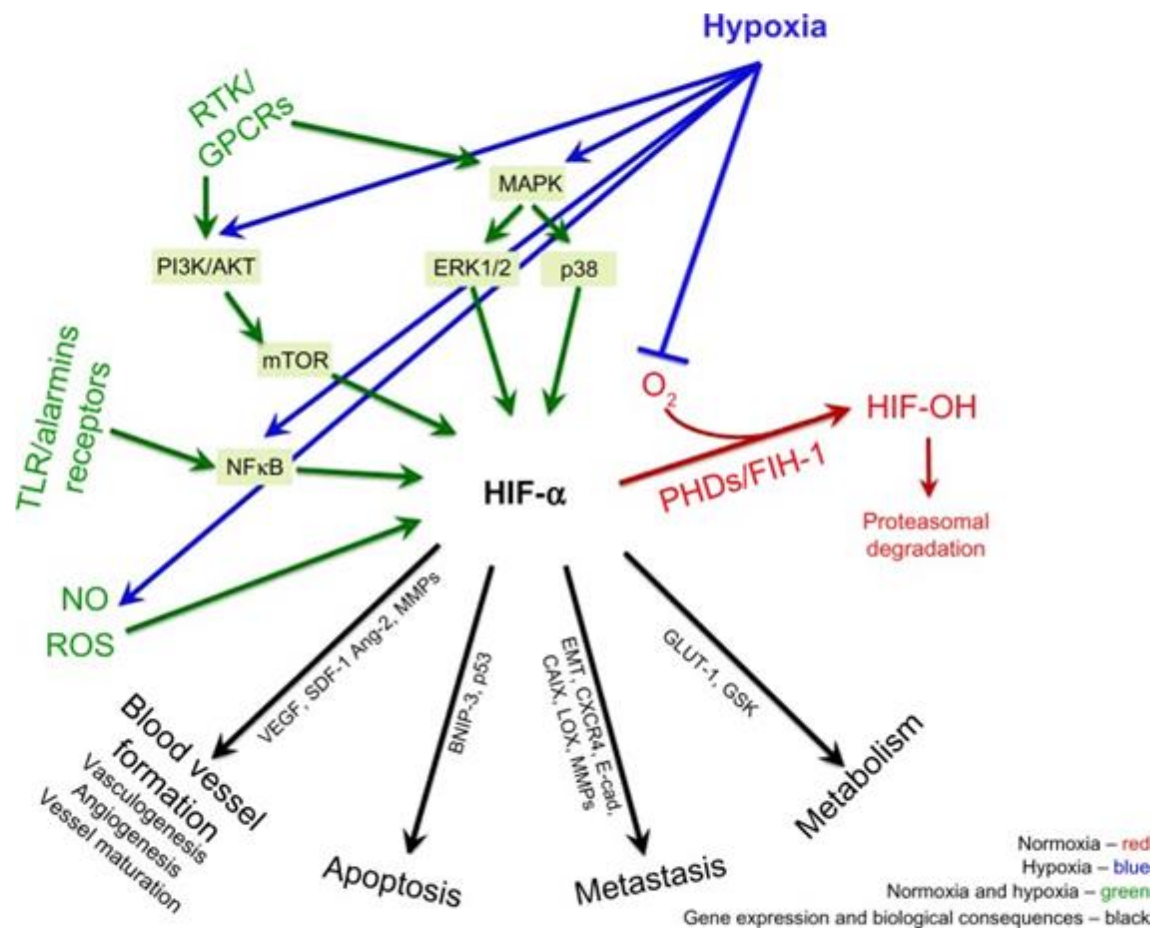

Figure 7. HIF accumulation and activation causes blood vessels formation alterations, apoptosis and metastasis and metabolism via different genes including epithelial-to-mesenchymal transition (EMT), E-cad, CXCR428 [30].

1.1.2.2.2. AXL Protein and Hypoxia

AXL is a receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm stimulating cell proliferation and survival, through downstream signaling through the PI3K/AKT/mTOR, MEK/ERK, NF-κB, and JAK/STAT pathways [31]. The expression of AXL was correlated with tumor hypoxia and HIF in TNBC, and was shown to induce metastasis, invasion, and bad prognosis leading to poor survival [31] (Fig. 8). AXL activates epithelial to mesenchymal transition cellular machinery causing tumor progression and metastasis [32].

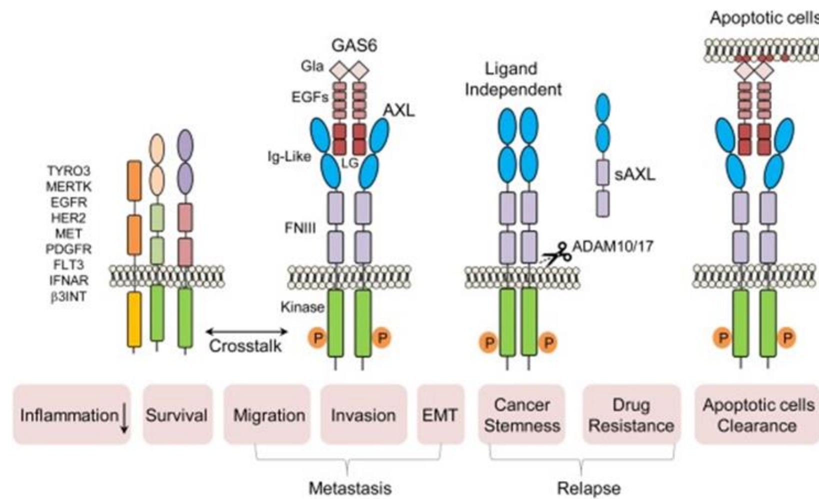

Figure 8. AXL activation, signaling, and cellular function [31].

1.2. Etiology of Breast Cancer

According to the global cancer statistics 2020, breast cancer has been a global leading cause of death among women in 110 countries around the world, while surpassing lung cancer as the leading cause of global cancer incidence in 2020, estimated 2.3 million cases of breast cancer will be in the same year [33]. Breast cancer constitutes 11.7% of all cancer cases.; and it is considered the fifth cause of death in the world [34].

According to the American Cancer Statistics Center, an estimated number of 290,560 new cases will be diagnosed with breast cancer in 2022 with an estimated death of 43,780 women [33]. Breast cancer in the USA is the highest for women compared to any other cancer besides lung cancer. About 13 % of women in the USA will develop IBC over life [35]. Death rates have shown a steady decrease in women <50 since 2007 and continued to drop in women >50 [35]. From 2013 until 2018 death rate from breast cancer was 1% per year. This drop in death rates is due to earlier detection with screening and molecular and genetic advanced techniques and advances in treatment [36].

TNBC has a prevalence of 13.7 cases per 10,000 people, and TNBC represents 10-20% of IBCs [37]. The rates of TNBC are very high among African American and Hispanic women, and among BRCA1 mutation carriers; The causes for high incidence

of TNBC among African American women are socio-demographic and healthcare characteristics, health behaviors, obesity, and smoking [37].

1.3. Current Therapies in Breast Cancer

The most important approach for treating breast cancer, in general, is full knowledge of the biological features and the extent of cancer. These two important factors help estimate the risk of cancer recurrence and provide information regarding the prediction of response to therapy. These factors include hormone receptors detection, and the presence or absence of HER2 [38, 39]. Immunohistochemical subtyping of TNBC provides better prognostications and election of appropriate targeted therapy for the subtypes of TNBC [39].

Since the TNBC subtype is characterized by negative expression of ER, PR, and HER2, patients cannot benefit from therapies targeting these markers [40]. Current therapies for TNBC patients involve the administration of chemotherapeutic drugs; however, 60% of TNBC patients have tumors highly resistant to chemotherapy leaving those patients with bad prognoses [40, 41]. Hence, there is a dire need for novel therapeutic strategies for patients with TNBC.

In the last decade, the gene therapy approach was a novel and promising technique for treating TNBC, RNA interference (RNAi) was used to regulate a wide set of genes involved in TNBC [41]. Although RNAi showed promising potential, none of the designed treatments was approved by the FDA [41]. Moreover, the use of RNAi agents is limited due to their degradation in the bloodstream before reaching the tumor [41].

1.3.1. Surgery

The primary objectives in treating nonmetastatic breast cancer are to eliminate the tumor from the breast and regional lymph nodes and to prevent the recurrence of metastasis [36]. Local therapy involves surgical removal of the tumor and sampling or removal of axillary lymph nodes, often followed by postoperative radiation. Systemic

therapy, which can be given before (neoadjuvant) or after (adjuvant) surgery, or both, is determined based on the breast cancer subtype [36], while endocrine therapy is administered for hormone receptor-positive (HR+) tumors, with some patients also requiring chemotherapy. ERBB2-directed antibody therapy with trastuzumab, along with chemotherapy, is given for ERBB2-positive (HER2+) tumors, and chemotherapy alone is used for TNBC. In metastatic breast cancer, the primary goals are prolonging life and relieving symptoms [36]. While metastatic breast cancer is generally incurable, the same systemic therapy approaches are employed as in the neoadjuvant/adjuvant setting. Local therapies such as surgery and radiation are typically used for palliative purposes in the metastatic disease [36].

1.3.2. Radiation and Chemotherapy

Various neoadjuvant and adjuvant chemotherapy regimens are available for early breast cancer treatment. Several major clinical trials have established standard modern regimens, as depicted in Fig. 9 [36]. Regimens like docetaxel/cyclophosphamide, adriamycin/cyclophosphamide, and cyclophosphamide/methotrexate/5-fluorouracil are also options for lower-risk patients, where chemotherapy benefits are relatively smaller and minimizing toxicities is crucial [36]. For high-risk patients, chemotherapy regimens combining anthracycline (e.g., adriamycin) and taxane (adriamycin/cyclophosphamide followed by taxane) provide the greatest risk reduction and remain the appropriate choice [36]. The use of anthracycline is particularly important in patients with more lymph node involvement and TNBC disease [36].

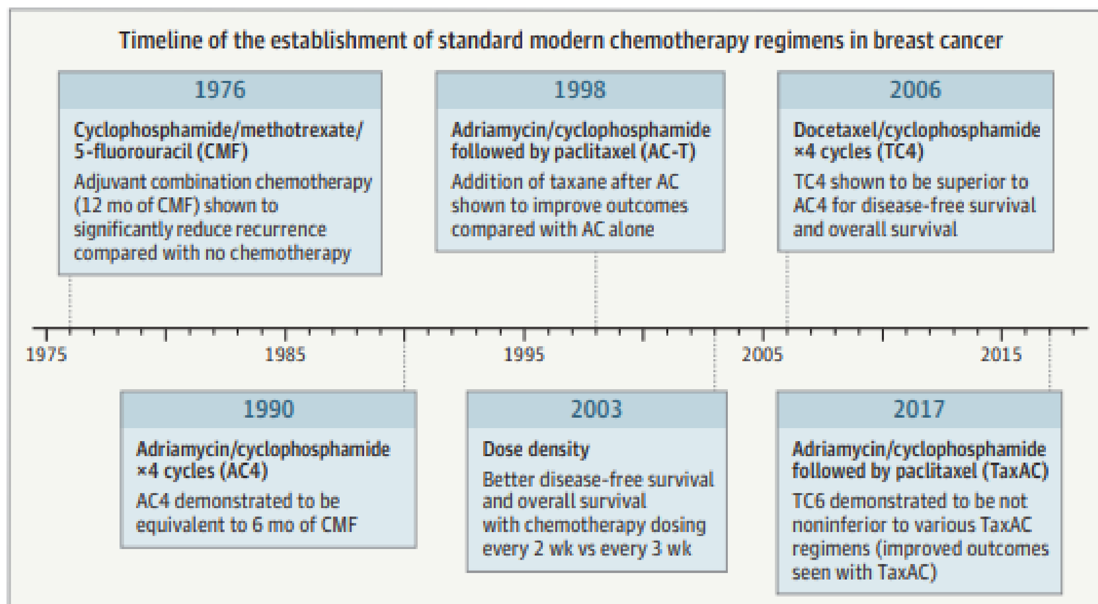

Figure 9. Development of modern and neoadjuvant chemotherapy regimens in breast cancer [36].

1.3.3. Immunotherapy

Cancer immunotherapy improves the ability of the immune system to recognize and combat cancer cells [42]. Immunotherapy can be based on both adaptive and innate immune cells [43]. Most of the clinically approved cellular-based immunotherapies are based on the adaptive system and particularly T cells. T cell-based immunotherapies, such as Chimeric Antigen Receptor T cells (CAR-T cells) and Bispecific T cell Engagers (BTCEs), were shown to induce long-term remission in cancer patients, especially in hematologic malignancies [44].

1.3.4. Chimeric Antigen Receptor T Cell Therapy

CAR-T cells are genetically engineered to target specific tumor markers on cancer cells [45]. Even though CAR-T therapy has been successfully used in hematological tumors, for solid tumors in general, and TNBC in particular, the development of CAR-T cell therapies has been less successful, mainly due to the lack of highly expressed unique tumor antigens [45, 46]. Besides these limitations, T cells have to be obtained by

extraction from the patient's blood, then activated, expanded, genetically engineered, ex vivo purified, and then reinjected into the patients; this whole process is complex, needs a lot of professional techniques and is highly expensive which can result in lack of availability in developing countries [47]. Moreover, the infiltration rate of CAR-T cells through multiple tissues before reaching solid tumors is inefficient. In addition, the tumor microenvironment is generally immuno-suppressive due to the upregulation of several immune-checkpoint proteins (such as PDL1, an immunoinhibitory molecule that suppresses the activation of T cells, and others) causing T cell exhaustion [47].

1.3.5. Bispecific T Cell Engagers

Bispecific T cell engagers (BTCEs) are novel therapeutic molecules composed of two single chain variable fragments (scFvs) linked together by a linker protein. These BTCEs function as double mono-specific antibodies but lack heavy chains. One of the scFv components is engineered to specifically bind to a target protein expressed on tumor cells, allowing for precise recognition of the desired antigen (marker) The other scFv component is a small single variable chain designed to recognize CD3, a specific marker present on T cells. (Fig. 10) [48]. The utilization of BTCEs facilitates a highly specific and tight interaction, forming an immune synapse between T cells and tumor cells. This interaction causes the establishment of an immunological synapse in the close contact zone, shifting the CD45-extracellular domain away from the T cell receptor. This activity stimulates T lymphocytes, allowing them to eliminate tumor cells more effectively [48].

Clinical investigations have shown that employing BTCEs to shift the cytotoxic activity of T lymphocytes to eradicate TNBC is effective. BTCEs have various advantages over CAR-T cells, including increased safety, off-the-shelf availability, and cheaper cost. However, there are certain drawbacks to BTCEs, including toxicity and the capacity to target only a single antigen. As a result, BTCEs are ineffective against cancers containing several antigens (polyclonal tumors). [48]. Furthermore, BTCEs exhibit a suboptimal pharmacokinetic profile, with a short half-life of approximately 2 hours. This necessitates multiple injections per week, leading to compromised patient quality of life.

Consequently, there is a pressing need to develop new T cell immunotherapies that can overcome these limitations and offer more effective and patient-friendly treatment options [48].

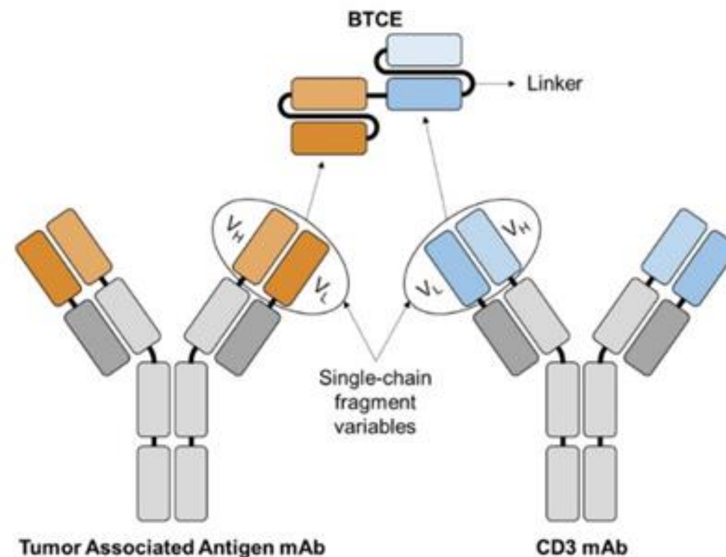

Figure 10. BTCE using scFvs from two monoclonal antibodies (mAbs) linked14 by a peptide linker [48].

1.3.6. Nanoparticle T Cell Engagers

Alhallak and Azab have developed a new generation of nanoTCEs; nanoparticle-based T cell engagers; Those PEGylated nanoparticles decorated with two antibodies; (i) anti-CD3 monoclonal antibodies (mAbs) targeting T-lymphocytes and (ii) mAbs targeting cancer antigens, were used to treat [48]. In multiple myeloma (MM), those nanoTCEs were successfully used as a therapeutic tool in treating leukemia (Fig. 12) [49].

Compared to BTCEs, those PEGylated nanoTCEs have a significantly improved half-life of 60 hours; hence they show a greater improvement to the current BTCE therapy and lower the risk of infections that may lead to deaths in some cases. According to Alhallak, there are significant differences between using marked nanoTCEs and isotype nanoTCEs (lack the marker mAbs-CD3- targeting T cells); and cells in 3D tissue-engineered bone marrow (3DTEBM) of MM (human BCWM.1 and MM.1S cell lines) in treating (killing MM cells in vitro and in vivo) MM cells were killed more efficiently by

nano BTCEs CD3 than the isotype nano BTCEs in vivo and in vitro [49, 50]. Using the same technique, targeted nanoTCEs efficiently bound to leukemia tumor cells in vitro and in vivo as well (Fig. 11) [49].

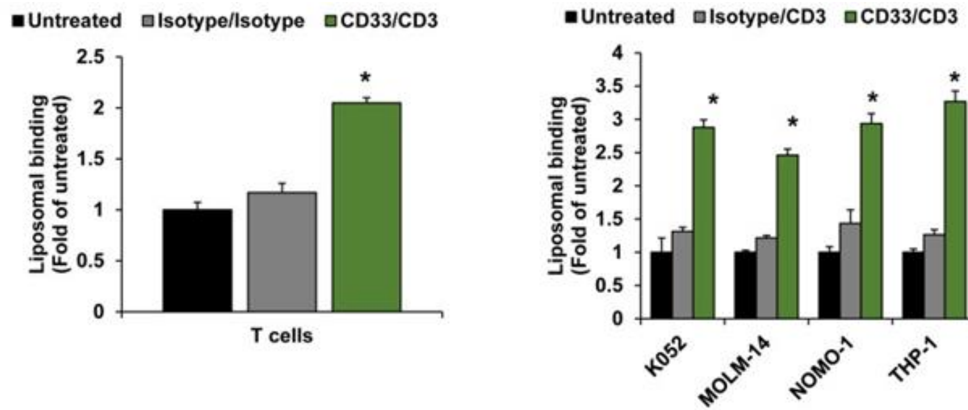

Figure 11. Binding of Isotype and CD33/CD3 nanoTCEs to T cells (left panel) and to AML cell lines (right lane) (* = $p < 0.05$) [49].

1.4. Three-Dimensional Cultures

Previous models have been created to replicate the three-dimensional (3D) microenvironment of the bone marrow (BM) using various materials such as collagen, Matrigel, acrylic polymers, silk, hyaluronic acid, and ossified tissues [51]. However, each of these models has its limitations. For instance, hydrogel systems like collagen, Matrigel, or synthetic polymers are simple and reproducible but do not mimic the physiological conditions of the BM [51]. Solid systems, such as ossified tissues, mimic the BM environment but are technically challenging to reproduce and adapt and rely on a normal BM microenvironment rather than the unique characteristics of the tumor cells such as MM cells [51] (Fig. 12).

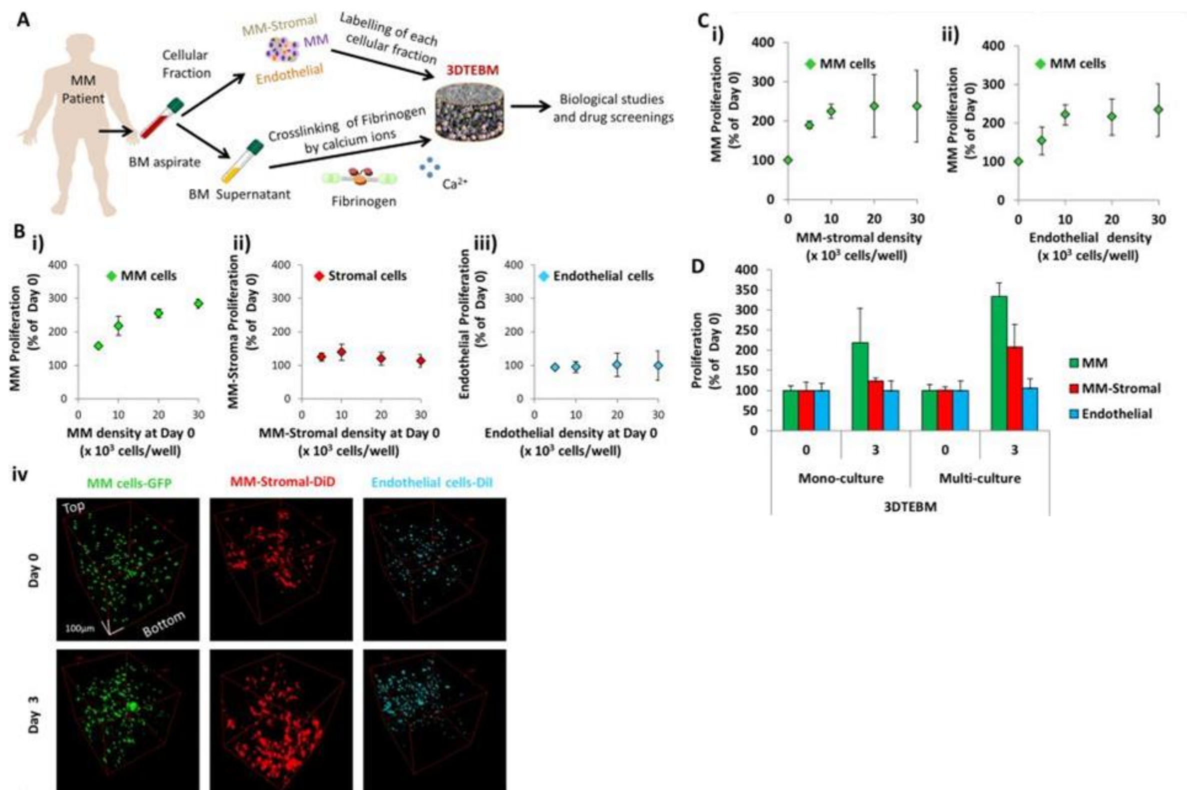

Figure 12. (A) 3DTEBM cultures were created by cross-linking fibrinogen, a component naturally found in the plasma of BM supernatant, with calcium. These cultures incorporated various cellular components, including MM cells, MM-derived stromal cells, and endothelial cells, which were pre-labeled and introduced into the cultures. (B) The impact of cell density (ranging from 5 × 10^3 to 30 × 10^3 cells/well) on the proliferation of i) MM cells, ii) MM-derived stroma, and iii) endothelial cells was examined in 3DTEBM mono-cultures at day 3. Confocal microscopy images of MM-GFP (green), MM-derived stroma-DiD (red), and endothelial cells-DiI (cyan) in mono-cultures within the 3DTEBM at days 0 and 3 were captured using Z-stack images in a rotated view. Scale bar = 100 µm. (C) The effect of co-culturing MM cells with i) MM-derived stromal cells (at varying densities of 0–30 × 10^3 cells/well) or ii) endothelial cells (at varying densities of 0–30 × 10^3 cells/well) on MM cell proliferation within the 3DTEBM was evaluated at day 3. (D) A summary of the proliferation rates of MM cells (30 × 10^3 cells/well), MM-derived stromal cells (10 × 10^3 cells/well), and endothelial cells (10 × 10^3 cells/well) in the 3DTEBM when cultured as mono-cultures or multi-cultures was provided at day 3 [51].

This study describes a 3D scaffold using BM supernatant from MM patients [51]. This scaffold incorporated various components found in the BM, including MM cells, stromal cells, and endothelial cells [51]. The model, referred to as a 3DTEBM culture, was hypothesized to enhance the growth of MM cells and provide a more relevant model for evaluating the effectiveness of drugs in MM. Please refer to Fig. 13 for an illustration

of the model [51] the same technique is used by Azab company Cellatrix for personalized treatment of MM patients (Fig. 13).

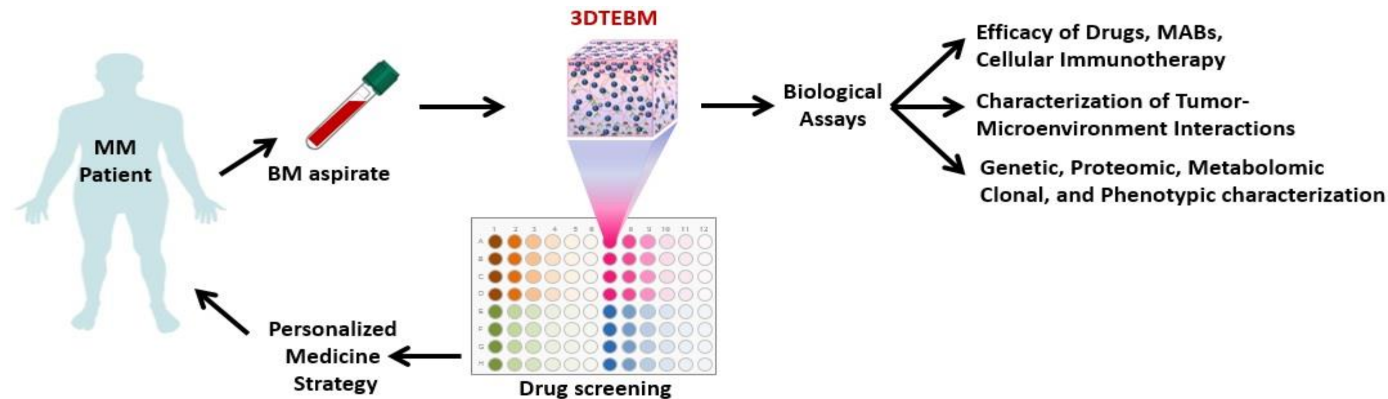

Figure 13. Using the 3D technique for personalized treatment. A patent by Abdel Kareem Azab [52].

1.5. The Hunt for Tumor Antigens for Immunotherapy in TNBC

Identifying specific tumor-associated antigens in TNBC has been a challenge and a bottleneck for the development of T cell-based immunotherapy [53], especially since TNBC is defined by the fact that it has "negative" expression of different markers. Several studies have demonstrated the use of CAR-T cells engineered against several "generic" markers expressed in solid tumors such as EGFR [54], ICAM [55], cMet [56] and MUC1 [57]. Unfortunately, none of these resulted in an efficacious treatment for TNBC. Therefore, there is an urgent need for finding a reliable tumor-associated antigen in TNBC.

2. Hypothesis and Aims

In this work, we hypothesized that AXL can serve as a tumor-associated antigen, and serves as a target for the development of nanoparticle-based T cell engagers against TNBC. Using our marked liposomes (nano BTCE) with their prolonged half-life will allow a more efficient tool to kill 4T1 TNBC cell lines due to the specific markers on the nanoTCE targeting the AXL of TNBC 4T1 cells in vitro by engaging those tumor cells to T cells by the mAbs targeting T cells.

2.1. Aim 1: To measure the expression of AXL on the surface of TNBC cells in vitro using targeted engager liposomes by testing those double-targeting antibodies with flow cytometry.

2.2. Aim 2: To target AXL using nano BTCEs liposomal engagers containing mAbs targeting AXL on TNBC cells and mAbs against the CD3 receptor on cytotoxic T cells that we harvested from healthy mice.

3. Materials and Methods

3.1. Materials

PAN cell Isolation was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Calcium chloride, tranexamic acid, and type 1 collagenase were obtained from Millipore-Sigma (Saint Louis, USA). Cell tracker DiD (excitation 635 nm; emission 655-730 nm) and counting beads were purchased from Invitrogen (Carlsbad, CA). RPMI-1640, 0.25% trypsin, L-glutamine, and penicillin-streptomycin were purchased from Corning (Corning CellGro, Mediatech, Manassas, VA). Fetal bovine serum, lipophilic tracers, collagenase, and counting beads were purchased from Life Technologies (Carlsbad, CA). 1,2-dipalmitoyl-sn glycerol-3-phosphocholine (DPPC), 1,2-distearoyl-snglycero-3-phosphoethanolamine-N-amino (polyethylene glycol)-2000] (DSPE-PEG2000), and extrusion membranes were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Millipore Sigma (Burlington, MA). Streptavidin conjugation kit was purchased from Abcam (Cambridge, United Kingdom).

3.1.1. Antibodies

The antibodies used in the experiments were analyzed by flow cytometry using MACSQuant Analyzer 10 (Milteny Biotec) with an excitation of 400 nm and emission 455 nm. The primary antibody was rat-anti-AXL-α−Biotin, and the secondary antibody was anti-biotin-VioBlue).

3.1.2. Cell Culture and 3D Culture

4T1 cell lines were generously given by Dr. Pilare from Washington University, and the cell lines were grown in RPMI-1640 media (Corning CellGro, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY), 2 mmol/L L-glutamine, 100 U/mL Penicillin, and 100 µg/mL Streptomycin (CellGro, Mediatech, Manassas, VA). The cells were cultured at a temperature of 37 °C and in an atmosphere of 5% CO2. Culturing was performed either under normoxic conditions (21% O2) using the NuAire water jacket incubator (Plymouth, MN), or under hypoxic conditions (1% O2) using a hypoxic chamber obtained from Coy (Grass Lake, MI).

Blood mononuclear cells were isolated from healthy donors using Ficoll-Paque PREMIUM (Millipore Sigma), and T cells were isolated using Pan T cell isolation kit. Cell lines were incubated in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, and 1% penicillin-streptomycin. Cell cultures were incubated either in NuAire water jacket incubators (NuAir, Plymouth, MN) at 37°C and in 5% CO2; or incubated in hypoxia (1% O2) using the hypoxia chamber purchased from Coy (Grass Lake, MI).

3.2. Methods and Technology

3.2.1. Cell Survival

GFP-dyed TNBC cell lines (4T1) cultured (plated on flat bottomed 96 well plate) in 3DTEBM. The 3DTEBM works as a breast-like microenvironment in vitro, generating a scaffold that generates a hypoxic gradient similar to breast tissue in the patient's body. Five replicates of cultured GFP-dyed-4T1 cells were incubated with T cells isolated from healthy mice and our DiD-dyed-AXL/CD3 nanoTCEs, and five other replicates of GPF-dyed 4T1 cell lines were treated with isotype/CD3 nanoTCEs, and five other replicates were untreated GPF-dyed 4T1 cell lines. All cells that were plated in the 3DTEBM on the same 96 well plate incubated in normoxia/hypoxia for 48 hours days, hours, 2 hours, and hypoxia in the same period. Following the incubation, the 3DTEBM gel was digested,

and counting beads were added to all samples. All samples were washed with PBS and analyzed by flow cytometry for GFP, the results of RMFB-relative mean fluorescent intensity were recorded for analysis.

3.2.2. TNBC Cell Cultures and 3DTEBM

Nanoparticle-based T cell engagers were prepared following the technology developed by Azab and Alhallak [49, 50]. The liposomes were loaded with two monoclonal antibodies attached to the liposomal nanoparticle surface; one antibody against cancer antigen in AXL in the 4T1 cells, while the other mAb targets cytotoxic T cells (Fig. 15C). The 3D cultures utilized in this study were derived from a model developed by Azab (Fig. 14). Specifically, 4T1 cells were cultured within 3DTEBM technology. The aim was to establish a model that closely mimics the pathophysiological conditions present in breast tissue, facilitating the ex vivo proliferation of TNBC cells. This 3D culture system offers a more accurate representation of the response of real TNBC tumor cells within their microenvironment in patients. The matrix used in this culture was generated by cross-linking fibrinogen, a natural component found in the breast's epithelial tissues. This environment better recapitulates the native conditions and interactions present in breast tissue, enhancing the relevance and reliability of the experimental findings.

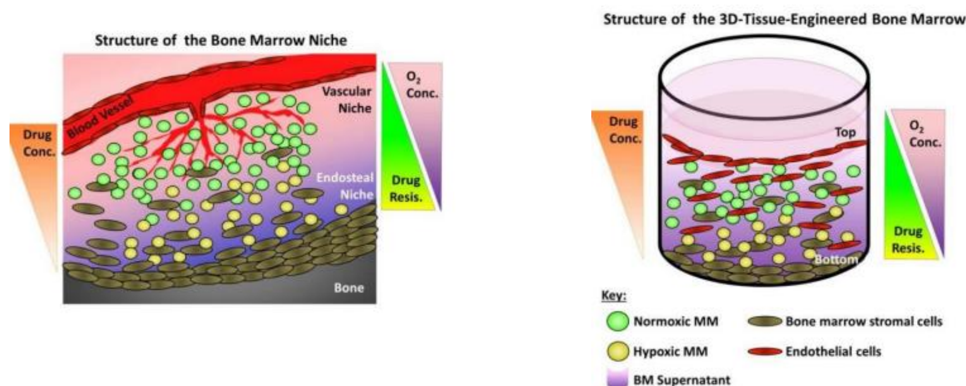

Figure 14. The 3DTEBM schematic involves the combination of blood plasma, RPMI-1640, $CaCl_2$, and tranexamic acid. Additionally, MM cells and treatment are introduced to the initial mixture. After a two-hour setting period, the mixture transforms into a gel-like scaffold. Subsequently, the gel can be digested using collagenase type II for two hours. The resulting mixture can then be treated as a conventional 2D in vitro sample.

3.3. Experiments

3.3.1. Preparation of Nanoparticle-Based AXL/CD3 T Cell Engagers

Liposomes were prepared from DPPC, cholesterol, and DSPE-PEG2000 with a ratio of 65:30:5, respectively. The lipids were mixed and solubilized in chloroform and then evaporated to form a thin film (Fig. 15A, B). The film was hydrated with PBS, and the suspension was extruded through polycarbonate membranes to yield unilamellar liposomes. Streptavidin was added to the amino groups on the surface of the liposomes following the protocol of the manufacturer (Abcam), for liposomes activation. Biotinylated antibodies were added to bind to streptavidin for targeting AXL. Zeta potential, diameter, and polydispersity were determined by Malvern Zetasizer Nano ZS90 (Malvern, Worcestershire, United Kingdom) for polydispersity, size, and zeta potential (Table 4). The activated liposomes are now termed nanoTCEs (Fig. 15C).

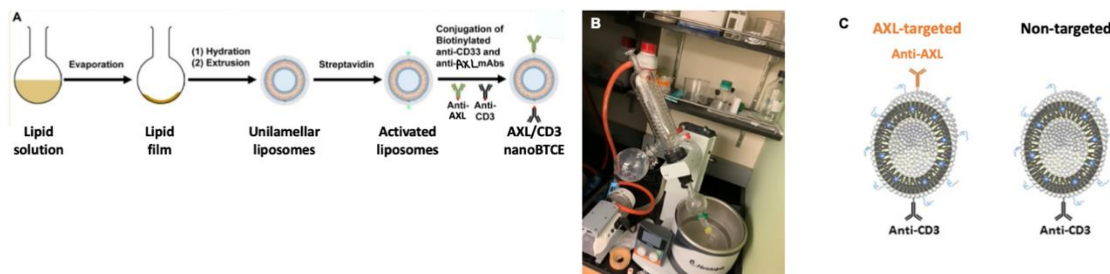

Figure 15. (A) Steps of nanoTCEs preparation. (B) Evaporating chloroform for lipids via the rotary evaporator. (C) AXL-targeted and non-targeted nanoliposomes for engaging 4T1 cells.

3.3.2. Cell Surface AXL Expression

In order to validate the presence of AXL on 4T1 cells, the culture was incubated in 3D normoxia and hypoxia for 4 hours, for 24 hours, and for 48 hours. Five duplicates for each treatment were performed. One group of cells was kept unstained and the other was incubated with Rat-anti-AXL-α-Biotin and then anti-biotin-VioBlue. The third group was incubated only with the secondary mAb anti-biotin-VioBlue. All antibody treatments were done at 4 ^{o}C for two hours. Cells were analyzed by flow cytometry to detect the quantity of anti-biotin-VioBlue using MACSQuant Analyzer 10 (Milteny Biotec) with an Ex/Em of 400 nm and 455 nm. Cells were gated using forward and scatter and analysis

was done for the mean fluorescent intensity (MFI) and relative mean fluorescence intensity (RMFI) of VioBlue using BD FlowJo Software.

3.3.3. AXL-Liposomes Binding

4T1 cell lines were treated with isotype/CD3 or AXL/CD3 nanoBTCE stained with DiD for two hours at 37 C. Spun down the cells, washed with PBS, and analyzed by flow cytometry with Ex/Em of 646 /670 ± 25. Cells were gated using forward and side scatter and analyzed for RMFI of DiD using BD FlowJo Software.

3.3.4. TNBC Survival in vitro

GFP-4T1 cells of TBNC cells were incubated with T cells from a healthy mouse in the 3DTEB (plated on a 3D flatbottomed 96 well plate) and treated with Isotype/CD3 and AXL/CD3 nanoBTCE for 4 days. The T cell count in the healthy mice sample was detected by using anti-CD3-PE with Ex/Em of 488/585±20 in BD FlowJo Software. The procedure used for staining T cells from the PBMCs begins with centrifuging the sample containing cells at 2000 rpm for 5 minutes. The supernatant liquid is then aspirated, and the cells are resuspended in phosphate buffer serum (PBS). Anti-CD3-PE was added to the cells, which were kept at 4 °C for one hour to stain the cells. The cells are then washed by adding 1mL of PBS, centrifuged, and aspirated again to clear the cells of unbound antibodies. The cells were resuspended and ready to be filtered before being analyzed by flow cytometry.

Knowing the number of T cells per µl, 4T1 cells were plated in our 3D culture and incubated with the T cells of the PBMCs (different immune cells are combined with the culture), and the T cells: 4T1 cells ratio was 1:1. Beads were be added to culture before matrix digestion. The cells will be then analyzed as GFP cells and normalized to the number of counting beads using BD FlowJo Software. After incubation for 4 days, the 3D culture was digested and cell survival was tested by flow cytometry gating for GFP.

3.4. Statistical Analysis

The experiments were replicated independently three times and conducted in quadruplicate. Data from our experiments were presented as means ± standard deviation. Statistical significance was determined using Student's t-test. *P* values less than 0.05 were considered indicative of statistically significant differences.

4. Results

4.1. Cell Surface AXL Expression

The AXL protein is found in abundance on TNBC cells making it a valuable target for treatment, this is why we needed to validate the presence of AXL on our 4T1 cell (representing TNBC), we measured the fluorescent mean intensity for mAb attached to AXL on the cells, we did this in hypoxia and normoxia and found the 4T1 cells to be expressing more AXL in hypoxia, this is an indication that the AXL can be a promising candidate for targeting and treating TNBC with our nanoBTCEs, the AXL stained cells show expression of 3.5 fold comparing to the isotype were only secondary mAb added (Fig. 16).

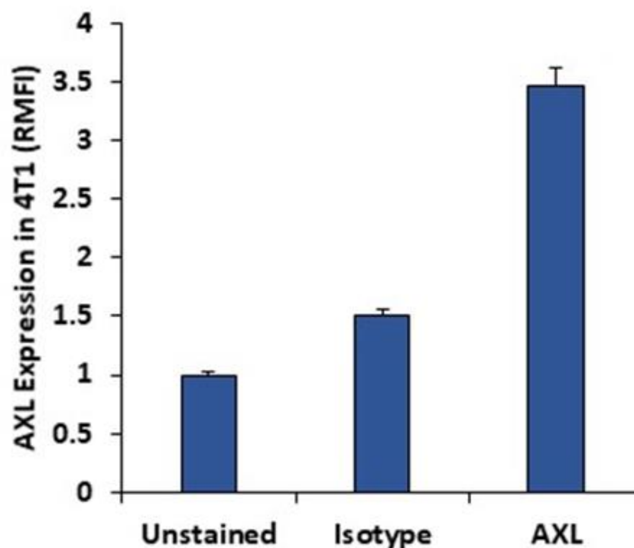

Figure 16. Expression of AXL on 4T1 cell lines was detected by Flow Cytometry using anti-AXL VioBlue-α-biotin antibody.

4.2. Preparation of Nano-Based AXL/CD3 T Cell Engagers

Characterization of the nanoTCEs physiochemical properties was performed using a Zetasizer. Liposomes were tested for diameter, polydispersity index (PDI), and zeta potential. The results are presented in Table 4 below. The produced nanoTCEs show uniformity in size (~140 nm) with low zeta potential close to neutral net charge, allowing them to successfully bind to AXL protein present on 4T1 cell membrane surfaces.

Table 4: Characterization of nanoTCEs

Formulation	Size (nm) Mean±SD	Polydispersity Index (PDI)	ζ-potential (mV) Mean±SD
AXL-Targeted TCEs	145 ± 7	0.04	-3.4 ± 0.4
Non-Targeted TCEs	139 ± 4	0.02	-4.1 ± 0.3

4.3. AXL Liposomes Binding

After that we tested the binding percentage of AXL/CD3 nanoBTCES to 4T1 cell lines, comparing it to isotype binding. The results show that the targeted AXL/CD3 nanoBTCEs bound at 4T1 cells were 75% compared to isotype/CD3 nanoTCEs (Fig. 17).

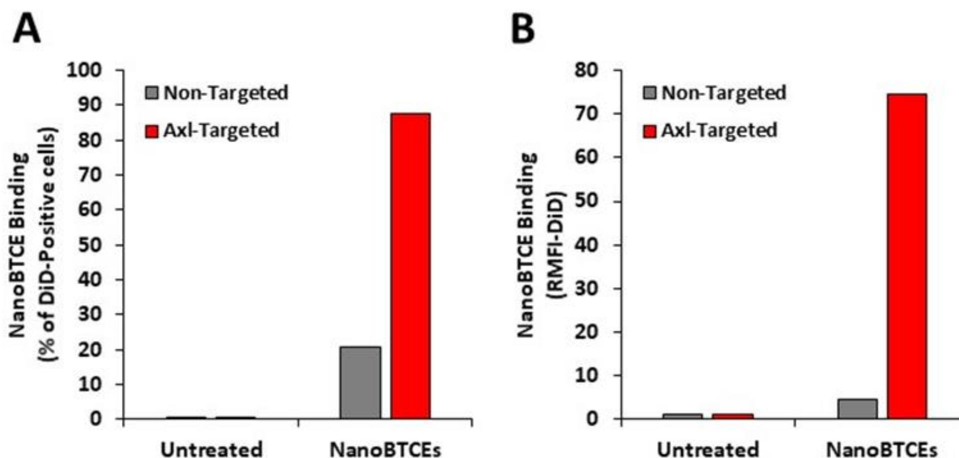

Figure 17. Binding of 4T1 cells to T cells via nanoBTCE. (A) Liposomal binding of isotype and CD3/AXL targeted-nanoBTCEs to 4T1 cells. (B) Relative mean fluorescence intensity representation of the binding of liposomal isotypes and CD3/AXL nanoBTCE targeted to 4T1 cells.

4.4. The Effect of AXL nanoTCEs on TNBC Survival

To assess the therapeutic potential of nanoBTCEs, the activation of T cells and the induction of cell killing was evaluated. In an in vitro 3DTEBM culture, the survival of cells was measured following non-targeted versus AXL-targeted nano-BTCE incubation. The 3DTEBM culture system replicates the solid tumor environment more accurately than conventional 2D systems, incorporating an O_2 gradient to mimic the hypoxic effects in the tumor core. Upon co-culturing T cells and 4T1 cells with nanoTCEs, activation of T cells that lead to the successful killing of 4T1 cells was observed (Fig. 18). These results demonstrate the efficacy of our nanoBTCEs in inducing the killing effect on 4T1 cells by facilitating their engagement with T cells. Notably, when using the isotype/CD3 alone, the nanoTCEs were unable to activate T cells, rendering the T cells ineffective in killing tumor cells.

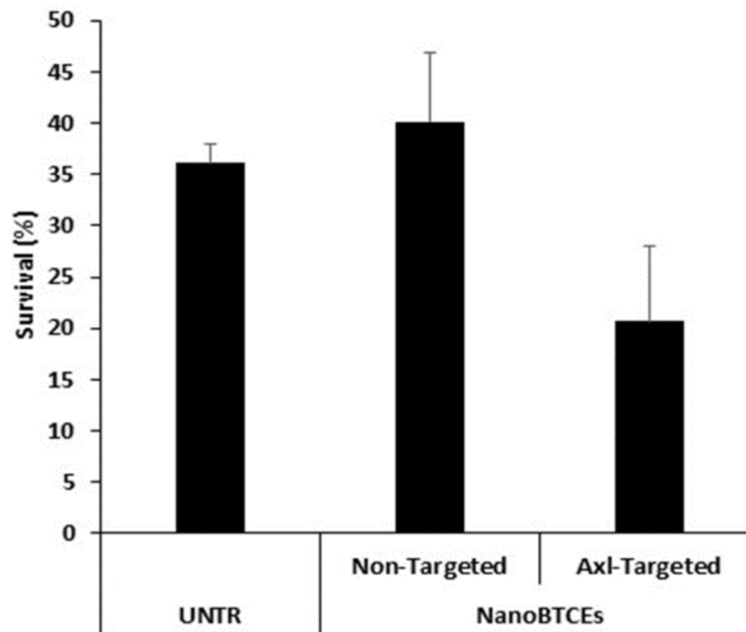

Figure 18. Efficacy of nanoBTCEs in vitro. AXL-targeted nanoBTCEs decrease the survival of 4T1 cells, compared to untreated and non-targeted nanoTCEs.

The interaction between 4T1 cells and T cells via nanoBTCEs aligns with the kinetic segregation model for T cell receptor triggering. Co-culturing 4T1 cells with T

cells using AXL/CD3 nanoBTCEs demonstrated an impressive 80% killing of 4T1 cells in vivo, surpassing the 63% killing observed in untreated cultures, and the 60% killing in 4T1 cells treated with non-targeted nanoTCEs (isotype) (Fig. 19).

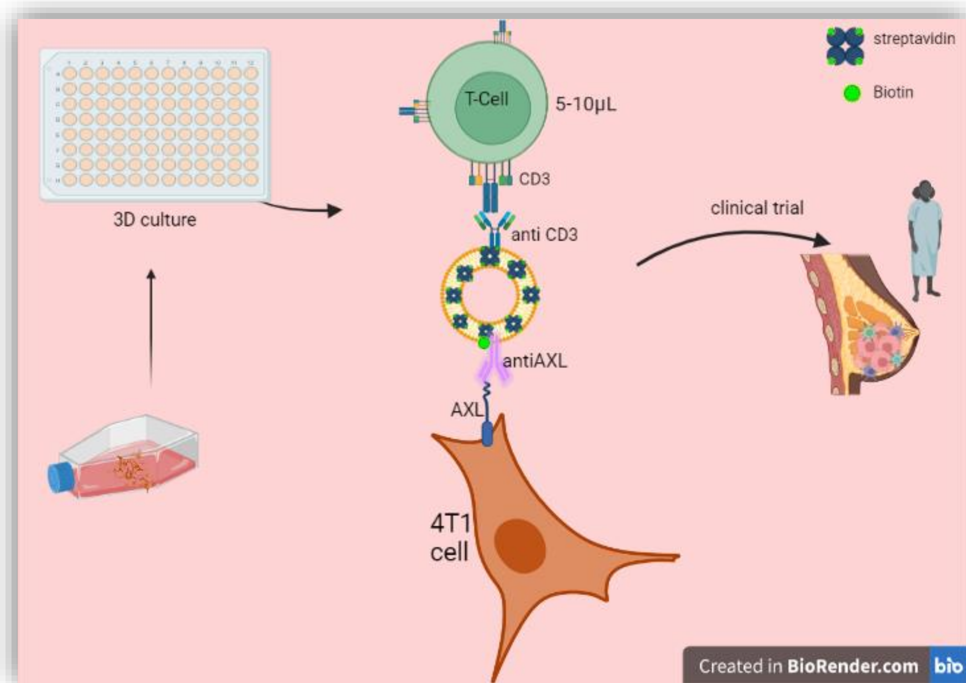

Figure 19. A schematic of the targeted 4T1 TNBC cell therapy by nanoBTCE. This figure was rendered using Biorender.com.

5. Discussion

TNBC is a specific type of breast cancer that relapses more quickly and has a relatively bad prognosis when compared to other breast cancers. The need for novel treatment for this type of TNBC. As a potential therapeutic strategy for TNBC, we created and tested the effectiveness of our AXL/CD3 nanoBTCE targeting AXL in TNBC cells after confirming that AXL is a notably prominent marker on TNBC cells in this work. Our findings demonstrate the potential of our nanoBTCE liposomes to overcome a variety of obstacles that currently available immunotherapies and CAR-T cells face, including high pricing, a short shelf life, and restricted accessibility.

Our AXL/CD3 nanoBTCE's ability to target TNBC cells specifically is a key benefit. Our nano TCE can attach directly to 4T1 cells and prevent contact with other cells in the tissue or circulating blood cells because AXL has been demonstrated to be extremely specific for TNBC cells. This specificity aids in lowering toxicity and improving the therapy's ability to target only cancer cells. Our liposomes also gain additional advantages from the use of PEG. PEG makes it more difficult for blood cells to phagocytose the liposomes, thereby improving their circulation time in the blood and increasing their potential to reach and target tumor cells effectively.

In combination with chemotherapy, our AXL/CD3 nanoBTCE demonstrated T cell activation and successful elimination of TNBC cells. This suggests that our technique has the potential to reduce chemotherapies, minimize relapse, and improve overall efficacy and efficiency in treating TNBC patients. However, upon analyzing the results of AXL expression in hypoxia, we encountered some confusing outcomes. Several possible explanations have been considered to understand the observed differences: First, the sensitivity of the 4T1 cell lines used in the experiment may be a limiting factor. The absence of normal niche cells found in breast tissue, which typically support the survival of these cells, might have influenced the results. Moreover, the 4T1 cells as solid tumor cells could be more sensitive to trypsinization compared to other tumor cells. To address this issue, future investigations could explore immunostaining techniques on slides containing the cells along with their surrounding cells from the "normal" tumor microenvironment, avoiding trypsinization. Secondly, our experimental conditions may not precisely replicate the hypoxic microenvironment seen in vivo within tumors. The absence of the diverse factors present in the tumor microenvironment and cellular interactions might have affected the response of the cells to the treatment. To improve the accuracy of our in vitro model, efforts should be made to mimic the tumor microenvironment more accurately, possibly by using co-culture techniques or three-dimensional models.

Additionally, the presence of shed AXL as a secreted protein could impact the effectiveness of our nanoBTCEs. Circulating AXL might interfere with the action of our TCEs by binding to them upon injection, hindering their effective targeting of tumor cells [8]. A comprehensive understanding of AXL shedding dynamics and its implications on TCE-based therapies is crucial for the success of future treatments. Moreover, while solid tumors exhibit acidic cores due to their rapid growth and inadequate blood supply, it is essential to ascertain whether the in vitro acidity accurately represents the in vivo tumor microenvironment. The patient's circulating blood system may play a role in resolving or buffering acidity differently inside the body, potentially affecting the efficacy of therapy. Further investigations are required to bridge this gap in knowledge and optimize therapeutic strategies.

6. Conclusions and Future Directions

In conclusion, our study has revealed promising results for our AXL/CD3 nanoBTCE as a targeted therapy for TNBC. However, further investigations and improvements are necessary to understand the factors influencing our results fully and to refine the approach for more accurate and effective TCE-based therapies in the future. Future research prospects may involve testing nano TCEs in combination with chemotherapy, utilizing double markers involving other receptors on TNBC cells, and conducting western blotting to assess shedding effects on tumor cells. Additionally, RNA microarrays can explore the involvement of AXL in other genes and oncogenes, shedding light on its broader impact on cancer biology. Overall, the development of our AXL/CD3 nanoBTCE represents a significant advancement in the field of clinical laboratory sciences, offering a targeted, specific, and potentially more accessible therapeutic option for TNBC patients.

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