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Review

Preclinical models of breast cancer: Two-way shuttles for immune checkpoint inhibitors from and to patient bedside

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Abstract The Food and Drug Administration has lately approved atezolizumab, anti-programmed death ligand 1 (PD-L1), to be used together with nanoparticle albumin-bound (nab) paclitaxel in treating patients with triple negative breast cancer (BC) expressing PD-L1. Nonetheless, immune checkpoint inhibitors (ICIs) are still challenged by the resistance and immune-related adverse effects evident in a considerable subset of treated patients without conclusive comprehension of the underlying molecular basis, biomarkers and tolerable therapeutic regimens capable of unleashing the anti-tumour immune responses. Stepping back to

Abbreviations: BC, breast cancer; CSC, cancer stem cells; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; GEM, genetically engineered model; HTS, High-throughput screening; ICI, immune checkpoint inhibitor; irAEs, immune-related adverse events; LAG3, Lymphocyte Activation Gene-3; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MSK-IMPACT, Memorial Sloan Kettering Cancer Center – Integrated Mutation Profiling of Actionable Cancer Targets; PD1, programmed cell death-1; PR, progesterone receptor; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM-3, T cell immunoglobulin and mucin containing protein-3; TNBC, triple-negative breast cancer; Treg, regulatory T cells; xGvHD, xenograft versus host disease.

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preclinical models is thus inevitable to address these inquiries. Herein, we comprehensively review diverse preclinical models of BC exploited in investigating ICIs underscoring their pros and cons as well as the learnt and awaited lessons to allow full exploitation of ICIs in BC therapy.

1. Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths in women [1]. BC is a heterogeneous disease which demands precise molecular classification and diagnostic techniques [2]. Based on estrogen receptor (ER), progesterone receptor(PR) and human epidermal growth factor receptor 2 (HER2) expression, BC is categorized into five subtypes [3]. Their treatment paradigms include surgery, radiotherapy and/or cytotoxic chemotherapies, endocrine or HER2-targeted therapies [2]. Compared with other subtypes, patients with triple-negative breast cancer (TNBC) ($ER^-/PR^-/HER2^-$)—which accounts for 10–20% of invasive BC—are associated with poorer prognosis given the lack of actionable druggable targets [3–6]. Additionally, despite considerable advances in treating other BC subtypes, tackling metastasis and drug-resistance (relapse) still constitute critical hurdles which ultimately contribute to the lower overall survival rates [3]. Novel efficacious therapeutic approaches are thus urged.

With the durable responses observed in a subset of treated patients with melanoma, non-small cell lung cancer and TNBC, immune checkpoint inhibitors (ICIs) are foreseen to revolutionize cancer history [7,8]. ICIs are monoclonal antibodies directed against inhibitory immune checkpoints expressed on diverse types of immune cells including cytotoxic T lymphocyte associated antigen-4 (CTLA-4), T cell immunoglobulin and mucin containing protein-3 (TIM-3), lymphocyte activation gene-3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), programmed death-1 (PD-1) or its ligand, programmed death ligand 1 (PD-L1) which is co-expressed by immune and tumour cells [9–11](Fig. 1).

Though several ICIs are in the pipeline of their pre/clinical development, only anti-CTLA-4, anti-PD-1 and anti-PD-L1 have been approved so far by the Food and

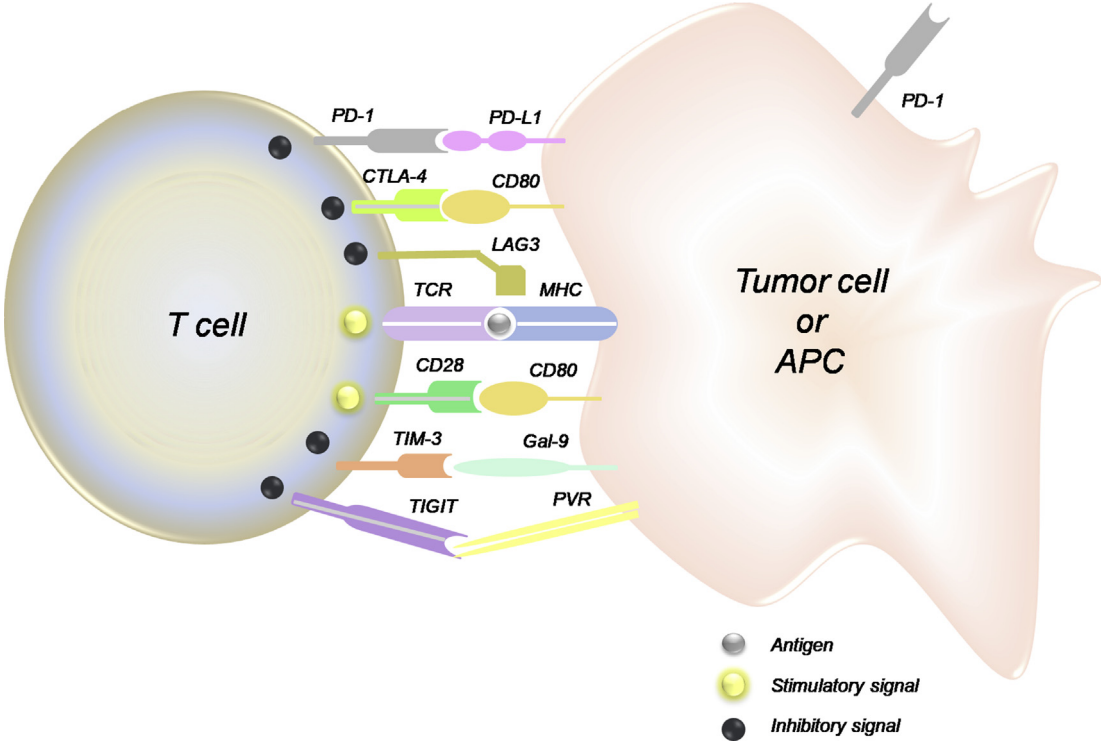

Fig. 1. **Ligand-receptor interactions between T lymphocyte and tumour or antigen presenting cells (APC).** The immune checkpoints CTLA-4 (cytotoxic T lymphocyte associated antigen-4), TIM-3 (T cell immunoglobulin and mucin containing protein-3), LAG3 (lymphocyte activation gene-3), TIGIT (T cell immunoreceptor with Ig and ITIM domains), PD-1 (programmed death-1), PD-L1 (programmed death ligand 1), APC (antigen-presenting cell as dendritic cell or macrophage); TCR (T-cell receptor), MHC (major histocompatibility complex), Gal-9 (Galectin-9) and PVR (poliovirus receptor).

Drug Administration (FDA) for cancer therapy [8,12]. T cell activation is a two-step process that involves the recognition of specific peptides presented by major histocompatibility complex (MHC) on the surface of tumour or antigen presenting cells (APC) via their T-cell receptor (TCR), coupled with a co-stimulatory signal delivered by CD28 receptor upon its binding with its ligands CD80 (B7.1) and CD86 (B7.2) [13]. CTLA-4 suppresses T-cell activation by outcompeting CD28 binding to B7 molecules [13](Fig. 1). PD-1 binding to its ligands (PD-L1 or PD-L2), predominantly expressed within inflamed tissues and tumour microenvironment (TME), triggers 'T-cell exhaustion' [13] (Fig. 1).

2. Immune checkpoint inhibitors for BC therapy

Unlike relatively longer clinical experience using ICIs in treating other tumours, analogous studies on BC have started relatively later because BC was originally thought to be non-immunogenic [14]. Nonetheless, accumulating evidence underscored its immunogenic nature especially TNBC subtype [15,16]. While acknowledging various PD-L1 positivity assessment strategies, systematic literature review revealed that PD-L1 expression greatly varied among diverse BC subtypes(luminal A [2.3–37%], luminal B [9–46%], HER2$^+$[0–33%] and basal-like/TNBC [5–80%]) [17,18]. High CD8$^+$ tumour-infiltrating lymphocytes (TILs) and CD8$^+$/FOXP3$^+$ ratio in residual tumours and following neoadjuvant chemotherapy correlated with improved prognosis in TNBC patients [19]. Transcriptomic analysis of more than 1000 BC patients revealed that high CD8$^+$ activation score was associated with an improved survival [20]. During chemotherapy, the repertoires of circulating CD8$^+$ T cells evolve and increased heterogeneity of such repertoire was associated with better clinical responses [19]. Breast cancer type 1 susceptibility protein (BRCA1)-mutated TNBCs harboured higher somatic mutational burden and increased PD-1^{hi} and CTLA-4^{hi} TILs as compared with BRCA1 wild-type TNBC patients [16].

The presence of tumour infiltrating CD8$^+$ T cells is associated with a reduced hazard of BC-specific mortality in both ER$^+$/HER2$^+$ and TNBC subtypes [16]. Single-cell proteomics atlas of human BC ecosystem revealed that PD-L1$^+$ tumour associated macrophages (TAMs) and exhausted T cells are prevalent in high-grade ER$^+$ and ER$^-$ BC [21]. The immune microenvironment during BC progression and metastasis is reported to be immunologically more inert compared with their paired primary BC samples [22]. These evidences stimulated the clinical evaluation of ICIs′ efficacy in BC.

The overall response rate of 27 TNBC patients treated with pembrolizumab (anti-PD-1) was reported to 18.5% [17]. Pembrolizumab monotherapy also demonstrated durable anti-tumour responses in treating a subset of previously treated metastatic TNBC patients [23]. Following IMpassion130 trial in which the response rate and complete response of PD-L1$^+$ TNBC patients treated with atezolizumab (anti-PD-L1) alongside nanoparticle albumin-bound (Nab)-paclitaxel was 58.9% and 10.3%, respectively, compared withto 42.6% and 1.1% in Nab-paclitaxel alone cohort, FDA has lately approved this combination for treating TNBC (>1% PD-L1$^+$ positivity) [8]. Nonetheless, ICIs are plagued by serious immune-related adverse events (irAEs) [24,25]. It is also worth noting that the molecular basis underlying ICIs resistance, predictive/prognostic biomarkers and tolerable therapeutic regimens capable of removing the brake from the immune system remain largely obscure.

3. Preclinical BC models

Preclinical models have served for ages as the backbone of anti-cancer drug discovery and development that led to historical clinical breakthroughs [26]. Nonetheless, most of the preclinically evaluated drugs (75–90%) failed to gain FDA approval [26] and for which preclinical models were blamed at least partially [23]. To provide a roadmap for performing more clinically relevant preclinical immunotherapeutic studies, in this section, we will review the opportunities alongside challenges associated with various preclinical BC models exploited for developing/characterizing ICIs.

3.1. Ex vivo BC models

Considerable efforts have been invested in developing *ex-vivo* systems considering the dynamic BC ecosystem to serve as a platform for high throughput screening (HTS) [27–29].

Ex-vivo microfluidic systems have been adapted to screen the capability of small molecules to unlock the resistance of murine and patient-derived organotypic tumour spheroids (MDOTS/PDOTS) to anti-PD-1 [28]. The TME comprising MDOTS/PDOTS and autologous stromal/immune cells retained tumour-infiltrating myeloid and lymphoid subpopulations and responded to anti-PD-1 within short-term (3–6 days) [28]. *Ex-vivo* responses to ICIs were evaluated using dual 'acridine orange/propidium iodide' labelling deconvolution fluorescence microscopy (to assess live/dead cells respectively) and cytokines profiling. Intriguingly, the responses of this *ex-vivo* system to ICIs matched the *in vivo* responses even preserving their heterogeneous responses [28].

Perfusion-based bioreactors – originally developed for tissue engineering and regeneration purposes – maintained the viability and functional activity of 27 surgically excised and metastatic BC tissues alongside their microenvironment including immune cells, for more than 2 weeks [29]. Perfusion-based bioreactors offered an advantage over static cultures because they permit continuous culture media flow/supplementation

to the tissues via porous scaffolds ultimately increasing the survival of BC specimens including their ecosystems [29]. Targeted next generation sequencing (NGS) did not reveal major alterations between cultured BC tissues and their original counterparts [29]. However, single nucleotide variations were observed [29].

Cultured ER^+ and $HER2^+$ BC tissues were responsive to fulvestrant (ER antagonist–selective ER degrader) and pertuzumab (anti-HER2) respectively [29]. Such responses were assessed by histomorphological and immunohistochemical analyses of the treated fixed paraffin-embedded BC sections. Massive cytotoxicity was evident in patient-derived TNBC tissues – but not normal breast tissues and lymphocytes – following anti-PD-L-1 or anti-CLTA-4 treatment *ex-vivo* [29].

In the precision/personalized medicine era, *ex-vivo* techniques could advantageously present a rapid animal-free preclinical screening platform for evaluating therapeutic regimens comprising ICIs [29]. Yet, the functional statuses, evolution and spatial orientation of diverse immune and stromal cells within these *ex-vivo* systems or assays remain largely unknown and need further systematic investigation. These approaches are also limited by their inability to simulate complex *in vivo* conditions impacting the tumour-immune system interaction and are restricted to pre-existing TILs and do not consider the newly to-be recruited immune cells. While their clinical predictability is still questionable, if deemed reliable, these *ex-vivo* systems could assist in stratifying potentially responsive patients while sparing others from enduring unnecessary toxicities.

3.2. In vivo models

In vivo murine models have long been used to validate *in vitro* or *ex-vivo* findings within a complex organism [30,31]. Nonetheless, improper selection, design and execution of *in vivo* studies would maximize the preclinical/clinical gap. Herein, we will review different syngeneic, genetically engineered model (GEM) as well as humanized mouse BC models and their use in characterizing ICIs.

3.2.1. Syngeneic murine models

These models involve the engraftment of murine tumour cell lines spontaneously or chemically induced into immunocompetent strain-matched mice [31]. In Table 1, we have shortlisted different syngeneic BC models including a brief description regarding their origin, genetic background, metastatic potential and response to monoclonal antibodies directed against the FDA-approved inhibitory immune checkpoints.

Syngeneic models have provided milestone insights into diverse aspects of BC carcinogenesis [32,33,35]. One of such key findings is Paget's '*seed and soil*' metastasis hypothesis [36]. In early 1990s, in an attempt to identify the selective events in metastasis, Aslakson and Miller evaluated the sequential dissemination of five BC subpopulation lines (namely 4T1, 66cl4, 168FARN, 67NR and 4TO7) isolated from a single spontaneously arising BC from the mammary fat pad of a BALB/cfC3H mouse [35](Table 1). Recapitulating human BC, 4T1 spontaneously metastasized to distant organs including lungs, liver, heart, spleen, kidneys and brain [35]. Intriguingly, some organs (e.g. lungs) were more conductive to disseminated '*seed*' tumour growth [35]. 4TO7 cells disseminated to the lungs and occasionally livers but failed to establish progressively growing metastatic nodules and were classified as '*metastatic antigenic*' [35]. The '*seed and soil*' concept has been recoined where a minor subset of disseminating '*seed*' tumour cells undergo epithelial-to-mesenchymal transition (EMT) and transform into cancer stem cells (CSC) generating premetastatic niche 'soil' in distant organs/sites. Conversely, dynamic mesenchymal-to-epithelial transition (MET) is required for effective colonization of tumour 'seeds' distantly [33]. Within this context, two different subsets of myeloid-derived suppressive cells (MDSCs): monocytic (mMDSC) and granulocytic (gMDSC) have distinct transcriptomic and functional profiles [33]. Unlike less invasive EMT6, metastatic 4T1 cells secrete higher levels of inflammatory chemokines/cytokines and trigger early induction and infiltration of mMDSCs intratumoural (promoting EMT/CSC phenotype) and gMDSCs in the lungs (suppressing EMT/CSC thereby favouring intrapulmonary '*seed*' proliferation) [33]. Most 4T1 orthotopically transplanted mice developed lung metastasis when surgical resection of the primary tumour was performed 2 weeks post-transplantation, where gMDSCs have expanded/infiltrated the secondary organs. Instead, no lung metastatic growth was detected upon resecting 4T1 primary tumours 1 week post-transplantation despite the existence of disseminated 'seeds' in the regional lymph nodes and lungs reflecting the so-called '*tumour dormancy*' [33]. Adoptive intravenous transfer of lung-derived gMDSCs awakened dormant seeds and thereby promoted lung metastasis [33]. MDSC-induced '*metastatic gene signature*' identified from syngeneic model has successfully predicted poor survival of cancer patients including BC [33].

Syngeneic models have also served as a faithful platform for evaluating ICIs [37–41] (Table 1). Besides being metastatic, 4T1 cells are poorly immunogenic. Indeed, anti-PD-1 or anti-CTLA-4 as monotherapies failed to elicit any therapeutic effects on the growth of primary 4T1tumour, metastasis and/or survival and anti-PD-L1 had limited efficacy [37–40,42]. EMT6 exhibited partially responsive phenotype to anti-CTLA-4 and anti-PD-L1 but not to anti-PD-1 [37,43].

To understand the molecular basis beyond differential responsiveness/resistance to ICIs and identify reliable biomarkers, comprehensive molecular/functional profiling of these models is needed. Using the clustering algorithm, Yang et al. have found that – with the

Table 1
Descriptive list of syngeneic murine models of breast cancer.

Murine breast cancer cell line	Genetic background	Metastatic sites/ efficiency (% of mice with lung metastases)	Mouse strain	Description	Response to ICIs as monotherapy			Ref
					Anti-PD-1	Anti-PD-L1	Anti-CTLA-4	
4T1	ER^- PR^- $HER2^-$ $CDKN2B^{-/-}$	Orthotopically transplanted cells spontaneously metastasizes to the lung, liver, bone, heart, kidneys, brain and spleen (100% efficiency)	BALB/c	TNBC, thioguanine-resistant variant selected from 410.4 subpopulation (without mutagen treatment) which was isolated from a single spontaneously arising BC in a BALB/cfC3H mouse	Resistant [37,38]	Very limited activity [39,42]	Resistant [38,40]	[32,35, 38–40, 42]
66cl4	ER^- PR^- $HER2^-$	Orthotopically transplanted cells spontaneously metastasizes to the lungs	BALB/c	TNBC, thioguanine-, ouabain-resistant variant of tumour subpopulation line 66 isolated from a single spontaneously arising BC	NA	NA	NA	[35]
168FARN	ER^- PR^- $HER2^-$	Rarely metastasizes spontaneously	BALB/c	TNBC, diaminopurine-, geneticin-resistant variant clone of line 168 obtained by transfecting 168FAR with bacterial pSV2 plasmid-containing neomycin resistance gene	NA	NA	NA	[35]
67NR	ER^- PR^- $HER2^-$	Rarely metastasizes spontaneously	BALB/c	TNBC, geneticin-resistant BC cell line obtained by transfecting 67 sublines isolated from murine spontaneous BC.	NA	NA	NA	[33,35]
4TO7	ER^- PR^- $HER2^-$	Rarely metastasizes spontaneously	BALB/c	TNBC thioguanine-, ouabain-resistant variant of 410.4 tumour subpopulation isolated from a single spontaneously arising BC from a BALB/cfC3H mouse	NA	NA	NA	[35]
EMT6	ER weak expression, PR^-	Some reported no metastasis while others demonstrated 100% lung metastatic efficiency with orthotopic transplantation [32,33]	BALB/c	Selected from the 25 passage/ transplantation generation of KHJJ BC following the implantation of a hyperplastic alveolar nodule	Resistant [37]	PR [35] (TVR% = 40%)	PR [43] (40% CR)	[32,33,37, 40]
D2A1	PR^-	Lung metastasis efficiency = 45% (with orthotopic transplantation)	BALB/c	Spontaneous BC derived from a D2 hyperplastic alveolar nodule line	NA	NA	NA	[32]

E0771	ER$^-$ PR$^-$ HER2$^-$ CDKN2B$^{-/-}$	Lung metastasis efficiency = 50% (with orthotopic transplantation)	C57BL/6	Spontaneous TNBC	PR [40] (TVR%~40%) PR [44] (TVR%~70%, Median survival increased from 25 to 55 days), Resistant [41]	PR [45] (Significantly prolonged the survival)	PR [46] (TVR% ~60%)	[32,40, 41, 45,46]
F3II	ER weakly positive, PR$^-$	Lung metastasis efficiency of 70% (with tail vein intravenous transplantation)	BALB/c	Sarcomatoid clone derived from spontaneous ER$^-$ negative BC (M3)	NA	NA	NA	[32]
TSAE1	ER weakly positive, PR$^-$	Lung metastasis efficiency = 100% (with tail vein intravenous transplantation)	BALB/c	Subclone of TS/A parental spontaneous BC cell line with epithelial morphology and higher metastatic potential	NA	NA	NA	[32]
EAC	NA	Metastasises to the lungs, liver, spleen, kidney, bone and adrenal glands	Swiss albino 129/SvJ	Spontaneous BC	NA	NA	NA	[30,47, 48]

BC = breast cancer; CR = % of mice with completely regressed tumour; EAC = Ehrlich Ascites Carcinoma; PR = partial response; TNBC = triple-negative breast cancer; TVR% = 100x (1-[tumour volume of ICI-treated cohort/tumour volume of control Ab-treated cohort]); NA = not available; PD-L-1 = programmed death ligand 1; PD-1 = programmed death-1; ICI = immune checkpoint inhibitor; CTLA-4 = cytotoxic T lymphocyte antigen-4; ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2.

exception of E0771 and D2A1 which are predominantly luminal B − an appreciated subset of syngeneic BC models belong to luminal A subtype [32]. The percent of GR1$^+$ granulocytes and CD3$^+$ T lymphocytes infiltrating EMT6 and F3II are higher compared with 4T1 model [32]. In 4T1 model, CD11b$^+$ tumour-associated myeloid cells constituted the major proportion of infiltrating immune cells which adversely affected CD8$^+$ infiltration/cytolytic potential [38]. Consistent with their relatively longer time of development, exome sequencing revealed higher single nucleotide variant burden/genome in spontaneous murine BC cell lines compared with GEM counterparts [32]. It remains to be investigated whether this correlates with variable responses to ICIs.

Syngeneic models have several limitations including the lack of some human targets, relatively lower mutational burden and rapid growth rates which does not allow the development of the chronic inflammatory microenvironment analogous to that of human BC [31]. Nonetheless, ease, low-cost coupled with the provision of an *in vivo* immunocompetent platform have all positioned them as the 'first-track' frontiers in immuno-oncology experiments [37–41].

3.2.2. Genetically engineered models

With the evolution of human-directed targeted therapies, syngeneic models were partially replaced by GEM and patient-derived tumour xenograft (PDX) models [31]. Genetic engineering enabled direct gene editing in germline and/or somatic cells [49]. Somatic cells-derived GEM are speculated to better recapitulate human tumours as they arise from few cells within the normal stromal microenvironment compared with germline GEM models. Timely and tissue-specific controlled genome editing is widely performed using transgenic mice expressing Cre-mutated alleles [31,49]. To model diverse BC subtypes, different promoters are utilized to direct oncogenic events to basal or luminal lineages [50]. Despite the use of lineage-specific promoters, their expression patterns have not been completely characterized and can result in confounding results [50]. Analogous to spontaneous models, several GEM models of BC were assigned to luminal A subtype (Table 2) [32].

BRCA1-mutated TNBC model has been generated to recapitulate an appreciated proportion of TNBC patients whose tumours are genomically more unstable compared with sporadic BC given the role of BRCA1 in cell cycle regulation and DNA damage repair [51,52]. Yet, among the hurdles limiting the preclinical use of this model is being time consuming (average BC latency ~7 months) [50]. Complementary to syngeneic models, GEM have been used to assess the efficacy of ICIs and identify predictive biomarkers [46,53](Table 2). Orthotopically MMTV-PyMT and subcutaneously transplanted MET mice displayed partially responsive and resistant phenotypes to anti-PD-1 respectively [53]. Depleting macrophages in these models increased CD8$^+$ intratumoural

Table 2
Descriptive list of genetically engineered mouse models (GEMs) of breast cancer.

Designation	Genetic background	Metastatic sites/efficiency	Mouse strain	Description	Response to ICIs			Ref
					Anti-PD-1	Anti-PD-L1	Anti-CTLA-4	
6DT1	PR^- Over-expressed Myc, $CDKN2A^-$ $CDKN2B^-$	Lung metastasis efficiency >90% (with orthotopic transplantation)	FVB/N	BC arising in MMTV-Myc transgenic mouse	NA	NA	NA	[32]
HRM-1	ER weakly positive, PR^- PIK3 CA,	Lung metastasis efficiency = 80% (with orthotopic transplantation)	FVB/N	Derived from a transgenic mouse model of breast cancer expressing PIK3CA (H1047R) in which the tumour reoccured following doxorubicin withdrawal	NA	NA	NA	[32]
M6	PR^-, deactivated p53 and Rb	Lung metastasis efficiency = 55% (with orthotopic transplantation)	FVB/N	BC arising in C3(1)/SV40 Large T-antigen (Tag) transgenic mouse	NA	NA	NA	[32]
MET1	ER^-, PR^-, $HER2^-$, activated PI3K	Lung metastasis efficiency = 90% (with tail vein intravenous transplantation)	FVB/N	MMTV-PyVT BC passaged in mammary fat pad	Resistant [53]	NA	NA	[32,53]
MVT1	PR^-, over-expressed Myc/VEGFA, $CDKN2A^-$ $CDKN2B^-$	Lung metastasis efficiency > 90% (with orthotopic transplantation)	FVB/N	BC developed in MMTV-Myc-VEGF bitransgenic mouse	PR (TVR% = 60%)	NA	NA	[32]
R3T	PR^-, potential contribution of activated Ras/Src pathway	Lung metastasis efficiency = 60% (with orthotopic transplantation)	129S3	Parental BC cell line derived from osteopontin KO mouse induced by medroxyprogesterone acetate followed by 7,12-dimethylbenz[a] anthracene mutagenic administration. Following their transformation with polyoma middle T (PyMT) oncoprotein and activated Ras, R3T cells were isolated from fat pad	NA	NA	NA	[32]

				tumours				
K14cre; $Cdh1^{F/F}$;$Trp53^{F/F}$	$Cdh1^{F/F}$ $p53^{F/F}$	Metastasis following mastectomy to the lungs, liver, spleen, pancreas and/or tumour-draining or distant lymph nodes	FVB/N	Conditional K14cre; $Cdh1^{F/F}$;$Trp53^{F/F}$ mouse model of invasive lobular BC which is based on stochastic loss of E-cadherin and p53 in the mammary epithelium	NA	NA	NA	[52]
$BRCA1^{F22-24/F22-24}$;$p53^{+/-}$	$BRCA1^{\Delta 22-24}$ $p53^{Null}$ ER^- PR^- $HER2^-$	NA	C57/BL6, 129/Sv	Basal-like BC generated by conditional deletion of BRCA1 exons 22−24 in the mammary gland using β-lactoglobin Cre and combined with heterozygosity for Trp53 mutation	NA	NA	NA	[50]
$BRCA1^{F5-13/F5-13}$;$p53^{F2-10/F2-10}$	ER^- $BRCA1^{\Delta 5-13}$ $p53^{\Delta 2-10}$	NA	FVB, 129/Ola	BC generated by K14 Cre-mediated conditional deletion of both BRCA1 exons 5−13 and Trp53	NA	NA	NA	[50]

BC = Breast cancer; metastatic efficiency = % of mice with lung metastasis; NA = not available; PR = partial response, TVR% = 100x (1-[$V_{treated}/V_{vehicle}$]). PD-L1 = programmed death ligand 1; PD1 = programmed death-1; ICI = immune checkpoint inhibitor; CTL4 = cytotoxic T lymphocyte antigen-4; ER = oestrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2. FVB/N = mice carry the *Fv1*^{b} allele that mediates their susceptibility to the B strain of Friend leukemia virus; MMTV = mouse mammary tumour virus; VEGF = vascular endothelial growth factor.

infiltration [53]. Exploiting MET1 and 66cl4 models, Eya3, a crucial regulator of embryogenesis, was found to promote immunosuppressive responses against TNBC via stabilizing Myc and increasing PD-L1 expression [46].

GEM of BC exhibit discrepant proliferative, angiogenic and immunogenic profiles [32,52]. For instance, the percent of $CD3^+$ T lymphocytes infiltrating R3T is higher compared to HRM1 [32]. Another invasive lobular BC (conditional K14cre; $Cdh1^{F/F}$;$Trp53^{F/F}$) is heavily infiltrated by macrophages and T-lymphocytes [52]. Investigating the responses of GEM with varying grades of immune infiltration to ICIs is needed.

Among the pros of GEM is their orthotopic *de novo* BC formation within a more physiological TME and histopathological recapitulation of human BC [52]. Nevertheless, these models are critically hampered by their costs, being time-consuming, relatively low metastatic incidence of some, low tumour mutational burden (originating from 1 to 2 mutated genes) and supra-physiological levels of the modified transgenes questioning their clinical relevance and the need for extensive expertise and infrastructure [31]. Perhaps, this all could explain their inferior exploitation in ICIs testing compared with syngeneic models.

3.2.3. Humanized mouse models

Lack of human targets as well as inherent interspecies immunological differences constitute major limitations of syngeneic and GEM models [54,55]. Accordingly, considerable efforts have been invested in humanizing the murine immune system (previously reviewed in Refs. [31,55–58]). One of these models is the immunoavatar model in which immunodeficient mice were reconstituted with human peripheral blood mononuclear cells (PBMCs), thus allowing human cancer cell lines or primary patient-derived tumours to be studied in an autologous or heterologous immunologic context [31]. Despite omitting the need of sophisticated purification techniques, prominent human xenograft versus host disease usually develops few weeks following the engraftment of human PBMCs and is thought to be because of the MHC mismatch between mouse and human T lymphocytes [31]. Alternatively, engrafting fully immunocompromised-(NOD scid gamma or NSG) mice with human haematopoietic stem and progenitor cells (HPSCs) ultimately gives rise to the multiple lineages of human haematopoietic and immune cells [56]. These so-called HuNSG mice developed partially functional human system. To date, human cord blood cells still represent the most commonly used source of HPSCs given their relatively feasible accessibility and better engraftment rates compared with those isolated from adult bone marrow [31,54]. Nonetheless, these models are restrained by their lack of fundamental cytokines involved in the maturation and differentiation of myeloid lineages. To favour better engraftment of haematopoietic stem cells (HSCs) and cell lineage differentiation, humanized models were further optimized (e.g. MITRG mice expressing genes encoding for human macrophage colony-stimulating factor [M-CSF], granulocyte macrophage colony stimulating factor [GM-CSF] and interleukin-3[IL-3] as well as NSG-SGM3 mice expressing human GM-CSF, IL-3 and stem cell factor) [31,59]. Herein, we will focus on the established humanized BC models and their application in ICIs testing (Table 3).

Six-to-eight weeks after engrafting 3–4 weeks old irradiated NSG mice with human $CD34^+$ HSCs, PDXs derived from five TNBC patients were orthotopically transplanted [60]. Distinct human haematopoietic cells and cytokines were readily detected in the peripheral blood, bone marrow, spleen and tumours of hNSG mice [60]. GM-CSF constitutively produced by TNBC-PDX seemed to compensate for its lack fostering the levels of $hCD33^+$ myeloid cells [60]. Besides their relatively good engraftment (80–85%) and lung metastatic capacity, TNBC-PDXs grew slightly slower in hNSG compared with non-hNSG mice [60]. This was attributed to the competent immunological status and partially matched human leukocyte antigen (HLA) typing between PDXs and the commercially available human $CD34^+$ HSCs used in that study [60]. Though the ideal scenario would be to isolate HSCs and PDX from the same patient, this might be practically difficult in large-scale preclinical studies considering both patient condition and long time (months-years) required to establish a PDX model. Hence, efficient growth of PDX in hNSG mice with partially HLA-matched allogeneic immune system might provide a more realistic/feasible alternative for establishing humanized models [56,60].

As clinically reported, some TNBC-PDXs responded to anti-PD-1 whereas others resisted [60] (Table 3). Anti-PD-1 dramatically reduced MC1 TNBC-PDX tumour volume promoting the cytotoxicity rather than number of TILs [60]. Conversely, MC1 did not respond to anti-CTLA-4, and two other TNBC-PDXs resisted anti-PD-1 [60]. Systematic dissection of the underlying mechanisms mandates further investigation.

Onco-HuNSG model was generated by the intravenous reconstitution of 3 weeks old irradiated female NSG mice with human fetal liver $CD34^+$ purified HPSCs [56]. With >25% of circulating $hCD45^+$ cells, Onco-HuNSG mice were considered humanized [56]. Unlike hNSG model, the growth pattern of HLA-partially matched engrafted TNBC tumours was comparable in Onco-HuNSG and non-humanized NSG mice [56,60]. Human immune cells infiltrating these PDXs were predominantly derived from $CD34^+$ HPSC donors rather than PDX-tumours and the intratumoural levels of $hCD45^+$, $hCD4^+$ and $hCD8^+$ T cells negatively correlated with TNBC volume *in vivo* [56].

Pembrolizumab significantly inhibited the growth of TNBC cell line and PDX-derived models [56](Table 3). Indeed, pembrolizumab evidently reduced the percent of tumour infiltrating $PD1^+$ $hCD45^+$ cells in MDA-

Table 3
Descriptive list of humanized models, their genetic background and responses to immune checkpoint inhibitors (ICIs).

Breast cancer cell line/PDX	Genetic background	Mouse Age/Strain	Source/ Transplantation route of human immune cells	Description	Response to ICIs as monotherapy	Ref
BT-474	$HER2^{+}$	New born NSG	Human cord blood purified $CD34^{+}$ cells/Intrahepatic	BC cell line derived from a 60 years old Caucasian female patient. BT-474 cells were grown in Herceptin (anti-HER2) for several weeks until the resulting cells (clones) became resistant to Herceptin. These resistant cells were then grown in standard culture media 'Herceptin -free' for many years. However, these cells remained resistant to Herceptin and retained HER2 overexpression	NA	[61]
MC1	ER^{-} PR^{-} $HER2^{-}$	3–4 weeks old NSG	Human $CD34^{+}$ HSCs/ Intravenous	Patient-derived primary TNBC cells	Anti-PD-1 significantly inhibited their growth (TVR% ~ 50%) and prolonged their survival. Anti-CTLA-4 did not confer therapeutic benefit.	[60]
BCM-4913	ER^{-} PR^{-} $HER2^{-}$	3–4 weeks old NSG	Human $CD34^{+}$ HSCs /Intravenous	Patient-derived primary TNBC cells	Pembrolizumab (anti-PD-1) significantly inhibited their growth (TVR% ~ 50%)	[60]

(*continued on next page*)

Table 3 (*continued*)

Breast cancer cell line/PDX	Genetic background	Mouse Age/Strain	Source/ Transplantation route of human immune cells	Description	Response to ICIs as monotherapy	Ref
BCM-4664	ER^- PR^- $HER2^-$	3–4 weeks old NSG	Human $CD34^+$ HSCs /Intravenous	Patient-derived primary TNBC cells	Resistant (irresponsive) to anti-PD-1 therapy	[60]
BCM-5471	ER^- PR^- $HER2^-$	3–4 weeks old NSG	Human $CD34^+$ HSCs /Intravenous	Patient-derived primary TNBC cells	Resistant (irresponsive) to anti-PD-1 therapy	[60]
MDA-MB-231	ER^- PR^- $HER2^-$	3-weeks old female NSG	Human fetal liver $CD34^+$ purified HPSCs /Intravenous	TNBC cell line derived from 51-year-old female Caucasian patient	Pembrolizumab (anti-PD-1) significantly inhibited the tumour growth (TVR % ~ 50%)	[56]
MDA-MB-231	ER^- PR^- $HER2^-$	New born BALB/c-$Rag2^{null}$ $Il2r\gamma^{null}$ $SIRP\alpha^{NOD}$ (BRGS)	Human cord blood purified $CD34^+$ cells/ Intravenous (facial vein)-if unsuccessful, intrahepatic	TNBC cell line derived from 51-year-old female Caucasian patient	Nivolumab (anti-PD1) significantly inhibited the tumour growth (TGI % = 61%)	[54]
BR1126	ER^- PR^- $HER2^-$	3-weeks old female NSG	Human fetal liver $CD34^+$ purified HPSCs /Intravenous	Patient-derived primary TNBC cells	Pembrolizumab (anti-PD-1) significantly inhibited their growth (TVR% ~70%)	[56]
BR0744	ER^- PR^- $HER2^-$	3-weeks old female NSG	Human fetal liver $CD34^+$ purified HPSCs /Intravenous	Patient-derived primary TNBC cells	NA	[56]
SKBr3	$HER2^+$	New born NSG	Human cord blood purified $CD34^+$ cells/Intrahepatic	BC cell line derived from the pleural effusion cells of a 43-years old Caucasian female patient who had been treated with radiotherapy, steroids, cytoxan and 5-fluorouracil	NA	[61]

BC = breast cancer; Tumour volume reduction percent (TVR%) = $100 \times (1\text{-}[V_{treated}/V_{vehicle}])$; Tumour growth inhibition percent (TGI %) = $100 \times (V_{treated\text{-}final} - V_{treated\text{-}initial})/(V_{vehicle\text{-}final} - V_{vehicle\text{-}initial})$; NA = not available; PR = partial response; TNBC = triple-negative breast cancer; PD-1 = programmed death-1; CTLA-4 = cytotoxic T lymphocyte antigen-4; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; HPSC = human haematopoietic stem and progenitor cells.

MB231 engrafted Onco-huNSG. Nonetheless, the prevalence of intratumoural $PD1^+$ leukocytes did not determine/correlate with the responsiveness to pembrolizumab [56]. In MDA-MB231 Onco-HuNSG model, pembrolizumab reduced the frequency of $CD45^+$ and $CD3^+CD4^+$ TILs and increased $CD8^+$ TILs migration at the center of the tumour which remained at the tumour periphery/margin in the vehicle-treated cohort [56]. Hu-CB-BRGS model engrafted with MDA-MB231 also demonstrated potent anti-tumour responses to nivolumab, another anti-PD-1, which was associated with increased frequencies and activation of $CD8^+$ TILs and decreased Tregs [54].

Variable responses of different $CD34^+$ HPSC donors to anti-PD-1 has also been observed in Onco-HuNSG mice which is in line with the differential response rates reported in anti-PD-1 treated patients [56]. While the exact underlying mechanisms still remain largely unknown, the authors speculated that variant T-cell repertoires across human population might attribute to such efficient anti-tumour immune responses observed in some but not all treated patients [56].

Instead of sequential human immune/tumour cells engraftment [56,60], Wege et al. concurrently transplanted HSCs and HER2 overexpressing BC cells in neonatal NSG mice which exhibited functional human immune system and metastases (Table 3) [61]. Nonetheless, the responses to ICIs within this setting remain to be investigated.

Despite the previously mentioned hurdles, ongoing attempts to optimally humanize these models shall pave the road for the emergence of a new generation of 'more' humanized models which are deemed to revolutionize cancer research.

3.2.4. Practical guidelines

Diverse experimental settings exploited while investigating the response of preclinical BC models (e.g.E0771) to ICIs (e.g.anti-PD-1) lead to heterogeneous responses (Table 4) [40,41,44,86]. Herein, we will provide an *in vivo* checklist which merits careful consideration.

3.2.4.1. Mouse or host-related factors. Mouse or host-related factors confer differential responses to immunotherapies (reviewed in Ref. [86]). These factors comprise choosing the species, strain, gender, age and body weight of the mice. Younger mice tend to respond better to ICIs compared to older mice (Table 4) [40,41,44]. The use of young, lean, and inbred mice is in sharp contrast with the heterogeneous population of BC patients with a median age of 61 years at diagnosis [62–64]. Correlating the global life expectancy of humans (~80 years) to the average lifespan of mice (~24 months) revealed that 1 human year is almost equivalent to 9 mice days [87]. 16–24 months-old-mice resembles elderly human in terms of thymic atrophy, chronic inflammation, and obesity. Thus, to provide more accurate prediction for the responses to ICIs, mice should be chosen based on the demographics of BC patients to be studied.

3.2.4.2. Transplantation site. Compared with subcutaneous site, orthotopic (into the mammary fat pad) or intraductal (into the mammary gland ducts through the nipples) transplantation provides more physiological TEM of BC [40,41,44,65].

3.2.4.3. Isotype and treatment schedule of ICIs. Aside from the antigen binding (F_{ab}) domain, distinct immunoglobulin constant (F_c) variants/isotypes of ICIs contribute to variable anti-tumour efficacies [66–68]. The timing of ICI administration is also critical since initiating treatment before or shortly after BC transplantation resembles more prophylactic therapies. In clinical practice, ICIs are mostly used as a treatment option whose administration starts after clear diagnosis (onset of symptoms). False-positive therapeutic effects of ICIs could simply be attributed to their action at early stage of disease compared with the clinical situation. Though some reported that the doses of ICIs tested preclinically were the maximum tolerated doses [44], many did not clarify the rationale beyond. Hence, more pharmacokinetic (PK)–pharmacodynamic (PD) studies outlining clinically relevant standardized treatment protocols/schedules of ICIs to be used preclinically are warranted [62].

3.2.4.4. Clinical problem-oriented experimental design and readout. While the effect of diverse therapeutic regimens on the growth of primary BC has historically been the primary focus of many preclinical studies, few have addressed the impact on the metastatic burden, drug resistance, safety and/or overall survival end-points [26,31]. Clinical responses to ICIs do not come in black/white or tolerant/responder but rather multicolor scenarios which need to be systematically considered preclinically(Fig. 2) [69,70]. Additionally, while investigating the anti-tumour efficacies of ICIs has been the main spotlight of preclinical investigations, fewer studies have investigated the serious off-target irAEs inflicted by ICIs which mandates further intensive interrogation of their toxicodynamics/kinetics as well as strategies alleviating such toxicities without compromising their anti-tumour immune responses [24,25,71–74].

BC metastasis is the end stage of a highly sophisticated multistep journey (invasion, intravasation, transport, arrest, extravasation and growth) [33,35]. Experimental BC metastasis models based on intravenous transplantation do not fully recapitulate BC invasion/intravasation but rather reflect the homing potential of circulating BC cells to secondary organs. These issues are partially resolved in orthotopically transplanted spontaneously metastasizing models

Table 4
Preclinical treatment schedule of E0771 syngeneic murine breast cancer model with anti-PD1.

E0771 transplantation	Age of C57/BL6 mice	Treatment schedule and notes	Response to anti-PD1 as monotherapy	Ref
Transduced E0771 cells expressing mCherry reporter gene (10^5 cells/20 μl) suspended in PBS and injected into the fourth mammary fat pad	8–12 weeks old	Anti–PD-1 was administered (250 μg/mouse, IP) on the 8^{th}, 11^{th}, 15^{th}, 18^{th}, 22^{nd} and 25^{th}day following tumour inoculation (n = 10/group). Tumour resection was carried out on day 15 post-tumour transplantation.	Resistant: no effect on primary tumour growth or metastasis	[41]
E0771 cells (2×10^5 cells) inoculated into the third mammary fat pad	6–8 weeks old	When tumours reached 3–4 mm in diameter, mice were randomly assigned and anti-PD-1 treated group was given (10 mg/kg, IP) every 3 days for a total of four doses. The sample size (n = 8/group) was be determined based on estimates from unpublished pilot experiments and previous experience to ensure that appropriate statistical tests could yield significant results.	PR (TVR%~40%)	[40]
E0771 cells were suspended at 10^7/ml in matrigel (50% v/v) in PBS and 100 μl injected into the mammary fat pad	4–6 weeks old	When tumours reached approximately 100 mm^3 (day 10), treatment was started with anti-PD-1(2.5 mg/kg,100 μl, IP) twice-weekly for a total of six doses (3 weeks) (n = 9–10/group). i.e. Mice were treated on days 10, 14, 18, 21, 25 and 28 post-tumour inoculation). Dose selection was based on unpublished preliminary maximum tolerated dose studies.	PR (TVR%~70%) Median survival increased from 25 to 55 days)	[44]

IP = intraperitoneal; PR = partial response; Tumour volume reduction percent (TVR%) = 100 x (1-[tumour volume of ICI-treated cohort/tumour volume of control Ab-treated cohort]); PD1 = programmed death-1; ICI = immune checkpoint inhibitor; PBS, phosphate buffered saline.

[33,35]. The reported controversial metastatic capabilities of some BC models could be attributed to discrepant sources, transplantation route, passage number and/or number of transplanted BC cells [32,33,35] (Table 1). Based on the inherent intramodel variability observed in twelve different BC models, *in-situ* model optimization is recommended for intervention studies [32].

4. Lessons learnt from preclinical BC models

4.1. Deciphering the molecular mechanisms of resistance to ICIs

Analyzing the immune cell landscape infiltrating two mouse tumour models with discrepant responses to ICIs revealed preferential infiltration of $CD11b^+$ myeloid cells on the expense of $CD8^+$ T cells which displayed compromised cytolytic activity in the ICI-resistant model [38,75]. Tumour-derived factors as GM-CSF also reshapes TME via fostering myelopoiesis and recruiting tumour associated myeloid cells (TAMCs) and blunting the efficacy of ICIs [38]. Combining J32, PI3K inhibitor with potent cytotoxicity on gMDSCs, with anti-CTLA-4 or anti-PD-1 eradicated 4T1 tumours in 80% of the treated mice [75]. Tumour associated macrophages form long-lasting interactions with $CD8^+$ T cells thereby trapping/excluding them from penetrating the center of the tumour. Depleting macrophages with PLX3397, an inhibitor of colony-stimulating factor 1 receptor, promotes the capacity of $CD8^+$ T cells to migrate and infiltrate tumour islets and hence improves anti-tumour immune responses to anti-PD-1 [53]. Consistently, inhibiting the myeloid highly expressed PI3Kγ using IPI-549 shifted immunosuppressive M2-like macrophages into inflammatory M1-like ones, increased $CD8^+$ infiltration and $CD8^+$/Treg ratio ultimately reducing the growth of 'TAMCs enriched' ICI-resistant tumour models [38]. Combining IPI-549 with ICIs as anti-CTLA-4 or anti-PD-1 elicited superior anti-tumour activity [38]. To this end, Phase I/Ib clinical trial has been launched to evaluate the safety and PK/PD of IPI-549 alone and when combined with nivolumab (anti-PD-1) in treating cancer patients including those with TNBC (ClinicalTrials.gov Identifier: NCT02637531).

ICIs are thought to expand cytotoxic effector T cells. Nonetheless, $CD8^+$ T cells are not fully functional till the suppressive brakes induced by gMDSCs are released

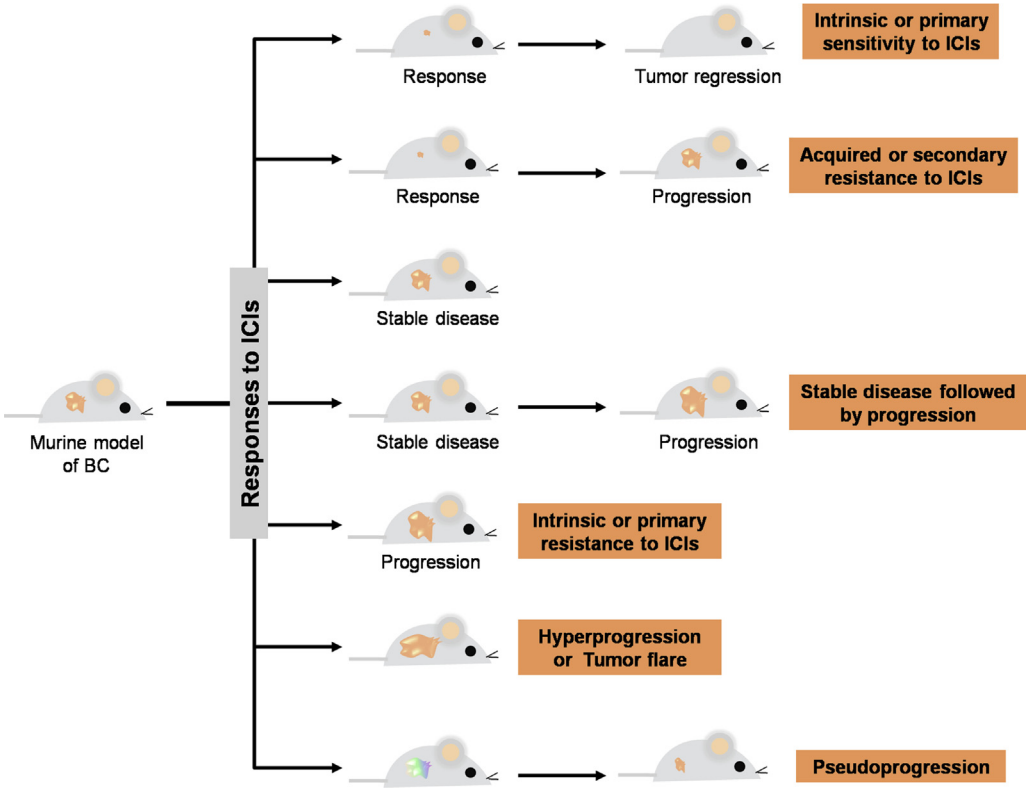

Fig. 2. **Illustrative figure depicting different scenarios of clinical responses to immune checkpoint inhibitors (ICIs) which need to be considered and further molecularly dissected in preclinical studies.** Clinical responses to ICIs comes in different forms including responders (who initially respond and undergo durable/complete remission), secondary resistant patients (who primarily respond and eventually progress/relapse; i.e. secondary or acquired resistance) stable disease, those who progress after a period of stable disease and intrinsically resistant (who do not exhibit any response .i.e. have primary or innate resistance). Moreover, hyperprogression (or tumour flare in which the rate of tumour growth or progression dramatically exceeds those untreated with ICIs) and pseudoprogression (which is characterized by initial immune-mediated increment of tumour size followed by tumour shrinkage) have been reported. BC, breast cancer.

by epigenetic modulators [75]. The promising preclinical anti-tumour activities stemming from the therapeutic regimens comprising anti-PD-1/anti-CTLA-4 with epigenetic modulators as 5-azacytidine or entinostat [75] instigated the initiation of a randomized Phase II trial on metastatic non–small-cell lung cancer patients treated with nivolumab alone or together with 5-azacytidine and entinostat (ClinicalTrials.gov Identifier: NCT01928576).

4.2. Biomarkers identification

4.2.1. Tumour mutational load/burden

Tumour mutational load/burden (TMB) promotes the production of neoantigens expressed on MHC which in turn boost the capability of the immunosurveillance system [75]. Exome sequencing of two preclinical syngeneic tumour models revealed heightened somatic mutations and TMB in the more ICIs-responsive colorectal cancer (CT26.WT) compared with the irresponsive TNBC (4T1) model [75]. BRCA1-mutated TNBC tumours with high TMB are more responsive to ICIs primed with chemotherapy suggesting that assessing BRCA1 status clinically could serve as a predictive biomarker [76]. Intriguingly, analyzing the genomic and clinical data of 1662 advanced cancer patients treated with FDA-approved ICIs revealed that higher TMB (calculated by normalizing the total number of somatic non-synonymous mutations to the total number of sequenced megabases—assessed using MSK-IMPACT assay) is associated with increased overall survival [88].

4.2.2. Tumour vessel perfusion

Tumour vascular normalization is a process where the tumour cell vasculature is remodelled to resemble that of normal tissues and increased vessel perfusion is a crucial component of this process [40]. Preclinical BC models have revealed that anti-CTLA-4 triggered vascular normalization in responsive tumours as evidenced by increased pericyte coverage, vessel perfusion and reduced tissue hypoxia [40]. Adapting Doppler 3D ultrasonography to estimate the tumour volume and percentage of perfused tumour vessel volume demonstrated that the overall increment in tumour perfusion promoted by ICIs could be measured even before detectable changes in tumour size and could predict the responsiveness to ICIs [40].

4.3. Devising therapeutic regimens to boost the anti-tumour immune responses to ICIs

4.3.1. ICIs combined with chemotherapy

Co-administering cisplatin with anti-PD-1 and anti-CTLA-4 substantially augmented the systemic as well as intratumoural immune responses against BRCA1-deficient TNBC tumours bearing mice robustly reducing their growth and prolonging their survival [76]. Vinorelbine, cyclophosphamide and 5-FU had significant effects on circulating and tumour-infiltrating immune cells in syngeneic TNBC and B-cell lymphoma models and hence demonstrated synergistic therapeutic effects with anti-PD-L1, though different types and sites of cancer generate significantly different atlases of intratumoural infiltrates [42]. Ixabepilone, FDA-approved microtubule inhibitor, synergized with anti-CTLA-4 inducing 100% complete tumour regression in EMT6 preclinical BC model [43]. These findings all support the initiation of clinical trials investigating the efficacy of chemotherapy-ICIs combos [77]. Indeed, the Phase II TONIC trial has demonstrated that short-term priming or induction with doxorubicin or cisplatin triggered favourable TME augmenting the response of metastatic TNBC patients to nivolumab (ClinicalTrials.gov Identifier:NCT02499367) [78].

4.3.2. ICIs and PARP inhibitors

Poly-ADP-ribose polymerase 1 (PARP1) and 2 (PARP2) are key sensors of DNA damage sensors [79]. BRCA1 and BRCA2 require a homologous chromosome or sister chromatid as a template to repair DNA double-strand breaks [50,51,76]. In 2018, Olaparib, PARP1/2 inhibitor, was approved by the FDA to treat patients with germline BRCA1/2 mutation or $HER2^-$ metastatic BC in which it triggers 'synthetic lethality' [81]. PARP inhibitors were found to upregulate PD-L1 expression and anti-PD-L1 co-administration resensitized BC cells to PARP inhibition via restoring $CD8^+$ TILs [80]. Within this context, multiple clinical trials were launched (ClinicalTrials.gov.Identifier: NCT02657889; NCT03639935; NCT02660034).

4.3.3. Neo-adjuvant versus adjuvant ICI therapy

Two BC models demonstrated the superior therapeutic value of neoadjuvant immunotherapies in eradicating distant metastases following mastectomy of primary tumour compared with adjuvant immunotherapies [85]. Conversely, paclitaxel—which promotes the proinflammatory cytokines from macrophages rather priming dendritic/T cells—conferred comparable beneficial effects when administrated as neoadjuvant or adjuvant chemotherapy [85]. Prolonged survival associated with neoadjuvant regimens might thus be restricted to therapies promoting T cell antitumour immune responses.

5. Future directions and conclusions

This review raises several questions, importantly: Can *ex-vivo* models serve as frontiers in HTS to select potential candidates to be next validated *in vivo*? Are preclinically tested treatment schedules of ICIs clinically relevant? Do the currently employed *ex-vivo* and *in-vivo* preclinical BC models recapitulate human BC heterogeneity? Are humanized murine models humanized

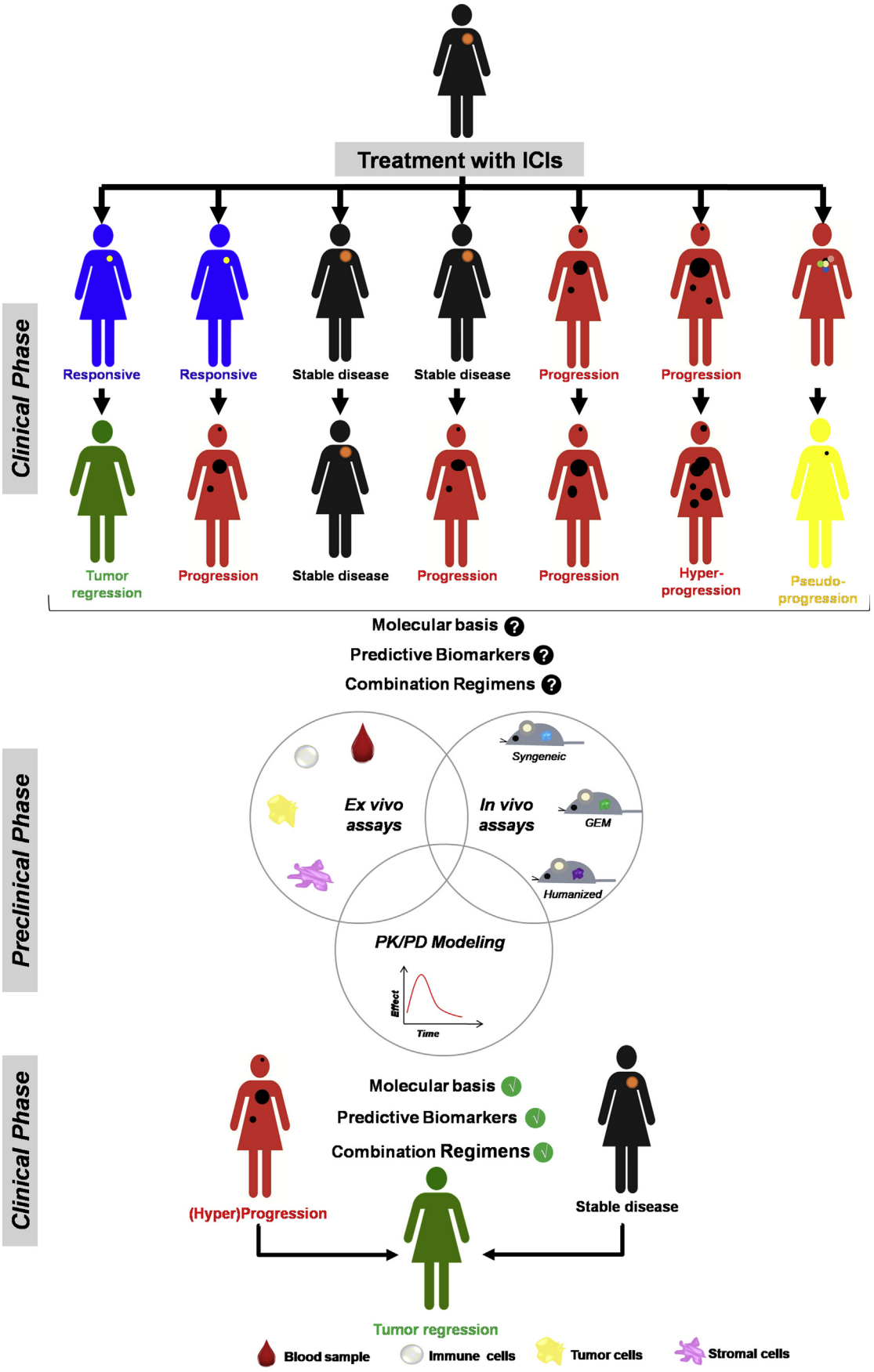

Fig. 3. Schematic overview of the proposed workflow of 'Clinical-Preclinical-Clinical' Development Cycle of ICIs. ICI = immune checkpoint inhibitor; PK, pharmacokinetic; PD, pharmacodynamic.

enough? Which preclinical models confer better clinical predictability in terms of therapeutic and toxic responses to ICIs?

Pharmacokinetics(PKs)/Pharmacodynamics(PDs) modelling could serve as valuable tool during both preclinical and clinical stages improving lead optimization and candidate selection, predicting needed drug dosage or exposure and combinations efficacies [82]. Notably, developing mathematical model to compute or determine the efficacy of a combination regimen composed of BET inhibitor and anti−CTLA-4 in treating BC correlated with the *in vivo* results [82]. Tumour-targeted delivery of ICIs could provide an avenue for uncoupling their anti-tumour responses from irAEs as demonstrated using a dual variable domain immunoglobulin of anti-CTLA-4 engineered to hide or shield the inner CTLA-4 binding domain by an external prostate tumour antigen targeting domain which released anti-CTLA-4 intratumoural while minimizing its systemic toxicity [74].

Single cell transcriptomic profiling of more than 16,000 immune cells obtained from almost 50 ICI-treated melanoma patients defined two distinct states of $CD8^+$ T cells associated with either tumour regression or progression [83]. Single cell RNA sequencing (scRNA-seq) and computational analyses of another of 33 melanoma specimens mapped ICI resistance program provoked by tumour cells before their exposure to immunotherapy that predicted the clinical responses to anti-PD-1 in more than 100 melanoma patients and suggested combination regimens to unlock the resistance to ICIs [84]. Indeed, exploiting scRNA-Seq could potentially resolve BC heterogeneity and define its immunogram.

In conclusion, while preclinical BC models have provided an amble amount of knowledge that supported the clinical development of ICIs, further optimization of these models is urged to better recapitulate human BC. Ideally, in the era of personalized/precision medicine, to-be-further-optimized humanized *ex-vivo* and *in vivo* models, PK/PD modelling, state-of-art NGS and tumour-targeted delivery technologies are all expected to provide an objective roadmap to dissect the molecular basis beyond the 'therapeutic as well as off-target toxic' responses to ICIs, identify predictive/prognostic biomarkers and devise durable and tolerable combination regimens for ICI-resistant and metastatic BC patients (Fig. 3).

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