

Immune cell response to external and internal signals: Regulation of breast tumor
infiltrating leukocytes by cell surface ADAM proteases and neutrophil precursor cells by the
mitochondrial CLPB

by

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B.S., Dalian Medical University, 2016
M.S., Emporia State University, 2019

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Department of Biochemistry and Molecular Biophysics
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Abstract

The immune system constitutes a dynamic network involving lymphoid organs, cells, humoral factors, and cytokines. Its crucial role becomes most evident when dysregulation occurs - insufficient activity of the immune system leads to severe infections and immunodeficiency-related tumors, and excessive activity of the immune system contributes to allergic and autoimmune diseases. There are two distinct categories of immune responses. Innate responses involve the use of phagocytic cells like neutrophils, monocytes, and macrophages, while acquired responses encompass the proliferation of antigen-specific B and T cells. Innate responses remain constant regardless of the number of encounters with the infectious agent, while acquired responses enhance upon repeated exposure to a specific infection. The goal of my research was to explore the regulation of the immune system in two diseases, cancer and neutropenia.

Several studies have now shown a correlation between the immune infiltrate in various human cancers, patient prognosis, and therapeutic responses. It has been shown that the anomalous infiltration of immune cells into tumors or normal tissues might facilitate tumor progression, invasion, and metastasis. The clinical success of cancer immune checkpoint blockade (ICB) has further redirected the focus onto tumor infiltrating immune cells. In my research, I investigated the composition of immunosuppressive cell populations, like TIMs (Tumor-Infiltrating Macrophages) and TINs (Tumor-Infiltrating Neutrophils), or PMN-MDSCs (Polymorphonuclear Myeloid-Derived Suppressor Cells), as well as adaptive immune cell populations like T cells, B cells, in the tumor microenvironment using a mouse model of claudin-low breast cancer. I showed that ADAM12 and ADAM9, which belong to the cell surface family of ADAM metalloproteases, affect the composition of tumor infiltrating immune cells. I found

that the deletion of ADAM12 in tumor cells results in reduced numbers of TINs/PMN-MDSCs and an increase in the numbers of tumor-infiltrating B cells and T cells. Importantly, the lack of ADAM12 in breast cancer cells enhances tumor sensitivity to the ICB therapy, but the initial response eventually leads to acquired therapy resistance. However, the deficiency of ADAM9 in cancer cells leads to an increase in both TINs/PMN-MDSCs and B cells, and this alteration does not enhance the efficiency of ICB therapy.

Neutropenias constitute a diverse group of hematological disorders marked by the impaired maturation of neutrophilic granulocytes. The human mitochondrial CLPB protein is a widely expressed homolog of caseinolytic peptidase B, belonging to the ATPases associated with diverse cellular activities (AAA+) family. Previous studies indicated that the presence of biallelic mutations in the *CLPB* gene is linked to a syndrome encompassing 3-methylglutaconic aciduria with cataracts, neurologic disease, and variable neutropenia. In contrast, a different group of monoallelic *CLPB* mutations is the cause of severe congenital neutropenia (SCN). In my study, I explored the role of CLPB in neutrophil differentiation and assessed its impact on mitochondrial function. I found a significant decrease in mitochondrial membrane potential (MMP) in cells with CLPB knockout. Additionally, we noted a reduction in cell viability during differentiation of neutrophil precursors with CLPB knockout. Collectively, these findings provide new insights into the complex roles of immune cells in the disease.

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Table of Contents

List of Figures	x
List of Tables	xii
Acknowledgements	xiii
Dedication	xiv
Chapter 1 - Introduction.....	1
1.1 Study the role of ADAM proteins in the breast cancer tumor microenvironment.....	1
1.1.1 Breast cancer classification.....	1
1.1.2 Breast cancers treatment strategies	3
1.1.3 Innovative approaches in treating triple negative breast cancer	5
1.1.4 ADAM proteins	9
1.2 Study the role of CLPB and its connections to congenital neutropenia	14
1.2.1 Severe congenital neutropenia	14
1.2.2 Human CLPB.....	16
1.3 The goals of the study	17
1.4 References.....	21
Chapter 2 - ADAM12 abrogation alters immune cell infiltration and improves response to checkpoint blockade therapy in the T11 murine model of triple-negative breast cancer	38
2.1 Abstract	39
2.2 Introduction.....	40
2.3 Materials and methods	42
2.4 Results.....	48
2.5 Discussion	57
2.6 Acknowledgments.....	61
2.7 Declaration of interest.....	61
2.8 Funding	62
2.9 Data availability statement.....	62
2.10 Reference	76

Chapter 3 - The effect of ADAM9 knockout on the tumor immune microenvironment in a mouse transplantation model of breast cancer	88
3.1 Introduction.....	88
3.2 Materials and methods	88
3.3 Results.....	90
3.4 Discussion.....	93
3.5 References.....	100
Chapter 4 - Mechanistic links between the CLPB gene and congenital neutropenia	102
3.1 Abstract.....	102
3.2 Introduction.....	103
3.3 Materials and Methods.....	105
3.4 Results.....	109
3.6 References.....	121
Chapter 5 - Conclusions.....	126
Appendix A - Copyright Permissions	1
Appendix B - Supplementary Information for ADAM12 abrogation alters immune cell infiltration and improves response to checkpoint blockade therapy in the T11 murine model of triple-negative breast cancer.....	1

List of Figures

Figure 1.1 Molecular classification of breast cancer.	19
Figure 1.2 Domain structure of the ADAM metalloproteases and dendrogram showing the relative peptide sequence similarities among widely expressed ADAM proteins.....	20
Figure 1.3 Neutrophil differentiation includes the following stages: GMP, myeloblast, promyelocyte, myelocyte, metamyelocyte, band and segmented neutrophil.	21
Figure 2.1 The effect of <i>Adam12</i> knockout on T11 tumor growth rates in mice.	63
Figure 2.2 Quantification of tumor-infiltrating immunosuppressive myeloid cells in A12-KO (g1) and control T11 tumors from BALB/c mice by flow cytometry.....	65
Figure 2.3 Quantification of tumor-infiltrating lymphocytes (TILs) in A12-KO (g1) and control T11 tumors from BALB/c mice by flow cytometry.	67
Figure 2.4 ADAM12 entrapment inside the cell exerts a similar effect on tumor-infiltrating T cells and B cells as <i>Adam12</i> gene knockout.	68
Figure 2.5 The effect of ADAM12 loss in cancer cells on chemotaxis of B cells <i>in vitro</i>	71
Figure 2.6 Abrogation of ADAM12 sensitizes T11 tumors to anti-PD1/anti-CTLA4 therapy....	72
Figure 2.7 Depletion of B cells eliminates the improved response to anti-PD1/anti-CTLA4 therapy in A12-KO T11 tumors.	74
Figure 2.8 . Correlations between human <i>ADAM12</i> mRNA levels and immune gene expression signatures in claudin-low TNBCs.	75
Figure 3.0.1 Detection of ADAM9 in T11 cells by Western blotting.	95
Figure 3.0.2 Quantification of tumor infiltrating CD45+ (A) and CD45+CD11b+ cells (B) in ADAM9-KO (g1) and control T11 tumors.	96
Figure 3.0.3 Quantification of tumor infiltrating immunosuppressive myeloid cells in ADAM9- KO (g1) and control T11 tumors.	97
Figure 3.0.4 Quantification of tumor infiltrating lymphocytes (TILs) in ADAM9-KO (g1) and control T11 tumors.....	98
Figure 3.0.5 The effect of anti-PD1/anti-CTLA4 therapy on ADAM9-KO T11 tumors.	99
Figure 4.1 CLPB knockout leads to a decreased mitochondrial membrane potential in HL60 cells.	115
Figure 4.2 CLPB loss in HL60 cells leads to the accumulation of S-OPA1 isoform e.	116

Figure 4.3.	117
Figure 4.4 Selected dominant-negative mutations in CLPB linked to severe congenital neutropenia.	118
Figure 4.5 Transient overexpression of dominant-negative CLPB mutants does not affect differentiation of HL60 cells.	119

List of Tables

Table 3.1 Reagents and antibodies.....	88
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Dedication

I dedicate my dissertation work to my loving parents, Zhiyan Wang and Qiu Wang, who have encouraged me attentively with their fullest and truest attention.

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Chapter 1 - Introduction

1.1 The role of ADAM proteins in the breast cancer tumor microenvironment.

1.1.1 Breast cancer classification

According to the International Agency for Research on Cancer (IARC), there has been a 66% rise in the worldwide count of cancer-related fatalities since 1960 [1]. Breast cancer accounted for 11.6% of all cancer cases, making it the second most frequently diagnosed cancer following lung cancer [2]. The occurrence rates of breast cancer among women surpassed those of other cancers, solidifying its status as the most commonly diagnosed cancer and the primary cause of cancer-related deaths in females worldwide [3]. The categorization of invasive breast cancer into molecular subtypes relies on mRNA gene expression levels. In 2000, Perou et al. identified four molecular subtypes—Luminal, HER2-enriched, Basal-like, and Normal Breast-like—by analyzing microarray gene expression data from a sample of 65 breast cancers [4]. The Normal Breast-like subtype has been excluded in subsequent analyses. Eventually, consensus clustering verified the presence of four primary intrinsic subtypes of breast cancer solely based on mRNA gene expression levels—Luminal A, Luminal B, HER2-enriched, and Basal-like [5]. Moreover, the identification of the 5th intrinsic subtype, the claudin-low subtype, occurred in 2007 through an integrated analysis of human and murine mammary tumors [6].

Luminal A and Luminal B breast cancers are known and characterized because of the presence of estrogen-receptor (ER). However, Luminal A tumors are also progesterone-receptor (PR)-positive and HER2-negative [7, 8]. In comparison, Luminal B tumors have worse prognosis than Luminal A tumors, they are PR-negative but HER2-positive [8, 9]. Unlike Luminal breast cancers, which constitute almost 70% of all breast cancer cases, the HER2-enriched subtype comprises 10-15% of breast cancers [10]. This subtype is identified by the high expression level

of HER2, and the absence of ER and PR. HER2-enriched cancers exhibit a faster growth rate compared to luminal cancers and previously had the worst prognosis among all subtypes until the advent of HER2-targeted therapies [11].

Breast tumors lacking ER, PR, and HER2 are categorized as triple-negative breast cancer (TNBC), and they constitute approximately 15% to 20% of all breast carcinomas (Figure 1.1) [12]. It has been noted that basal-like tumors frequently exhibit the triple-negative phenotype, however, not all triple-negative breast cancers belong to the basal type [13]. It has been shown that approximately 75% to 80% of TNBCs display a basal phenotype, so it is crucial to avoid using these two terms interchangeably, as they refer to distinct types of lesions [14]. Basal-like breast cancers can be recognized through various immunohistochemical markers, including cytokeratins (CK5, CK6, CK14, or CK17), epidermal growth factor receptor (EGFR), smooth muscle actin (SMA), P-cadherin, p63, or c-kit antigen [15]. TNBCs represent approximately 20% of all breast cancers, TNBC is more prevalent in women under the age of 40 and among African American women [16]. Through advancing transcriptomic studies, seven distinct clusters were identified within TNBCs: basal-like (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR), along with an unspecified group (UNS) [13, 17]. Of note, the clinical significance of the subtyping remains uncertain, and further research is imperative to elucidate its influence on decision-making in the treatment of TNBCs [18].

The latest recognized tumor category is the claudin-low subtype. This subtype is characterized by low expression levels of numerous claudin genes, including claudin 3, 4, and 7 [19]. Claudins are essential transmembrane proteins, which play a crucial role as integral components of epithelial cell tight–tight junctions (TJs). TJs are pivotal in regulating paracellular

permeability and preserving the polarity of epithelial cells [20]. Additionally, this subtype is enriched in stem-like markers and features associated with epithelial-mesenchymal transition (EMT) [21]. Claudin-low tumors are often triple-negative, they are associated with a poor prognosis and high-grade invasive ductal breast carcinoma, and they constitute 7–14% of all invasive breast cancers [7, 22].

1.1.2 Breast cancers treatment strategies

In the case of nonmetastatic breast cancer, the primary objectives of the therapy include eliminating the tumor from the breast and regional lymph nodes, as well as preventing the recurrence of metastasis [23]. In the case of metastatic breast cancer, the therapeutic aims involve extending the life and alleviating symptoms. Presently, metastatic breast cancer remains incurable in almost all individuals affected by the condition [23].

Surgery

The treatment of early-stage invasive breast cancers typically involves surgical removal of the tumor mass through mastectomy or breast-conserving surgery (BCS) [24]. Breast-conserving surgery is preferred, because this therapy involves the removal of the tumor along with a margin of surrounding normal tissue, without the need to remove excessively the healthy breast tissue [25]. While breast-conserving surgery demonstrates high efficacy in managing early-stage breast cancer, the selection of treatment should be guided by factors such as tumor size, surgical feasibility, clinical phenotype, and the patient's inclination to preserve the breast [26].

Radiation therapy

Radiotherapy is a localized treatment that follows breast-conserving surgery and/or chemotherapy, this is beneficial for higher-risk breast cancer cases and aims at ensuring the elimination of all cancerous cells to minimize the risk of recurrence [27]. An analysis of 10 randomized controlled trials demonstrated that the inclusion of radiation therapy substantially lowered the five-year local recurrence rate and potentially diminished the 15-year breast cancer mortality risk [28]. The selection of the radiation therapy type is contingent upon the preceding surgical intervention and specific clinical conditions. Common techniques include breast radiotherapy (following breast-conserving surgery), chest-wall radiotherapy (usually following mastectomy), and the 'breast boost' (entailing high-dose radiotherapy) [29]. Resistance to radiation therapy may manifest in advanced invasive breast cancer, especially within a hypoxic tumor microenvironment, where the HIF-1 α (hypoxia-inducible factor 1 alpha) protein assumes a significant role [30]. Overall, radiation therapy significantly contributes to improved survival rates and reduced recurrence risk in breast cancer patients [26].

Chemotherapy

Chemotherapy is a systemic approach to treating breast cancer. It can be given in either the neoadjuvant or adjuvant setting, depending on the specific characteristics of the tumor [29]. Neoadjuvant chemotherapy (NAC) is implemented on non-metastatic but inoperable breast cancer with unreachable tumors [31]. TNBC and HER2+ subtype breast cancers can be sensitive to chemotherapy, making NAC an effective strategy to achieve a pathologic complete response in these breast cancer subtypes [32]. Breast cancer patients with lymph node metastases or a high risk of recurrence are prescribed adjuvant chemotherapy [33]. Anthracyclines or taxanes are often preferred. Anthracyclines work by DNA intercalation, which induces double-stranded chromosomal DNA breaks and inhibits transcription and replication, results in cell cycle arrest

and apoptosis. Taxanes attach to microtubules, preventing their depolarization, ultimately causing cell cycle arrest and triggering apoptosis [34, 35]. Although chemotherapy is acknowledged for its effectiveness, it frequently induces various side effects, such as hair loss, nausea/vomiting, diarrhea, mouth sores, fatigue, heightened susceptibility to infections, bone marrow suppression, leucopenia, anemia, and bruising or bleeding [29].

1.1.3 Innovative approaches in treating triple negative breast cancer

TNBC is the most aggressive form of breast cancer, accounting for approximately 15-20% of all cases. It is more common among younger women and is characterized by distinct biological features and a poor prognosis. To enhance the prognosis and survival rates for patients with TNBC, various new treatments have been developed and refined, including antibodies-drug conjugates, targeted therapies and immunotherapy.

Antibodies-Drug Conjugates (ADC)

Antibody drug conjugates (ADCs) deliver a cytotoxic drug into the tumor cell by specifically binding an antibody to a cell surface molecule [36]. The newly formed ADC-antigen complex undergoes internalization and subsequent degradation by lysosomes, releasing the payload and eventually leading to the induction of tumor cell death [37]. At present, regulatory approval has been granted for 15 ADC drugs, 3 out of 15 approved ADC drugs can be used as breast cancers treatment, and a substantial number of ADC drugs are currently in various stages of clinical trials [37]. For example, sacituzumab-govitecan merges an antibody directed against trophoblast antigen 2 (Trop-2, expressed in 90% of triple-negative breast cancers) with a topoisomerase I inhibitor, SN-38 [38]; Trastuzumab-emtansine (T-DM1) is a conjugate of trastuzumab and a cytotoxic molecule, DM1. T-DM1 can bind to HER2, releasing DM1

metabolites into the cytoplasm and eventually causing cell death [39]. Although three generations of modifications have been made to ADC drugs, existing limitations persist. Further research on ADC drugs is essential to enhance their therapeutic efficacy, address drug resistance, and mitigate adverse reactions.

Targeted therapies

A comprehensive understanding of the molecular pathology and identifications of biomarkers associated with TNBC allow the development of effective targeted agents for TNBC [40]. Thus, several targeted therapies have been developed and these strategic therapies are being clinically tested.

Epidermal Growth Factor Receptor (EGFR) inhibitors

EGFR is a receptor with tyrosine kinase activity located on the cell surface, it is encoded by the erythroblastic leukemia virus oncogene homologue B1 (ErbB1), and it belongs to the human epidermal growth factor receptor (HER)/ErbB family [41]. EGFR can bind various extracellular ligands, which act as agonists for the receptor, resulting in the activation of multiple downstream signaling events, including the Ras/Raf/MAPK, PI3K/Akt/mTOR, and STATs pathways [42]. EGFR is abundantly expressed in various malignancies, including TNBC. Activation of this gene triggers both primary tumorigenesis and metastasis [43]. Gefitinib is an EGFR inhibitor that has been approved for clinical trials. It can reduce cancer cell proliferation and enhance the cytotoxic effects of carboplatin and docetaxel [44].

Mammalian Target of Rapamycin (mTOR) Inhibitors

The mTOR protein is a serine/threonine kinase encoded by the *FRAP1* gene in humans, it is a subunit of two separate multi-protein complexes known as mTORC1 and mTORC2 [45]. While both mTORC1 and mTORC2 can be activated by cell stimulation by growth factors:

mTORC1 promotes cell growth and survival by enhancing nutrient uptake and metabolism. It also stimulates cell growth by promoting ribosome production and protein synthesis while inhibiting protein degradation; mTORC2 plays a significant role as a kinase that phosphorylates and activates Akt to support cellular survival [45]. Incorrect regulation of mTOR signaling, particularly the phosphoinositide-3 kinase (PI3K)/Akt/mTOR pathway, is associated with cancer cell growth, cell proliferation and angiogenesis [46]. Furthermore, increased levels of Akt, a protein kinase, are also linked to tumor metastasis and invasion [47]. The mTOR inhibitor everolimus and the Akt inhibitor ipatasertib have been tested in clinical trials, and patients with HER2+ BC showed better progression-free survival than TNBC patients [48, 49].

TGF- β signaling pathway inhibitors

TGF- β 1 is a member of the TGF- β superfamily of cytokines and is encoded by the *TGF- β 1* gene. While it was initially identified in human platelets, TGF- β 1 is broadly expressed and plays a vital role in both wound healing and immune system regulation [50]. Studies have demonstrated that TGF- β 1 inhibits the secretion and blocks the activities of different cytokines like IFN- γ , TNF- α , and IL-2 [51]. TGF- β 1 plays a significant role in breast cancer stem cells, which overexpress TGF- β 1 and the TGF- β receptor 1 [52]. TGF- β 1 inhibitors have the ability to block the proliferation of chemotherapy-resistant tumor-initiating cells (TIC) *in vivo*, providing a basis for combined chemotherapy approaches for TNBC patients [52]. Moreover, TGF- β 1 induces EMT in mammary cells, resulting in the acquisition of stem cell-like characteristics [53]. The reversal of EMT can be achieved using TGFBR1/2 inhibitors, which promote mesenchymal-to-epithelial (MET) differentiation in mammary epithelial cells [53].

Immunotherapy

The immune system plays a crucial role in breast carcinogenesis and the disease outcome. Several studies have shown that advanced tumors can evade adaptive immune responses, primarily through pathways like immune checkpoints, which play crucial roles in regulating immune tolerance and minimizing collateral tissue damage [54]. In normal breast tissue, immune cells are primarily found within the epithelial components of breast ductal lobules. In contrast, transition to breast cancer is characterized by a higher infiltration of immune cells in both the tumor parenchyma and stroma, including CD4⁺ and CD8⁺ granzyme B⁺ cytotoxic T cells, B cells, macrophages, and dendritic cells [40]. The immune cell infiltration in breast cancer encompasses various cell types, such as CD3⁺ T cells (including CD4⁺ and CD8⁺ subtypes), B cells, monocytes/macrophages, dendritic cells, and natural killer (NK) cells [55]. In recent years, substantial efforts have been dedicated to developing novel strategies aimed at instigating tumor cell death by activating the immune system to identify and eliminate malignant cells [56]. For instance, immune checkpoint blockade (ICB) stands out as the most extensively researched form of immunotherapy in breast cancer [57]. Another effective immune-based strategy is the adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells [58].

Investigations of different immune checkpoint receptor-ligand interactions are underway as potential tools for cancer treatment. The objective is to inhibit the immune checkpoints and to restore the anti-tumor immune system function. The pathways involving Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA4, CD152) and Programmed Cell Death Protein 1 (PD-1, CD279)/Programmed Cell Death Protein 1 Ligand (PD-L1, CD274) have proven to be effective targets for innovative cancer treatments [59, 60]. Consequently, multiple CTLA and PD-1/PD-L1 checkpoint inhibitors have received clinical approval. PD-1 is a receptor found on activated T-cells, serving as an immune checkpoint. Its ligand, PDL-1, is expressed on the

surface of many cells in the tumor microenvironment, including cancer cells, dendritic cells, and macrophages. When PD-1 interacts with PDL-1, it suppresses the T-cell response [61]. CTLA-4, expressed on T-cells, also hinders T-cell activation by binding to CD80/CD86, consequently reducing the immune response [62].

Unfortunately, the outcome of ICB therapies varies in patients. While monotherapy using these immune checkpoint inhibitors benefits some patients, it is insufficient to elicit a profound and enduring response in the majority of patients [63]. Therefore, it is necessary to combine ICB therapies with other treatments to achieve a synergic effect. One strategy is to target the tumor immunosuppressive microenvironment. In addition to cancer cells, various immune and non-immune cells contribute to the formation of the tumor microenvironment [64]. Some of these cells have immunosuppressive properties, such as type 2 tumor-infiltrating macrophages (TIMs), regulatory T lymphocytes (Treg), tumor-infiltrating neutrophils (TINs), Myeloid-Derived Suppressor Cells (MDSCs), or cancer-associated fibroblasts (CAFs) [65]. The strategy to target these immunosuppressive populations or the cytokines they produce may lead to the reduction of immunosuppression within the tumor, sensitizing it to the immunotherapy through checkpoint inhibitors.

1.1.4 ADAM proteins

ADAMs (A Disintegrin And Metalloproteases) form a large family of transmembrane proteins. They consist of several conserved domains, including an N-terminal prodomain, a Zn^{2+} -dependent metalloproteinase catalytic domain (M-domain), a disintegrin domain, a cysteine-rich domain, an epidermal growth factor domain-like (EGF-like) domain, a transmembrane region, and a cytoplasmic tail [66]. To date, approximately 40 family members have been recognized in

the mammalian genome, with 22 of them being expressed in humans, out of which 13 are proteolytically active [67]. ADAMs can be categorized into four different types, with type I being the most prevalent one, which has the classical structure shared by most members. Type I ADAMs include ADAM-1, 8, 9, 12, 13, 15, 19, 20, 21, 24, 26, 28, 30, 33, and 40. Type I ADAMs feature a catalytic Zn^{2+} binding signature (HEXXHXXGXXH) in their M-domain, a D-domain characterized by the XCD sequence, and an EGF-like region. Type II includes ADAM-2, 7, 11, 18, 22, 23, 27, 29, and 32. In contrast to type I ADAMs, type II ADAMs lack the characteristic catalytic Zn^{2+} binding signature in their metalloproteinase domain. Type III is specific to ADAM-15, with its M-domain retaining the characteristic catalytic- Zn^{2+} binding signature, but its disintegrin domain containing the RGD sequence instead of the XCD sequence. Type IV is specific to ADAM-10 and 17, which do not possess the EGF-like region (Figure 1.2).

The activation of ADAMs can be achieved by various factors such as cytokines, proteinases, protein kinase C activators, G-protein coupled receptor (GPCR) agonists, and Ca^{2+} ionophores [68, 69, 70]. In contrast to matrix metalloproteinases (MMPs), which are often activated in the extracellular compartment [71], ADAMs are activated in the intracellular space [72]. Pro-protein convertases in the Golgi system remove the pro-domain of ADAMs by cleaving the Rx(R/K)R motif between the pro-domain and the metalloprotease domain [73, 74].

ADAMs main function is cell adhesion and the proteolytic processing of ectodomains of various cell surface receptors and signaling molecules. Therefore, ADAMs are involved in a wide array of biological processes, including signal transduction, inflammation, immunity, neurodegenerative disease, and cancer biology [75, 76, 77].

ADAM12

The human ADAM12 gene is located on chromosome 10q26 and produces two distinct

isoforms: a long variant (ADAM12-L) with the conventional transmembrane modular structure, and a secreted form (ADAM12-S) that lacks the transmembrane and cytoplasmic domains. An analogous scenario is observed for ADAM9 [78, 79]. Both ADAM12-L and ADAM12-S have the proteolytic activity and impact distant cell physiological targets by modulating the shedding of various membrane-bound ectodomain substrates from the cell surface [80].

Regulation of ADAM12 expression is a complex process that involves different pathways. Studies have identified cellular mechanisms leading to the induction of ADAM12 that include NF- κ B and TGF- β 1 signaling pathways. NF- κ B, identified in 1986, is a transcription factor initially recognized as a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain in activated B cells [81]. While NF- κ B is essential for normal mammary gland development, breast cancers often exhibit abnormal and elevated expression of NF- κ B subunits, including c-Rel, p65, and p50 [82, 83]. It has been revealed that the interaction between NF- κ B and the ADAM12 promoter, along with increased NF- κ B levels in breast cancer cells, results in the upregulation of ADAM12 expression [84]. Another study demonstrated that ADAM12 expression is stimulated by Notch activation of NF- κ B through a CSL (CBF1/Su(H)/Lag-1)-dependent mechanism. This NF- κ B activation leads to downregulation of microRNA-29, which is a negative regulator of ADAM12 mRNA, and thus results in increased ADAM12 expression [85]. Since only ADAM12-L mRNA contains a miR-29 target site in its 3'-UTR, only this isoform is susceptible to post-transcriptional regulation by miR-29. In contrast, ADAM12-S lacks the miR-29 target site and cannot be regulated by miR-29 [86].

ADAM12 is highly expressed in breast tumors, and reduced ADAM12 expression is correlated with a more favorable survival prognosis [90]. Recently reported findings indicate an elevated expression of ADAM12 within the claudin-low subset of TNBC when compared to

other subtypes of breast cancer. This distinction is evident at both the mRNA level in tumor tissues and at the protein level in cell lines [91]. ADAM12 is induced through EMT, a trait commonly found in claudin-low breast carcinomas expressing markers of cancer stem cells (CSC). Another study explored the role of ADAM12 in the development of the CSC phenotype (CD44^{hi}/CD24^{-/lo}) in breast carcinoma cells. Cell populations with elevated ADAM12 expression showed increased levels of CSC markers and enhanced ability to form mammospheres [92]. On the contrary, knocking down ADAM12 reduced cell migration, invasion, and resistance to anoikis, as well as impeded mammosphere formation.

EGFR and its ligands support endothelial cell proliferation and angiogenesis in solid tumors [87]. Ligand binding to the extracellular domain of EGFR triggers the formation of homo- and/or heterodimers, leading to the phosphorylation of tyrosine residues within the receptor's intracellular domain [88]. ADAM12-L, EGFR, and heparin-binding EGF-like growth factor (HB-EGF, an EGFR ligand) exhibit a positive correlation with each other in TNBC. Interestingly, HB-EGF serves as an ADAM12 substrate, and it was shown that enhanced expression of ADAM12-L increased the phosphorylation of EGFR in a mouse xenograft model of primary breast cancer due to elevated levels of soluble HB-EGF [89]. In contrast, ADAM12 knockdown reduced the basal activation of EGFR. RNA sequencing revealed a significant overlap between ADAM12 and EGFR-regulated genes [92], and incubating cells with exogenous EGF counteracted the decrease in the CD44^{hi}/CD24^{-/lo} cell population induced by ADAM12 knockdown [92].

In my study, I found that the loss of ADAM12 in breast cancer cells increases B cell chemotaxis *in vitro*. This effect can be blocked by inhibiting CXCR4 on B cells, which is the receptor for CXCL12, or by using anti-CXCL12 blocking antibody. Importantly, when

ADAM12 is deleted in mouse T11 cancer cells, tumors become more responsive to combination therapy targeting PD1/CTLA4 immune checkpoints, although the initial responsiveness is later followed by acquired resistance to the therapy. Depletion of B cells in mice abrogated the enhanced response to immune checkpoint blockade in T11 tumors lacking ADAM12 [93]. This indicates that ADAM12 could potentially contribute to promoting immunosuppression within the tumor microenvironment of TNBC.

In summary, these findings suggest that ADAM12 plays an active role in strengthening the CSC phenotypes in claudin-low breast carcinoma cells by regulating the EGFR pathway.

Taken together, ADAM12 might be a viable drug target, as targeting ADAM12 could provide a benefit in cancer treatment by reducing tumor cell progression and increasing their sensitivity to applied drugs. However, achieving specificity in targeting the metalloproteinase domain may meet challenges due to the structural similarities among enzymes in the ADAM and MMP families. Therefore, more research is needed to investigate each ADAM individually, along with their unique functions and their roles in cancer development.

ADAM9

ADAM9, another member of the ADAM family, was initially discovered in breast carcinoma in 1996 [94]. Like ADAM12, ADAM9 has a broad expression in human tissues, is catalytically active, and it participates in shedding of the ectodomain of HB-EGF [95]. ADAM9 plays a significant role in tumor proliferation, angiogenesis, metastasis, and, notably, immune evasion [96]. In breast cancer, there is an up-regulation of ADAM9 expression compared to normal tissue. Additionally, ADAM9 contributes to the progression of the disease by enhancing tumor extravasation and cancer cell migration [97]. In certain subtypes of TNBC, deregulation of methylation leads to the overexpression of EGFR, amplifying cell proliferation and survival.

Interestingly, ADAM9 shares the same methyltransferase as EGFR [98]. Furthermore, nuclear receptor-binding SET domain protein 2, a member of the histone methyltransferase family, up-regulates the expression of both ADAM9 and EGFR. This dual up-regulation promotes resistance to EGFR inhibitors in TNBC cells [98]. Moreover, ADAM9 is not only reported to undergo up-regulation in tumor cells but also within the tumor microenvironment, comprising blood vessels and immune cells. Interactions among these microenvironmental components contribute to the increased potential for tumor metastasis and epithelial–mesenchymal transition [99, 100]. Increased ADAM9 expression correlates with adverse outcomes in cancer patients.

The roles of ADAM9 in tumor proliferation, angiogenesis, metastasis, and immune evasion have been thoroughly investigated in recent years [96]. Consequently, directing attention to ADAM9 as a therapeutic target could offer a novel approach to treating cancers.

1.2 The role of CLPB in congenital neutropenia

1.2.1 Severe congenital neutropenia

Neutropenia is a hematologic condition characterized by abnormally low count of neutrophils in the blood. Neutropenia in adults is classified according to the absolute neutrophil count (ANC), with the designation of severe when the blood ANC falls below $0.5 \times 10^9/L$, while a healthy adult typically has a neutrophil count between $2.5 - 7 \times 10^9/L$ of blood. Broadly, neutropenia can be categorized as either congenital or acquired. Congenital neutropenia arises due to autosomal dominant or recessive inheritance, typically manifesting in early childhood [101]. In severe congenital neutropenia (SCN), the frequently observed occurrence is a maturation arrest of granulopoiesis at the level of promyelocytes in the bone marrow [102].

Mutations in various genes give rise to SCN, and alterations in the ELANE gene are the primary reasons for the most common occurrences [103]. The increasing use of Next Generation Sequencing (NGS) or copy number analysis in diagnostic procedures enables the identification of novel genes responsible for neutropenia. Further categorization of mutated genes associated with SCN is usually based on the functionality of their protein products. The isolation of different gene subgroups has the potential to facilitate the identification of the mechanisms leading to the loss of neutrophil function, thereby contributing to the understanding of the disease.

SCN is associated with an elevated risk of developing acute myeloid leukemia or myelodysplastic syndromes. Mutations in the ELANE and GATA2 genes are specifically correlated with leukemic transformation, whereas the risk is relatively low in the presence of mutations in other genes, such as GATA1 [104, 105]. Recently, it has been reported that some patients with SCN are carriers of heterozygous alterations in the CLPB gene, and they may also experience the development of myelodysplastic syndromes (Figure 1.3) [106].

The two primary methods of treating neutropenia patients involve the use of antibiotics to combat infections and medications that facilitate neutrophil production. In certain cases, bone marrow transplantation may be considered the optimal treatment [102]. In nearly all cases of severe congenital neutropenia, the primary treatment choice is the use of granulocyte colony-stimulating factor (G-CSF) [107]. This therapeutic agent stimulates the production of neutrophils and induces hematopoietic stem cell mobilization by initiating the dimerization of the granulocyte colony-stimulating factor receptor (G-CSFR) [106]. Although G-CSF treatment can efficiently alleviate neutrophil deficiency, some patients lack or have poor response to the

treatment. In such cases, patients should consider bone marrow transplantation, which markedly enhances the likelihood of the patient's survival [102].

1.2.2 Human caseinolytic peptidase B protein (CLPB)

Different protein-disaggregase systems are present in different cellular compartments, working to disassemble protein aggregates by extracting individual polypeptides and returning them to their soluble, functional state [107]. Non-metazoan mitochondria contain the AAA+ (ATPases associated with various cellular activities) protein disaggregase, Hsp78, within the mitochondrial matrix. This enzyme disaggregates and reactivates proteins that are trapped in aggregates [108]. In contrast, multicellular animals do not possess Hsp78. While protein aggregates inside mitochondria can be isolated and removed through selective mitophagy, the system for disaggregating and reactivating proteins within their mitochondria remain unclear [109].

Human caseinolytic peptidase B protein homolog (CLPB)/suppressor of potassium transport defect 3 (SKD3) belongs to the HSP100 family proteins. HSP100 proteins, on the other hand, belong to a larger AAA+ ATPase family. All AAA+ proteins utilize ATP hydrolysis to produce energy when demanded. They usually consist of an N-terminal domain (NTD) and one or two ATPase domains (also known as nucleotide-binding domains, NBD), and they commonly operate as hexameric structures [110]. CLPB's N-terminus comprises a mitochondrial targeting signal (MTS), succeeded by four ankyrin repeat (ANK) domains, and concludes with a single C-terminal nucleotide-binding domain (NBD). It has been reported that the activation of CLPB occurs through the proteolytic cleavage by the mitochondrial presenilins-associated rhomboid-like protein (PARL) [111]. PARL is involved in maintaining mitochondrial quality control by

regulating processes like apoptosis, as well as modulating mitochondrial dynamics, including fission and fusion [112].

CLPB has recently been postulated to function as a protein disaggregase within the intermembrane space (IMS) of mitochondria [112]. Autosomal dominant mutations in CLPB lead to the development of SCN. Several mutations associated with the disease have been demonstrated to eliminate the disaggregase function of CLPB, such as N499K, E557K, R561G, or R620C [113]. In contrast, autosomal recessive or distinct biallelic mutations in CLPB, such as T268M, R408G, R475Q, A591V, R650P, are the cause of 3-methylglutaconic aciduria type 7 (MGCA7). These mutations also compromise CLPB disaggregase activity, but they do not exert a dominant-effect on the wild-type CLPB [102]. We believe that more research is needed to gain insight into the biological function of human CLPB and reveal new perspectives of the disease-causing mutations.

1.3 The goals of the study

- To study the role of ADAM12 and ADAM9 in breast cancer tumor microenvironment. Previous studies have shown that ADAM12 and ADAM9 expression is highly upregulated within breast cancers belonging to the claudin-low subtype and they are associated with a poor prognosis for survival. However, the understanding of ADAM proteins that involved in the activation of intratumoral immune cells within solid tumors, including TNBC, as well as their impact on modulating ICB responses, remains limited. Therefore, my goal was to examine how ADAM12 and ADAM9 suppress anti-tumor immunity and contribute to inhibiting ICB responses in the mouse transplantation model of claudin-low breast cancer.

- To study the role of mitochondrial CLPB during neutrophil differentiation. Although

recent studies showed that the expression of dominant-negative variants of CLPB impairs granulocytic differentiation of human hematopoietic progenitor cells, the role of CLPB during neutrophil differentiation remains unclear. My aim is to investigate the involvement of CLPB in both neutrophil differentiation and the preservation of mitochondrial functions.

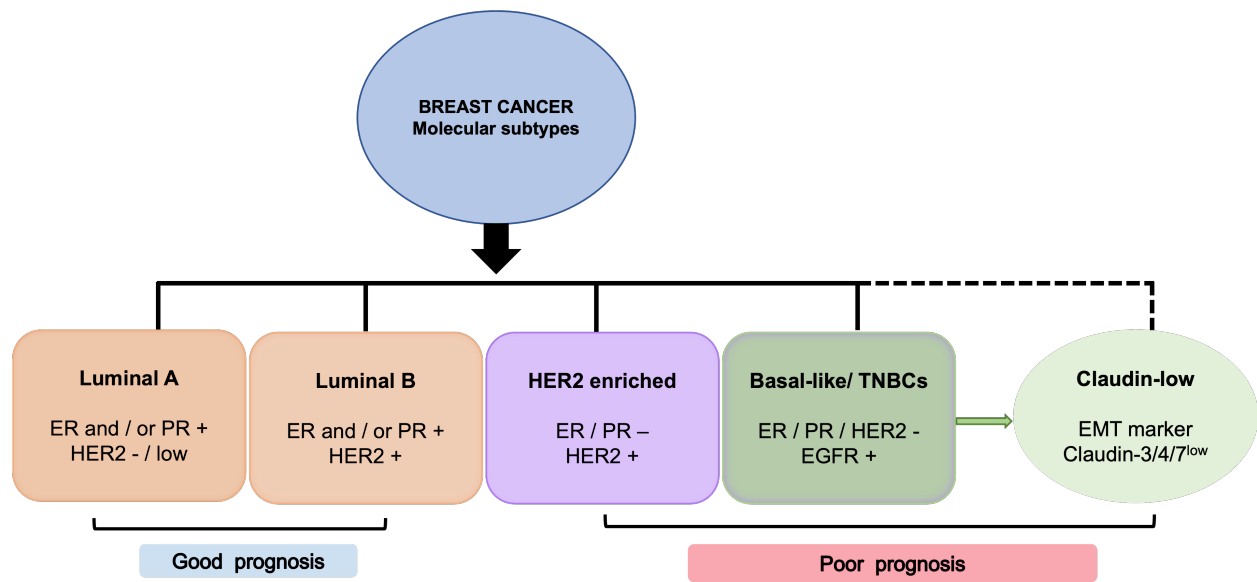


Figure 1.1 Molecular classification of breast cancer.

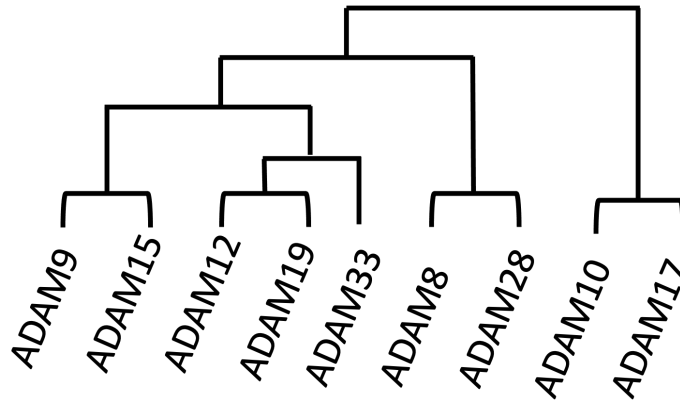
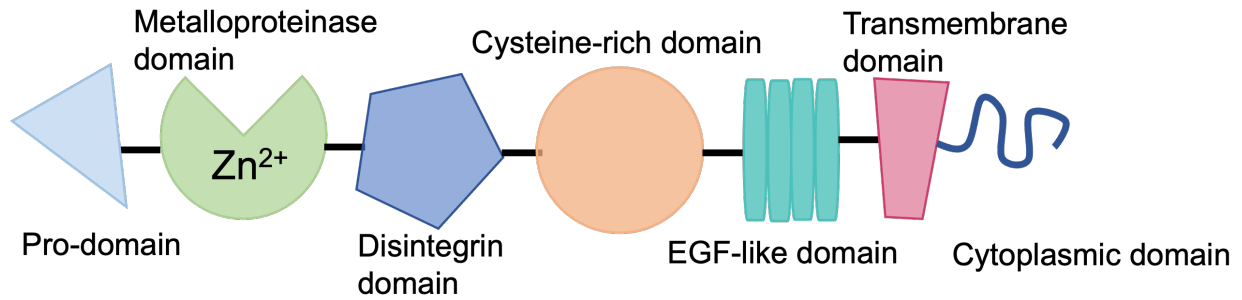


Figure 1.2 Domain structure of the ADAM metalloproteases and dendrogram showing the relative peptide sequence similarities among widely expressed ADAM proteins.

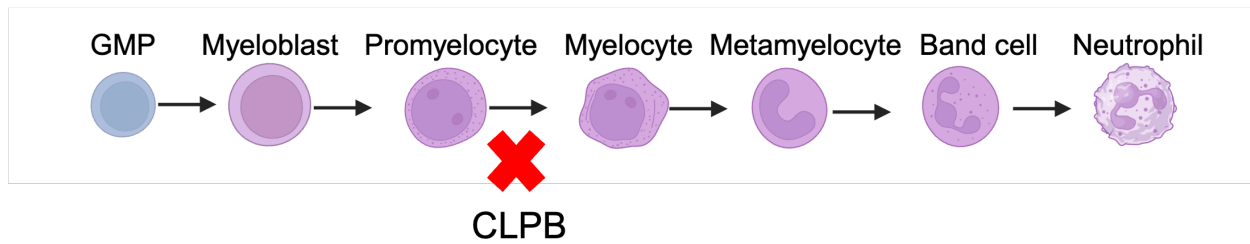


Figure 1.3 Neutrophil differentiation includes the following stages: GMP, myeloblast, promyelocyte, myelocyte, metamyelocyte, band and segmented neutrophil.

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Chapter 2 - ADAM12 abrogation alters immune cell infiltration and improves response to checkpoint blockade therapy in the T11 murine model of triple-negative breast cancer

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2.1 Abstract

Immunosuppressive tumor microenvironment (TME) impedes anti-tumor immune responses and contributes to immunotherapy resistance in triple-negative breast cancer (TNBC). ADAM12, a member of cell surface metalloproteases, is selectively upregulated in mesenchymal/claudin-low TNBCs, where its expression is largely restricted to tumor cells. The role of cancer cell expressed ADAM12 in modulating the immune TME is not known. We show that *Adam12* knockout in the T11 mouse syngeneic transplantation model of claudin-low TNBC leads to decreased numbers of tumor-infiltrating neutrophils (TINs)/polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and increased numbers of tumor-infiltrating B cells and T cells. ADAM12 loss in cancer cells increases chemotaxis of B cells *in vitro* and this effect is eliminated by inhibition of CXCR4, a receptor for CXCL12, or anti-CXCL12 blocking antibody. Importantly, ADAM12 loss in T11 cancer cells sensitizes tumors to anti-PD1/anti-CTLA4 combination therapy, although the initial responsiveness is followed by acquired therapy resistance. Depletion of B cells in mice eliminates the improved response to immune checkpoint blockade of *Adam12* knockout T11 tumors. Analysis of gene expression data for claudin-low TNBCs from the METABRIC patient cohort shows significant inverse correlations between *ADAM12* and gene expression signatures of several anti-tumor immune cell populations, as well as a significant positive correlation between *ADAM12* and gene expression signature of TINs/PMN-MDSCs. Collectively, these results implicate ADAM12 in immunosuppression within the TME in TNBC.

Keywords

Triple negative breast cancer, mouse models, ADAM, checkpoint inhibition, tumor-infiltrating immune cells, tumor microenvironment, B cells, CXCL12, mesenchymal tumors

2.2 Introduction

Triple negative breast cancer (TNBC; estrogen receptor-, progesterone receptor-, and HER2-negative) accounts for ~15% of all breast cancers and is associated with an earlier onset, a more aggressive clinical course, and worse prognosis than other types of breast cancer [1, 2]. Recently, notable progress has been achieved in applying immune checkpoint blockade (ICB) therapy to treat TNBC [3, 4]. Two blocking antibodies, pembrolizumab (anti-PD1 mAb) and atezolizumab (anti-PD-L1 mAb), have been FDA-approved for use in combination with chemotherapy in patients with unresectable or metastatic PD-L1-positive TNBC [5-7]. Furthermore, the results of several phase 3 and phase 2 clinical trials involving patients with early stage TNBC now indicate that the addition of ICB to neoadjuvant chemotherapy increases the rate of pathologic complete response (pCR) at the time of surgery and improves event free survival (EFS) [8-10]. Despite these advances, several clinical challenges remain unresolved, including identification of predictive biomarkers for ICB response, optimization of treatment strategies, and preventing disease recurrence after exposure to ICB [11, 12].

TNBC comprises a heterogeneous group of cancers, with differences at the histologic, genomic, and immunologic levels [13, 14]. The mesenchymal subtype, or the closely related claudin-low subtype, represents one of the molecular subtypes of TNBC [15-17]. Claudin-low tumors are characterized by high expression of epithelial-to-mesenchymal transition (EMT) markers and/or stromal genes [15-17], as well as high infiltration with tumor-infiltrating macrophages (TIMs) [18] and regulatory T cells (Tregs) [19]. All these attributes - EMT, fibrotic stroma, TIMs, and Tregs - have been linked to the immunosuppressive tumor microenvironment (TME) and resistance to ICB [20-24]. Thus, deciphering molecular mechanisms responsible for the acquisition of these immunosuppressive traits is critical for the success of ICB therapy in

TNBC.

ADAM metalloproteases are the family of cell surface proteolytic enzymes expressed in cancer cells and/or in other cells in the TME [25-28]. Since ADAMs have the capability to release or post-translationally modify various chemokines and cytokines [29-32], they are well positioned to modulate anti-tumor immune responses. Yet, the role of ADAMs in the recruitment, expansion, or activation of intratumoral immune cells in solid tumors, including TNBC, and in the modulation of ICB responses is poorly understood. In this study, we investigate the role of ADAM12, a prominent member of the ADAM family [33, 34], in suppressing anti-tumor immunity and inhibiting ICB responses in the T11 mouse transplantation model of claudin-low breast cancer. T11 is a mouse cell line derived from a *Tp53*^{-/-} mammary tumor syngeneic transplant [35, 36]. T11 cells have a mesenchymal appearance. When orthotopically injected into BALB/c mice, T11 cells form triple negative breast tumors with claudin-low features [37] which are resistant to ICB treatment [18, 19, 38].

Among breast cancer patients, *ADAM12* gene expression is elevated in claudin-low tumors [39, 40], where it is upregulated by several pro-tumor signaling pathways/programs, including EMT [39], hypoxia [41, 42], TGF- β [43], and the Notch [42, 44] pathway. In return, ADAM12 protein stimulates epidermal growth factor receptor (EGFR) [39, 42], TGF- β [45], and Notch [46] signaling. We now show that *Adam12* knockout in T11 cells, or *Adam12* gene editing resulting in the loss of a functional ADAM12 enzyme at the surface, alters the composition of immune cells infiltrating T11 mammary tumors and sensitizes T11 tumors to the ICB therapy, suggesting an important role of ADAM12 in modulating anti-tumor immunity.

2.3 Materials and methods

Cell culture

T11 cell line was provided by Dr. Jeffrey M. Rosen (Baylor College of Medicine). Phoenix-Eco cells were a gift from Dr. Garry P. Nolan (Stanford University). 4T1 cells were obtained from American Tissue Culture Collection (ATCC). Cells were cultured in DMEM (T11 and Phoenix-Eco cells) or RPMI-1640 medium (4T1 cells) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO₂.

Generation of *Adam12* knockout in T11 and 4T1 cells

T11 cells were co-transfected with an *Adam12*-specific gRNA vector (OriGene Technologies), using Turbofectin transfection reagent. Two different gRNA vectors were used, with *Adam12*-targeting sequence 5'-GATGACCAAGTACGTAGAGC-3'(gRNA1) or 5'-CCAAGGAACCACCATCGGCA-3' (gRNA2). To generate *Adam12* knockout in 4T1 cells, recombinant *S. pyogenes* HiFi Cas9 Nuclease V3 (IDT) was mixed with sgRNAs to generate ribonuclear protein complexes, followed by nucleofection into 4T1 cells using the Neon system (Invitrogen). *Adam12*-targeting sequences were 5'-CCAAGGAACCACCATCGGCA-3' (gRNA2) or 5'-AGAGCATGACGAACATCCAA-3' (gRNA3). Single cell clones of T11 and 4T1 cells were isolated using glass cloning cylinders and analyzed by genomic PCR using primers flanking the predicted target sites in the *Adam12* gene, Sanger sequencing of the PCR products (GENEWIZ/Azenta), and ADAM12 immunoblotting.

Immunoblotting

Cells were incubated without or with 5 ng/ml of mouse transforming growth factor β

(TGF- β ; R&D Systems) for 48 hours and then treated with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 10 mM 1,10-phenanthroline). Cell lysates were centrifuged for 15 minutes at 17,000xg at 4°C. Supernatants were incubated with concanavalin A-agarose (50 μ l resin per 1 ml cell lysate) for 4 hours at 4°C to enrich samples for glycoproteins. The resin was then washed three times with lysis buffer and glycoproteins were eluted with 3xSDS gel-loading buffer. Proteins were resolved using SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% milk, membranes were probed with anti-ADAM12 [47] or anti- β 1 integrin antibody and horseradish peroxidase-conjugated IgG secondary antibodies. Signal detection was performed using the SuperSignal West Pico PLUS chemiluminescent detection kit (ThermoFisher) and Azure c500 digital imaging system.

Mutagenesis and stable overexpression of ADAM12

Retroviral expression vector *Adam12*-pBabePuro was used for the expression of ADAM12-wildtype (ADAM12-WT) protein [48]. The *Adam12*- Δ 6 construct was generated using the QuickChange kit (Agilent Technologies). Phoenix-Eco cells were transfected using calcium phosphate precipitation method (20 μ g DNA/100-mm plate), in the presence of 25 μ M chloroquine. Viral supernatants were harvested 48 hours later, supplemented with 8 μ g/ml polybrene, and used without further dilution for infection of T11 cells. After 48 hours, cells with stable overexpression of ADAM12-WT or ADAM12- Δ 6 were selected with 2 μ g/ml puromycin for 8 days.

Cells surface biotinylation

Cells grown in 6-well plates were washed with Dulbecco's phosphate-buffered saline (DPBS), incubated at 4 °C for 60 minutes with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific), and then washed with ice-cold 100 mM Tris-HCl/DPBS. Cellular proteins were extracted with lysis buffer, as described above. Cell extracts were centrifuged at 17,000xg for 15 minutes at 4 °C. The supernatants were incubated for 1 hour at 4 °C with NeutrAvidin agarose (Thermo Scientific; 50 µl of resin/0.5 ml cell extract). The resin was washed three times with lysis buffer, eluted with 3xSDS gel-loading buffer. Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting.

ELISA assay

Cells were incubated in 6-well plates for 48 hours. Conditioned media and cell lysates were collected and centrifuged for 15 minutes at 17,000xg. CXCL12 concentrations in the supernatants were measured using mouse CXCL12/SDF-1 α ELISA kit (R&D Systems, the kit has some cross-reactivity with CXCL12/SDF-1 β) and BioTek H1M microplate reader. CXCL12 concentrations in cell lysates were normalized to equal protein concentrations, measured with the BCA assay kit (ThermoFisher).

Orthotopic T11 cell transplantation

All animal experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Kansas State University. Female 5-6-week-old BALB/c and NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice were purchased from The Jackson Laboratory. Animals of 6-10 weeks of age were used as the recipients of cell transplantation.

Cells were detached, washed, suspended in DPBS, counted, and adjusted to 2×10^5 cells/ml (for tissue harvesting experiments) or 4×10^5 cells/ml (for immunotherapy experiments). Cells (50 μ l) were then injected into the left 4th inguinal mammary fat pad of mice under isoflurane sedation (a single injection per animal). Mice were monitored for tumor growth daily; tumor size (length x width) was measured with a caliper.

Isolation of tumor-infiltrating immune cells

Mice were euthanized and tumors were excised and placed in ice-cold DPBS. Tumors were weighed, transferred to 5 ml Hank's balanced salt solution (HBSS) with calcium and magnesium at room temperature, cut into small pieces using razor blades, and transferred to 50 ml tubes containing 10 mg/ml collagenase type IV, 200 units/ml DNase type IV, and 1,500 units/ml hyaluronidase type IV-S (all from Sigma). Tubes were shaken at 130 rpm for 1 hour at room temperature. Single cell suspensions were obtained by filtering through a 70- μ m cell strainer. The digestion reaction was stopped by adding 1 mM EDTA/0.1% BSA, followed by centrifugation for 3 minutes at 800 x g, resuspension in 2 ml ACK lysis buffer (Lonza), incubation on ice for 1 minute, centrifugation, and washing with DPBS. Cell suspensions were enriched for lymphocytes by centrifugation in 42%/78% Percoll (GE Healthcare) gradient in DMEM medium, at 1000 x g for 20 minutes with no brakes on deceleration. Approximately 4 ml solution was collected from the interface between the two Percoll layers. Isolated lymphocytes were washed with DPBS and resuspended in FACS buffer (DPBS containing 3% bovine serum albumin).

Flow cytometry

Single-cell suspensions of tumor-infiltrating leukocytes were prepared as described above. Cells were first incubated with 2.5 µg/ml TruStain FcX Plus (BioLegend) in FACS buffer for 10 min on ice. Cells were subsequently stained with fluorochrome-conjugated antibodies (Supplementary Table S1; BioLegend) in FACS buffer for 30 minutes on ice in the dark, followed by a wash with FACS buffer and resuspension in 400 µl of FACS buffer supplemented with 1 µg/ml propidium iodide. In some experiments, the absolute numbers of tumor-infiltrating immune cells were determined using the Precision Count Beads™ (BioLegend). When staining for FoxP3 in Tregs, cells were first incubated with 1 µl eFluor 450 fixable viability dye (Invitrogen) for 30 minutes on ice in the dark, followed by FcR block and cell surface marker fluorochrome-conjugated antibodies staining. Stained cells were then fixed and permeabilized using BioLegend True Nuclear Transcription Factor Buffer Set and stained with anti-FoxP3 antibody. Samples were analyzed using a LSRFortessa X-20 flow cytometer (BD), and data analysis was performed using FCS Express 7 (DeNovo Software). Gating strategies are shown in Supplementary Figures B. S1-4.

B cell migration assays

Cancer cells were plated at $\sim 0.5 \times 10^6$ cells/ml into lower chambers of Transwell 6-well plates® (Corning) and incubated for 48 hours. Splenocytes were isolated from BALB/c mice by mincing the tissue, passing sequentially through 18, 21, and 26 gauge needles, then through a 70-µm cell strainer, and centrifuging at 800 x g for 3 minutes. Cell pellets were resuspended in T11 or 4T1 cell culture media, cells were counted, and 2.5×10^6 cells were seeded into transwell upper chambers (inserts) containing 3.0-µm pore membrane. After incubation for 4 hours at 37°C, cells migrated into lower chambers were stained with anti-CD45 and anti-CD19 antibodies

and analyzed by flow cytometry. In some experiments, splenocytes were pre-incubated with 10 µg/ml of AMD3100, a CXCR4 inhibitor, for 30 minutes prior to the migration assay.

Alternatively, cancer cells in the lower compartment were pre-incubated for 1 h with 2.5 µg/ml CXCL12-blocking or isotype-matched antibodies. The initial content of B cells among splenocytes (input) was determined after seeding splenocytes into wells without inserts. The amount of migrated B cells was expressed as a percentage of the input.

Immune checkpoint blockade

Cells were implanted orthotopically, as described above. When tumors reached 10-20 mm² in size, mice were randomized, and 125 µg anti-CTLA4 (clone 9D9) and 200 µg anti-PD1 (clone RMP1-14) antibodies were delivered intraperitoneally every 3-4 days, for a total of 3-4 doses. Control animals received an equal amount of isotype-matched antibodies (mouse IgG2b clone MPC-11 and rat IgG2a clone 2A3). B cell depletion was achieved by injecting 250 µg anti-CD20 mAb (Ultra-LEAF purified, clone SA271G2, reported depletion of 30 or more days) into a lateral tail vein 3 days prior to ICB; isotype-matched rat IgG2b (clone RTK4530) was injected as control. In a parallel experiment, B cell depletion was confirmed by isolating splenocytes 7 days after anti-CD20 treatment and quantifying B cells by flow cytometry.

Data mining and correlation analyses

Adam12 expression data were retrieved from Gene Expression Omnibus (GEO) at <https://www.ncbi.nlm.nih.gov/geo/>; GSE series numbers are provided in the text. Expression data for human *ADAM12* and immune genes in 106 claudin-low TNBCs from the METABRIC dataset [49] were accessed via the cBioPortal for Cancer Genomics [50, 51]

(<http://www.cbioportal.org/public-portal/>). The list of core claudin-low tumors was obtained from ref. 17; sample IDs for these tumors are listed in Supplementary Table S2. Gene expression signature scores for selected immune cell types were calculated as the average gene expression z-scores for marker genes associated with each cell types (Supplementary Table S3). Correlation analysis between *ADAM12* expression and each immune cell signature score was performed using GraphPad Prism 9 software; Pearson *r* correlation coefficients and *P* values (two-tailed) are reported for each analysis. Expression data (ultra-low input RNA-Seq) for different human *ADAMs* in purified immune cell populations were accessed via ImmGen data portal at <https://www.immgen.org/>.

Statistical analyses

Data were analyzed with GraphPad Prism 9 software. When comparing two groups, normality tests were performed first. If the data followed a Gaussian distribution and if the F-test confirmed the same variances, statistical analysis was performed using unpaired two-tailed t-test. When the data did not follow a Gaussian distribution (as some groups in Figs. 2.3 and 2.4), the Mann-Whitney nonparametric test was used. For survival analysis, Kaplan-Meier curves were generated and compared using the log-rank test.

2.4 Results

We analyzed *Adam12* microarray gene expression data for 341 unique murine tumors representing different molecular subtypes of breast cancer, including 25 claudin-low tumors, and 18 normal murine mammary gland samples [37] (retrieved from Gene Expression Omnibus (GEO) at <https://www.ncbi.nlm.nih.gov/geo/>, datasets GSE3165, GSE8516, GSE9343,

GSE14457, GSE15263, GSE17916, GSE27101, and GSE42640; individual samples are listed in Supplementary Table B. S4). We noticed that *Adam12* expression levels were significantly higher in claudin-low tumors than in other tumor types or in normal mammary glands (Fig. 2.1a). Analysis of RNA sequencing (RNA-seq) results for four murine cell lines representing the mesenchymal/claudin-low subtypes of TNBC, namely 67NR, E0771, PyMT-M, and T11 [18] (retrieved from GEO:GSE104765) further showed that *Adam12* mRNA levels were the highest in T11 cells (Fig. 2.1b), and this cell line was selected for our studies.

Recent investigations have shown that while T11 syngeneic orthotopic tumors are resistant to ICB, they become ICB-sensitive upon increasing the mutation burden in T11 cells via overexpression of Apobec3 or UV exposure [38]. We have retrieved *Adam12* expression data for T11, T11-Apobec3, and T11-UV cells (GEO:GSE124821) and determined that *Adam12* mRNA levels were significantly lower in ICB-sensitive than in ICB-resistant tumors (Fig. 2.1c).

To investigate whether abrogation of *Adam12* expression in T11 cells might impact anti-tumor immunity and response to ICB, we performed CRISPR/Cas9-mediated *Adam12* gene knockout (KO) in T11 cells. Initially, cells were co-transfected with pCas-Guide CRISPR-gRNA1 vector targeting exon 7 in the *Adam12* gene (Fig. 2.1d). Several single cell clones were selected and analyzed by genomic PCR using primers flanking the donor integration site and PCR product sequencing (to validate *Adam12* gene KO) and by immunoblotting (to confirm the lack of ADAM12 protein). Since ADAM12 expression is upregulated by TGF- β [43, 52], cells were incubated for 48 h with TGF- β to further confirm band identities (Fig. 2.1e). Two individual clones lacking ADAM12 expression (referred to as A12-KO (g1)) were combined and used in further analyses.

There was no difference between proliferation rates of A12-KO and parental T11 cells *in*

vitro. In contrast, breast tumors formed after orthotopic transplantation of A12-KO (g1) cells into immunocompetent BALB/c mice grew significantly slower than tumors formed by parental T11 cells (Fig. 2.1f). The difference in tumor growth was largely reduced in immunodeficient NOD/SCID gamma (NSG) mice (Fig. 2.1g) that lack the adaptive immune system (mature T, B and NK cells are absent) and have deficiencies in the innate branch of the immune system (macrophages and dendritic cells are defective, complement is absent)[53]. Thus, A12-KO (g1) tumors might be susceptible to anti-tumor attack by tumor-infiltrating lymphocytes (TILs) and/or certain components of the innate immunity. The remaining small, but significant, difference between growth rates of A12-KO (g1) and T11 tumors in NSG mice (Fig. 2.1h) might be linked to the function of residual myeloid cells in NSG mice or to changes in the TME not related to the immune system.

We next compared the numbers and composition of tumor-infiltrating immune cells in A12-KO (g1) *versus* control T11 tumors. When tumors reached a similar size, they were resected, enzymatically digested, and single cell suspensions were stained and analyzed by flow cytometry. First, we observed that the total number of tumor-infiltrating CD45⁺ immune cells in A12-KO (g1) tumors was decreased by 25-30% compared to T11 tumors (Fig. 2.2a). This decrease was due to the reduction of the myeloid CD11b⁺ cell population, from $\sim 2.4 \times 10^6$ to $\sim 1.6 \times 10^6$ cells/g, or from $\sim 90\%$ to $\sim 83\%$ of all CD45⁺ cells (Fig. 2.2b). In contrast, the absolute number of CD11b⁻ cells was slightly, but not significantly, higher in A12-KO (g1) cells and amounted to $\sim 3 \times 10^5$ cells/g of tumor tissue. The percentage of CD11b⁻ cells among CD45⁺ rose from 10% in T11 tumors to 16% in A12-KO (g1) tumors, and this increase was significant (Fig. 2.2c).

It has been previously reported that the T11 tumor model belongs to a macrophage-

enriched subtype (MES) of TNBC, in which the tumor-infiltrating myeloid compartment is dominated by TIMs, with significantly lower frequencies of tumor-infiltrating neutrophils (TINs) [18]. Here, we have quantified the TIM and TIN populations, defined as CD45+CD11b+Ly6G-Ly6C-F4/80+ and CD45+CD11b+Ly6G+Ly6C^{med/low}, respectively [18]. Importantly, such designation of the TIN population also comprised polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), as mouse TINs and PMN-MDSCs are currently not distinguishable based on phenotypic cell surface markers [54]. The CD45+CD11b+Ly6G-Ly6C^{hi} population defined monocytic MDSCs (M-MDSCs) [54]. Representative flow cytometry plots are shown in Fig. 2.2d, and gating strategies are included in Supplementary Figure B. S1.

The absolute number of TIMs did not differ significantly between T11 and A12-KO (g1) tumors, but TIM frequency among all CD45+ cells increased from ~54% in T11 tumors to 59% in A12-KO (g1) tumors, and this increase was significant (Fig. 2.2e). Interestingly, the absolute number of TINs/PMN-MDSCs dropped from 7.2×10^5 per gram in T11 tumors to 2.8×10^5 per gram in A12-KO (g1) tumors, or from 26% to 15% of CD45+ cells, respectively (Fig. 2.2f). The population of M-MDSCs represented only a small fraction (~1%) of all tumor-infiltrating cells and did not differ between T11 and A12-KO (g1) tumors (Fig. 2.2g).

We next compared tumor-infiltrating lymphocytes (TILs) in control and A12-deficient T11 tumors. Since the CD45+CD11b- population comprising the bulk of TILs amounted to only ~10% of all tumor-infiltrating immune cells in T11 tumors (Fig. 2.2c), TILs were enriched by centrifugation in a discontinuous Percoll gradient [55] prior to flow cytometry analysis and quantified as percent of CD45+ cells collected from the 42%/78% Percoll interface. Notably, A12-KO (g1) tumors contained higher percentages of CD3+ T cells (median values 32.4% *versus* 25.4%; Fig. 2.3a,b) and CD19+ B cells (median values 14% *versus* 4.4%; Fig. 2.3a,c)

than T11 tumors. In contrast, the frequencies of CD3-CD49b⁺ NK cells, the third major class of lymphoid cells, were similar in A12-KO (g1) and control T11 tumors (Fig. 2.3d).

The frequencies of cytotoxic CD8⁺ T cells did not significantly differ between A12-KO (g1) and T11 tumors (Fig. 2.3e). Accordingly, the increase in T cell frequencies observed in A12-KO (g1) tumors was mainly due to the expansion of CD4⁺ T cells (median value 19.6% in A12-KO (g1) tumors *versus* 14.7% in T11 tumors; Fig. 2.3f). Among CD4⁺ T cells, the relative abundance of immunosuppressive regulatory T cells (Tregs; CD45⁺CD3⁺CD4⁺CD25⁺Foxp3⁺) *versus* non-Tregs remained similar in A12-KO (g1) and T11 tumors and amounted to ~20% (Fig. 2.3g).

Our results so far have suggested that ADAM12 loss in T11 tumor cells may alter certain populations of tumor-infiltrating myeloid and lymphoid cells. In this work, we wanted to further investigate the consequences of ADAM12 loss on TILs, in particular on B cells. First, to validate the results obtained for A12-KO (g1) tumors, we used a different pCas-Guide CRISPR vector to knockout ADAM12 in T11 cells, with gRNA2 targeting exon 10 in the *Adam12* gene (Fig. 2.4a). We isolated two different clones: A12-KO (g2), which contained biallelic disruption of the *Adam12* gene and lacked ADAM12 protein (Fig. 2.4b), and A12-Δ6, in which one *Adam12* allele was disrupted and the second allele contained an in-frame deletion of an 18-nt fragment next to the Cas9 cleavage site, resulting in a 6-amino acid deletion in the metalloprotease domain of the ADAM12 protein (Fig. 2.4a).

In the predicted model of the wild-type ADAM12 metalloprotease domain, the six amino acids missing in A12-Δ6 were positioned close to the catalytic site (Fig. 2.4c). Therefore, we hypothesized that A12-Δ6, generated fortuitously during non-homologous DNA end joining (NHEJ) after Cas9-mediated DNA cleavage, might show defects in folding, post-translational

processing, or intracellular trafficking. Typically, nascent full-length forms of ADAMs are catalytically inactive and retained in the intracellular compartments. In contrast, mature processed forms of ADAMs lack the N-terminal inhibitory domain, are catalytically active, and are present at the cell surface [25, 56]. To examine possible defects in A12- Δ 6 processing/trafficking, the wild-type ADAM12 (A12-WT) or A12- Δ 6 were overexpressed in T11 cells. Cells were subjected to surface biotinylation using a membrane-impermeable biotinylation reagent, followed by isolation of glycoproteins on concanavalin A agarose (a routine enrichment step for all ADAM12 isoforms) or by isolation of biotinylated proteins on streptavidin agarose. Consistent with previous reports, in A12-WT overexpressing cells, only the ~90-kDa mature form was biotinylated and the ~120-kDa nascent form was not. In A12- Δ 6 overexpressing cells, in which the ~120-kDa nascent form was predominant and the ~90-kDa mature form was largely missing, no biotinylated ADAM12 was detected (Fig. 2.4d), confirming defects in the processing/trafficking of A12- Δ 6 and the absence of an active A12- Δ 6 enzyme at the cell surface.

Despite the different status of ADAM12 in A12-KO (g2) and A12- Δ 6 cells (a complete loss of ADAM12 *versus* the absence of catalytically active ADAM12 at the cell surface), both A12-KO (g2) and A12- Δ 6 tumors contained significantly higher frequencies of T cells than T11 tumors (median values 42.6%, 42.1%, and 30.0%, respectively; Fig. 2.4e). The frequencies of tumor-infiltrating B cells in A12-KO (g2) and A12- Δ 6 tumors were also significantly higher than in T11 tumors (median values 7.4%, and 6.9%, and 4.4%, respectively; Fig. 2.4f). These results indicate that *Adam12* gene knockout in T11 cancer cells increases the frequencies of T cells and B cells among tumor-infiltrating immune cells and this effect is largely due to the lack of functional ADAM12 at the cell surface.

Between tumor-infiltrating T cells and B cells, the relative effect of *Adam12* knockout on the frequencies of B cells was particularly prominent (Figs. 2.3c and 2.4f). Therefore, we wanted to determine whether ADAM12 loss in cancer cells had a direct effect on B cell chemotaxis. We performed transwell cell migration assays, in which cancer cells were plated in lower transwell chambers, freshly isolated splenocytes were seeded in upper chambers, and the amount of B cells migrated into the lower chambers was measured by flow cytometry (Fig. 2.5a). We observed that the presence of T11 in the lower chambers increased B cell migration when compared to migration toward empty wells without cancer cells. Importantly, B cell migration toward A12-KO (g1) or A12-KO (g2) T11 cells was significantly higher than toward wild-type T11 cells (Fig. 2.5b and 2.5c, respectively).

We then asked whether *Adam12* knockout in a different murine breast cancer cell line would exert a similar effect on directional B cell migration. We tested 4T1 cells, which are commonly used as a model of basal-like triple-negative breast cancer [18]. As in T11 cells, ADAM12 expression in 4T1 cells was strongly stimulated by TGF- β . We used two different gRNAs to successfully disrupt the *Adam12* gene in 4T1 cells, gRNA2 and gRNA3 (Fig. 2.5d; see also Materials and Methods). Unexpectedly, there was no difference between B cell migration toward wild-type 4T1 cells, *Adam12* knockout 4T1 cells, or empty wells (Fig. 2.5e,f).

The CXCL12 chemokine is a known chemoattractant for B cells *in vitro* and *in vivo* [57, 58]. Expression profiling by high throughput RNA sequencing previously revealed a significantly higher *Cxcl12* mRNA level in T11 than in 4T1 cells [18] (Fig. 2.5g). Here, we performed ELISA assays to measure CXCL12 protein concentrations in conditioned media and cell lysates from T11 and 4T1 cells. Consistent with gene expression profiling, there was a very

significant difference between CXCL12 levels in T11 and 4T1 cells, with the latter ones below the ELISA detection limit (Fig. 2.5h). This suggested that CXCL12 secreted by T11 cells might have bound to its receptor CXCR4 in B cells and stimulated directed B cell migration in transwell assays. Also, ADAM12 loss in T11 cells might have potentiated the chemoattractant properties of CXCL12 (see Discussion), and the lack of CXCL12 expression in 4T1 cells might be responsible for the lack of stimulation of B cell migration. In line with this hypothesis, adding AMD3100, a CXCR4 inhibitor, to the upper transwell chambers (Fig. 2.5i-k) or anti-CXCL12 blocking antibody to the lower chambers (Fig. 2.5l-n), efficiently inhibited B cell migration toward T11, A12-KO (g1) T11, and A12-KO (g2) T11 cells. Pre-incubation of cancer cells with an isotype-matched control antibody did not have any effect on B cell migration (Supplementary Figure B. S5).

We next explored whether alterations in tumor-infiltrating immune cells observed in A12-KO (g1) and A12-KO (g2) tumors translated into improved tumor responses to the ICB therapy. A12-KO (g1), A12-KO (g2), or control T11 tumor-bearing mice were treated with anti-PD1/anti-CTLA4 antibodies (Fig. 2.6a). Control T11 tumors were resistant to ICB (Fig. 2.6b), in agreement with published reports using the same T11 model [18, 38]. Injection of the same doses of isotype-matched control antibodies did not have any effect on the growth of A12-KO (g1) tumors (Fig. 2.6c). Remarkably, A12-KO (g1) tumors stabilized and did not progress for 7-8 days after the first dose of antibodies (Fig. 2.6d). Median progression-free survival (PFS) was extended from 3 days in untreated mice to 9 days in treated mice (Fig. 2.6d). Progression was defined as a 20% increase in tumor diameter, or 1.44-fold increase in tumor area. Similar effects were observed for A12-KO (g2) tumors, which stabilized for ~5 days after the first dose of antibodies (Fig. 2.6e). Median PFS was extended from 2 days in untreated mice to 5 days in

treated A12-KO (g2) mice (Fig. 2.6e). Importantly, while both A12-KO (g1) and A12-KO (g2) tumors showed initial response to ICB, all tumors relapsed after ~8 days (Fig. 2.6d,e) and grew with the rates similar to those before the treatment, which was indicative of acquired resistance to ICB.

To explore whether increased frequencies of tumor-infiltrating B cells in ADAM12-deficient tumors contributed to improved tumor responses to the ICB therapy, we performed B cell depletion experiments. Mice bearing A12-KO (g1) or A12-KO (g2) tumors received one dose of B cell-depleting anti-CD20 antibody or an equivalent dose of isotope-matched control antibody, followed by three injections of anti-PD1/anti-CTLA4 antibodies. B-cell depletion was confirmed by flow cytometry analysis of splenic B cells (Fig. 2.7a). In both A12-KO (g1) and A12-KO (g2) tumors, B cell depletion significantly reduced the efficacy of anti-PD1/anti-CTLA4 treatment (Fig. 2.7b,c).

Using data mining approaches, we then examined whether our results linking the loss of ADAM12 to anti-tumor responses and ICB susceptibility in the T11 mouse model may be applicable to human TNBC patients. First, expression profiling of different immune cell populations by RNA-Seq suggests that *ADAM12* is expressed at very low levels in immune cells (Fig. 2.8a, data retrieved at www.immgen.org). In contrast, most other catalytically active *ADAMs*, except for *ADAM9*, are expressed at high levels in various types of immune cells (Fig. 2.8a). This means that *ADAM12* expression values obtained during mRNA profiling of bulk tumors should predominantly reflect *ADAM12* expression in cancer cells, with a minimal contribution from tumor-infiltrating immune cells.

We then retrieved bulk tumor expression data for *ADAM12* and different immune genes from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset

[49], accessed via the cBioPortal for Cancer Genomics [50, 51] (<http://www.cbioportal.org/public-portal/>). Among 106 claudin-low TNBC tumors profiled in the METABRIC study, *ADAM12* expression was significantly higher in 31 so-called “core claudin-low” tumors [17], with strong features of EMT in carcinoma cells, than in the remaining 75 claudin-low TNBCs (Fig. 2.8b). Importantly, among all 106 claudin-low TNBCs from the METABRIC dataset, there were significant inverse correlations between *ADAM12* mRNA levels and immune gene signatures associated with anti-tumor responses (CD4⁺ Th1 T cells [59], CD4⁺ Tfh T cells [38, 59], B cells [38, 60, 61], and TLSs [62]) (Pearson *P* values <0.01; Fig. 2.8c). In contrast, *ADAM12* mRNA levels were positively correlated with the pro-tumor TIN/PMN-MDSC [18] gene signature (Pearson *P* = 0.0002; Fig. 2.8c). There was no significant correlation between *ADAM12* mRNA and the TIM signature [63] (Fig. 2.8c).

2.5 Discussion

Results presented in this study demonstrate that *Adam12* knockout in claudin-low T11 breast cancer cells had a significant impact on the composition of tumor-infiltrating immune cells. In particular, the frequencies of B cells were significantly elevated in ADAM12-deficient tumors, and this effect was observed for two different guide RNAs used in CRISPR-Cas9-mediated *Adam12* gene knockout. Tumors arising from A12-Δ6 T11 cells lacking the catalytically active ADAM12 at the cell surface also contain higher frequencies of B cells, suggesting that the presence of catalytically active ADAM12 at the surface of cancer cells is vital for its role in modulating B cell infiltration. An inverse correlation between *ADAM12* mRNA levels and gene expression signatures for B cells was also detected in claudin-low TNBCs from the METABRIC dataset, further supporting the notion that high expression of ADAM12 limits

the accumulation of B cells in the breast TME.

New research indicates that B cells play a prominent role in promoting anti-tumor responses in various types of cancer [64-67]. Immunological mechanisms, clinical impact, and therapeutic potential of tumor-infiltrating B cells have been recently discussed in several excellent review articles [58, 68-72]. For TNBC explicitly, it has been reported that clonally expanded, IgG isotype-biased B cell immunity is associated with improved clinical outcomes [73]. Furthermore, using mouse models of claudin-low or basal subtypes of TNBC, it has been demonstrated that increasing the mutation burden in cancer cells by overexpressing APOBEC3B, a cytidine deaminase, or by exposure of cancer cells to short-wave UV radiation prior to transplantation, expanded intratumoral B cells and potentiated the effects of anti-PD1/anti-CTLA4 therapy [38]. In our studies, ADAM12-deficient T11 tumors stabilized, albeit transiently, after administration of anti-PD1/anti-CTLA4 antibodies, whereas control T11 tumors did not respond to the ICB treatment. Importantly, B cell depletion prior to ICB eliminated the improved response to ICB, indicating that the susceptibility of ADAM12-deficient tumors to ICB was, at least in part, mediated by increased frequencies of intratumoral B cells.

In addition to B cells, we observed increased frequencies of T cells in ADAM12-deficient tumors, although the effect of ADAM12 loss on tumor-infiltrating T cells was more modest than on B cells. Among T cells, only the CD4⁺ subset was significantly expanded in ADAM12-deficient tumors, whereas the frequencies of CD8⁺ cytotoxic T cells were not affected. Finally, among CD4⁺ cells, the ratio of immunosuppressive Tregs to non-Treg cells was not significantly changed upon the loss of ADAM12, arguing against the release of Treg-mediated immunosuppression in ADAM12-deficient tumors. This conclusion is important, because it has

been reported previously that Treg depletion alone reduces immunosuppression and potentiates ICB therapy in claudin-low breast cancer [19, 74].

Along with changes within the lymphoid compartment, we observed an altered composition of tumor-infiltrating myeloid immune cells in ADAM12-deficient T11 tumors. Specifically, tumors derived from A12-KO (g1) cells contained significantly less TINs/PMN-MDSCs than tumors derived from control T11 cells, and a positive correlation between *ADAM12* mRNA levels and the TINs/PMN-MDSC gene expression signature has been detected in claudin-low TNBCs from the METABRIC dataset. Implications of these findings are currently not clear. While PMN-MDSCs are unequivocally immunosuppressive [54], TINs span neutrophil populations with tumor-promoting or tumor-suppressing activities [75, 76]. Thus, a detailed functional characterization of the TIN/PMN-MDSC population from ADAM12-deficient *versus* wild-type T11 tumors is needed to obtain a better understanding of the role of ADAM12 in regulating immunosuppression within the myeloid compartment. Of note, lower numbers of tumor-promoting TINs in ADAM12-deficient tumors might explain the fact that A12-KO (g1) tumors grew slightly slower than T11 tumors in NSG mice that retain their neutrophil population, as certain tumor-promoting activities of TINs may be executed even in the absence of the adaptive immune system⁷⁶. Furthermore, it has been shown that a conversion of the macrophage-enriched subtype (MES) to the neutrophil-enriched subtype (NES) in several mouse models of TNBCs mediated acquisition of ICB resistance in initially sensitive MES models [18]. Since ADAM12-deficient T11 tumors become eventually resistant to ICB after the initial period of therapy response, future enumeration of TIMs and TINs/PMN-MDSCs at different time points after ICB administration will be particularly informative.

TIMs play tumor-promoting functions in breast cancer and are generally associated with a poor prognosis [77]. It has been recently demonstrated that TIM inhibition/depletion, combined with a low dose of chemotherapeutic agent cyclophosphamide, induces T cell and B cell infiltration and durable regression in the T11 and related T12 mouse models of TNBC [78]. In our experiments, absolute numbers of TIMs did not differ significantly between A12-KO (g1) and control T11 tumors, and the percentage of TIMs among all tumor-infiltrating CD45⁺ cells was even slightly increased in A12-KO (g1) tumors. Therefore, it seems unlikely that increased B cell infiltration in ADAM12-deficient T11 tumors was mediated via TIMs.

In search for potential mechanisms of increased accumulation of B cells in A12-KO (g1) and A12-KO (g2) tumors, we have discovered that B cells migrated faster toward T11 cells lacking ADAM12 than toward control T11 cells in *in vitro* transwell assays. Several lines of evidence then suggested a key role for CXCL12 in this process: (i) increased B cell migration toward ADAM12-deficient cells was observed for CXCL12-positive T11 cells but not for CXCL12-negative 4T1 cells, (ii) B cell migration was significantly reduced by a CXCR4 inhibitor, and (iii) B cell migration was significantly diminished by an anti-CXCL12 blocking antibody. Importantly, CXCR4 inhibition or anti-CXCL12 antibody reduced B cell migration toward ADAM12-deficient T11 cells essentially to the basal level, indicating that the CXCL12/CXCR4 interaction was the main driver of B cell migration, at least in our transwell assays. While the contribution of other cytokines to the increased accumulation of B cells in T11 tumors *in vivo* is also possible, we propose the hypothesis that ADAM12 is a negative regulator of cancer cell-derived CXCL12 and that ADAM12 loss in T11 cells increases the activity of CXCL12.

Of note, CXCL12- α and CXCL12- β , two major splice variants of CXCL12 in mice and

humans, can undergo N-terminal truncation by several members of the ADAM-related matrix metalloproteinases (MMPs), including MMP-1, -2, -3, -9, -13 and -14. These MMPs remove the first four amino acids in CXCL12, leading to its complete inactivation [79, 80]. While ADAM12 activity toward CXCL12 has not been investigated, it is feasible that ADAM12 mediates a similar cleavage and inactivation of CXCL12. In such a case, a combination of ICB with ADAM12 inhibition may represent a rational approach to improve the ICB efficacy in claudin-low TNBC.

In summary, our results establish a paradigm in which a cell surface ADAM in breast cancer cells modulates the repertoire of tumor-infiltrating immune cells and improves ICB responses. This invites a comprehensive examination of ADAM12 and other cancer cell-expressed ADAM metalloproteases as potential regulators of anti-tumor immunity and/or immunosuppression in the TME.

2.6 Acknowledgments

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2.7 Declaration of interest

The authors declare no conflicts of interest.

2.8 Funding

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2.9 Data availability statement

The data that support the findings of this study are openly available in Gene Expression Omnibus at <https://www.ncbi.nlm.nih.gov/geo/> (GSE3165, GSE8516, GSE9343, GSE14457, GSE15263, GSE17916, GSE27101, GSE42640, GSE104765, and GSE124821), Human Cell Atlas Immune System at <http://immunecellatlas.net/>, and cBioPortal at <https://www.cbioportal.org>.

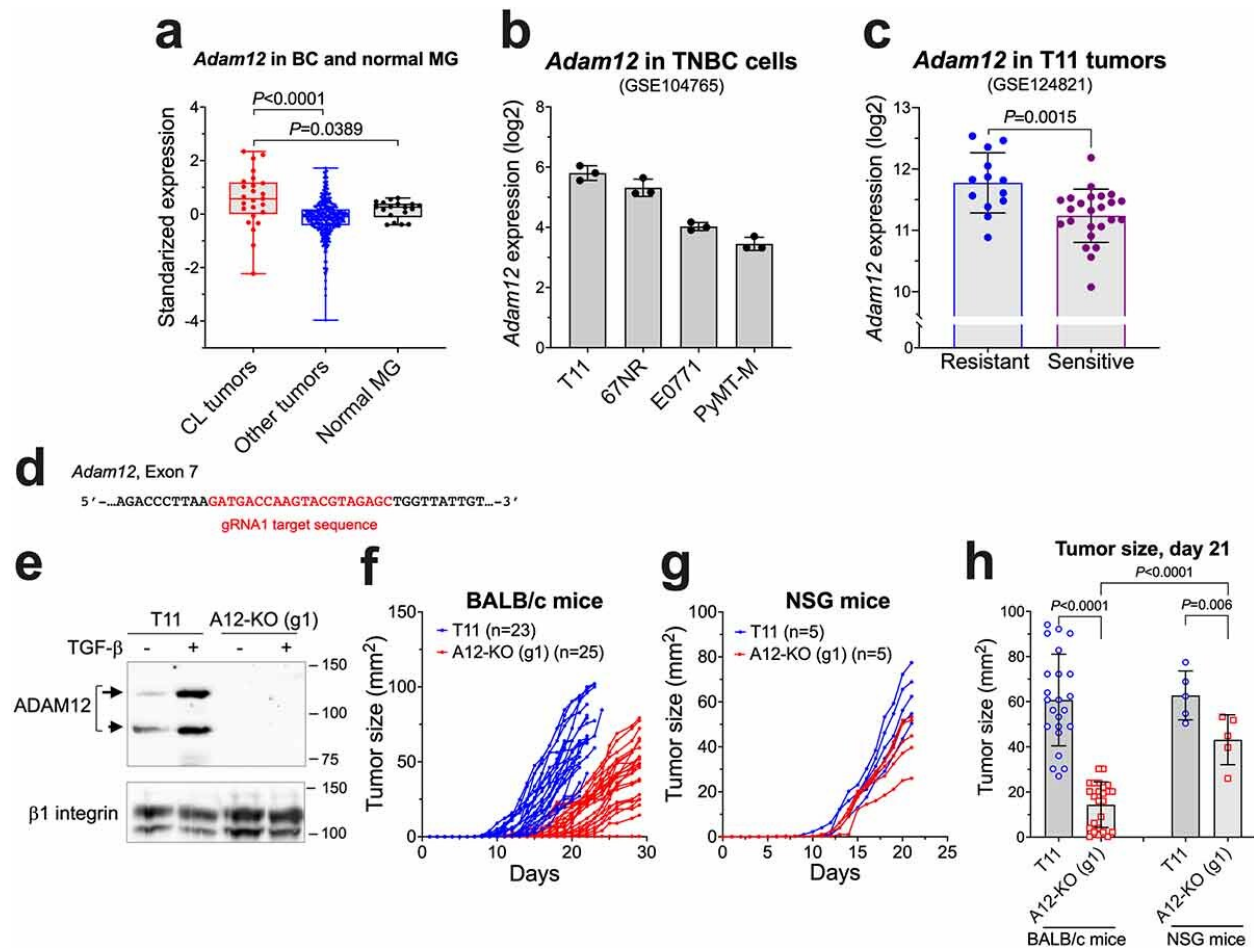


Figure 2.1 The effect of *Adam12* knockout on T11 tumor growth rates in mice.

(a) *Adam12* expression levels in murine claudin-low (CL) tumors, other tumor types, and normal mammary glands. The data were retrieved from GEO: GSE3165, GSE8516, GSE9343, GSE14457, GSE15263, GSE17916, GSE27101, and GSE42640; the list and classification of individual samples are based on ref. 37 and are included in Supplementary Table S4. The Mann-Whitney nonparametric test was used to calculate *P* values. (b) *Adam12* mRNA levels in four murine mesenchymal breast cancer cell lines, as reported in ref. 18. The data were retrieved from GEO:GSE104765. (c) Expression of *Adam12* in ICB-resistant control T11 breast tumors and in ICB-sensitive breast tumors derived from T11 cells with stable overexpression of Apobec3 or treated with UV light, as described in ref. 38. The data were retrieved from GEO:GSE124821.

(d) Position of the gRNA1 target sequence in the *Adam12* gene. (e) Detection of ADAM12 by Western blotting in control T11 cells and in T11 cells in which *Adam12* was knocked out using gRNA1 (A12-KO (g1)). To facilitate the detection of ADAM12, cells were treated for 2 days with 5 ng/ml TGF- β ; cell lysates were enriched for glycoproteins on concanavalin A agarose prior to the analysis. Nascent and mature forms of ADAM12 are indicated with long and short arrows, respectively; β 1 integrin is gel loading control. (f,g) A12-KO (g1) and control T11 tumor growth rates. 1×10^4 cells were injected into the 4th mammary inguinal mammary fat pad in BALB/c (f) or NSG mice (g). Tumor sizes (length x width) were measured daily with a caliper. In (f), data represent pooled values from five different experiments. (h) Comparison of tumor sizes 21 days after cell injection. Mean values $\pm$ S.D. are shown; *P* values were determined by unpaired two-tailed Student's *t* test.

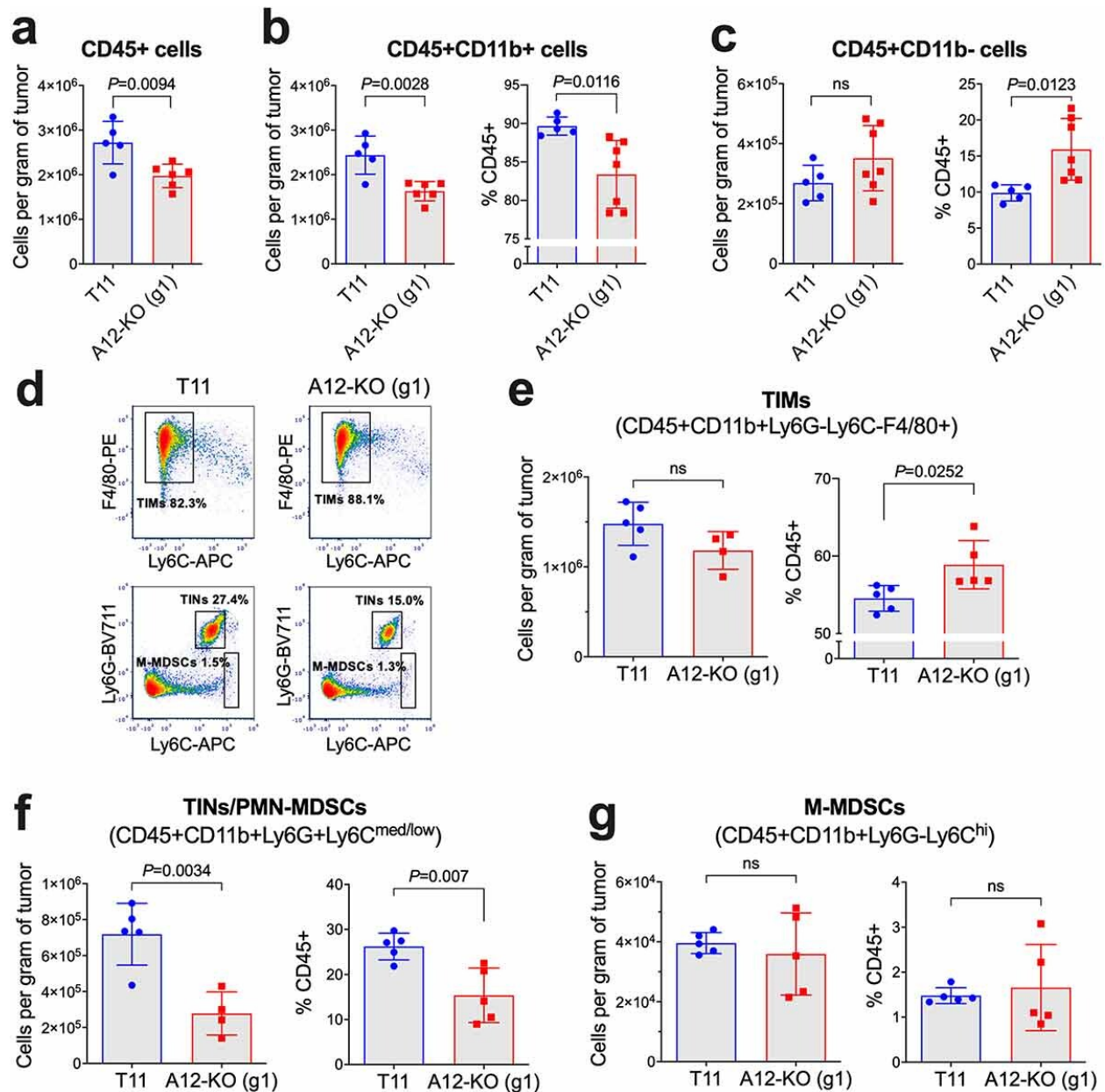


Figure 2.2 Quantification of tumor-infiltrating immunosuppressive myeloid cells in A12-KO (g1) and control T11 tumors from BALB/c mice by flow cytometry.

Absolute numbers of all tumor-infiltrating CD45+ immune cells (a), CD45+CD11b+ cells (b), and CD45+CD11b- cells (c) per gram of tumor tissue were determined using Precision Count beads. In (b, c), percentages of each population among CD45+ cells are also shown. (d)

Representative FACS analyses of tumor-infiltrating macrophages (TIMs), tumor-infiltrating

neutrophils (TINs)/polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), and monocytic myeloid-derived suppressor cells (M-MDSCs). Plots are gated on CD45⁺CD11b⁺Ly6G⁻ cells (top) and CD45⁺CD11b⁺ (bottom). (e-g) Absolute numbers and percentages among CD45⁺ cells are shown for TIMs (e), TINs/PMN-MDSCs (f), and M-MDSCs (g). Gating strategies are included in Supplementary Figure S1. The unpaired t-test was used to calculate P values; ns, non-significant, with $P > 0.05$.

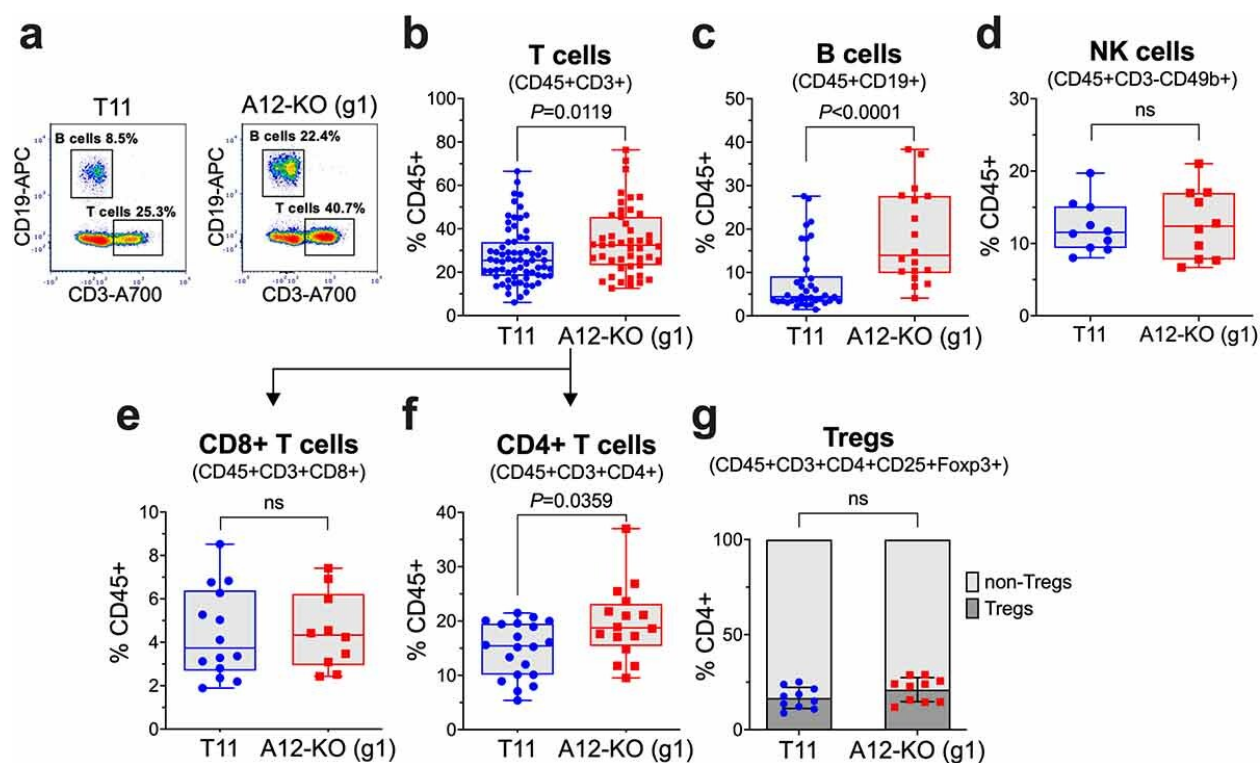


Figure 2.3 Quantification of tumor-infiltrating lymphocytes (TILs) in A12-KO (g1) and control T11 tumors from BALB/c mice by flow cytometry.

Frequencies of TILs were determined after cells enrichment by Percoll gradient centrifugation.

(a) Representative FACS analysis of tumor-infiltrating T cells and B cells. Plot is gated on CD45+ cells. Frequencies of T cells (b), B cells (c), NK cells (d), CD8+ T cells (e), and CD4+ T cells (f) among CD45+ cells and frequencies of regulatory T cells (Tregs) among CD4+ T cells (g) are shown. Gating strategies are shown in Supplementary Figures S2 and S3. Data were pooled from two (d,g), four (e,f), seven (c), or fifteen (b) different experiments, each experiment included 3-5 tumors per group. In (b-d, f), the data did not follow a Gaussian distribution and the Mann-Whitney nonparametric test was used to calculate P values; box and whiskers plots are shown for all data sets.

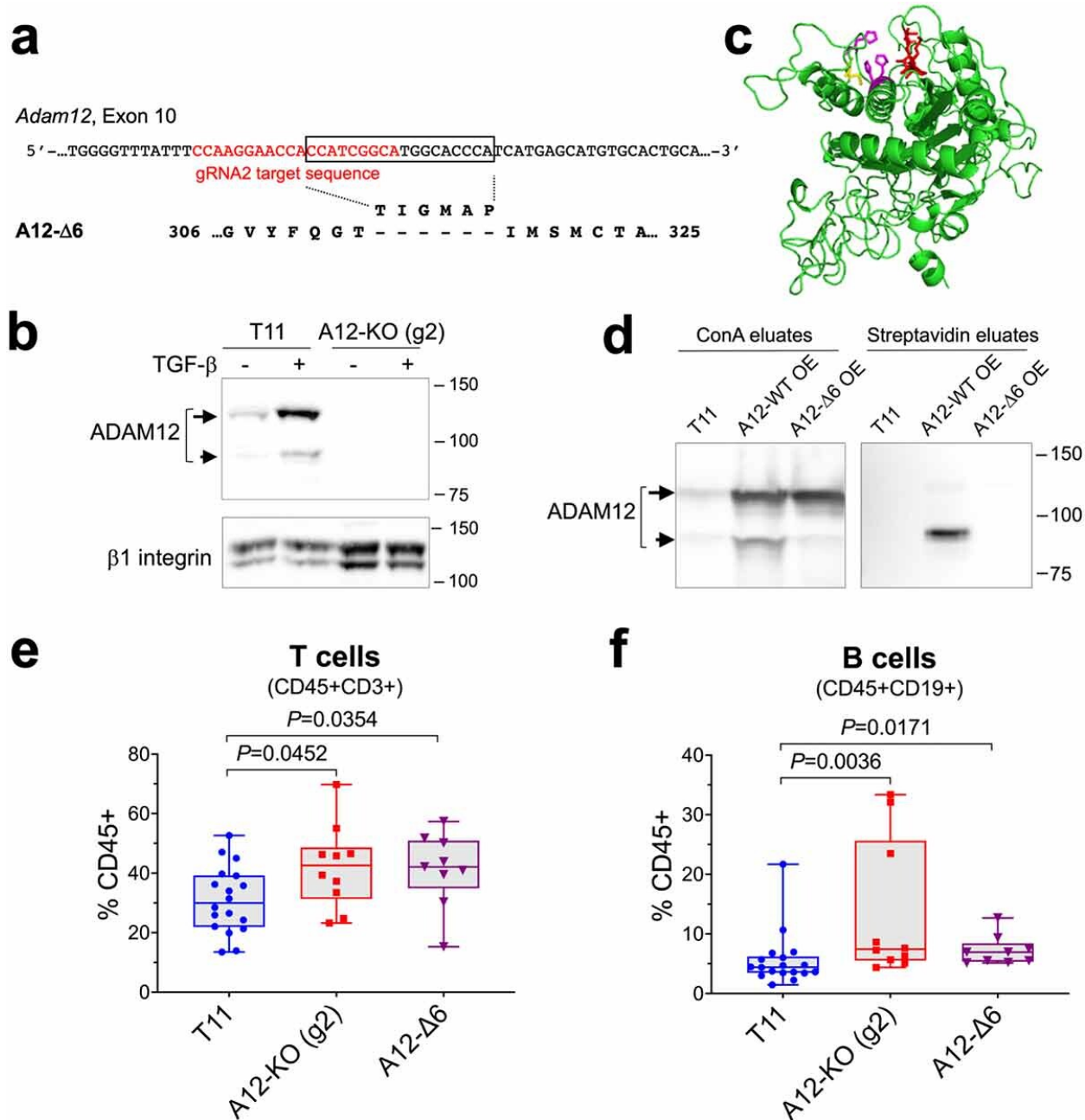


Figure 2.4 ADAM12 entrapment inside the cell exerts a similar effect on tumor-infiltrating T cells and B cells as *Adam12* gene knockout.

(a) Position of the gRNA2 target sequence in the *Adam12* gene is shown in red. A12-Δ6 is an ADAM12 variant created during CRISPR-Cas9 editing, this variant lacks the T-I-G-M-A-P sequence encoded by the 18-nt fragment in the *Adam12* gene (boxed). (b) Detection of

ADAM12 in control T11 cells and in T11 cells in which *Adam12* was knocked out using gRNA2 (A12-KO (g2)). To facilitate the detection of ADAM12, cells were treated for 2 days with 5 ng/ml TGF- β . Nascent and mature forms of ADAM12 are indicated with long and short arrows, respectively; β 1 integrin is gel loading control. (c) A model of the metalloprotease domain of ADAM12 showing the proximity of the TIGMAP peptide (red) and the catalytic residues Glu349 (yellow), His348, His352, and His358 (magenta). The model was generated using the I-TASSER protein structure prediction server at <https://zhanggroup.org/I-TASSER/>. (d) Cell surface biotinylation of wild-type ADAM12 (A12-WT) and A12- Δ 6 deletion mutant. A12-WT or A12- Δ 6 were stably overexpressed (OE) in T11 cells. Intact cells were treated with sulfo-NHS-LC-biotin, followed by incubation of cell lysates with concanavalin A agarose (left) or streptavidin agarose (right). The eluates were analyzed by Western blotting. (e, f) Quantification of tumor-infiltrating T cells (e) and B cells (f) in A12-KO (g2), A12- Δ 6, and control T11 tumors from BALB/c mice by flow cytometry. Data were pooled from two (A12-KO (g2) and A12- Δ 6) or four (T11) different experiments, each experiment included 3-5 tumors per group. The data did not follow a Gaussian distribution and the Mann-Whitney nonparametric test was used to calculate *P* values.

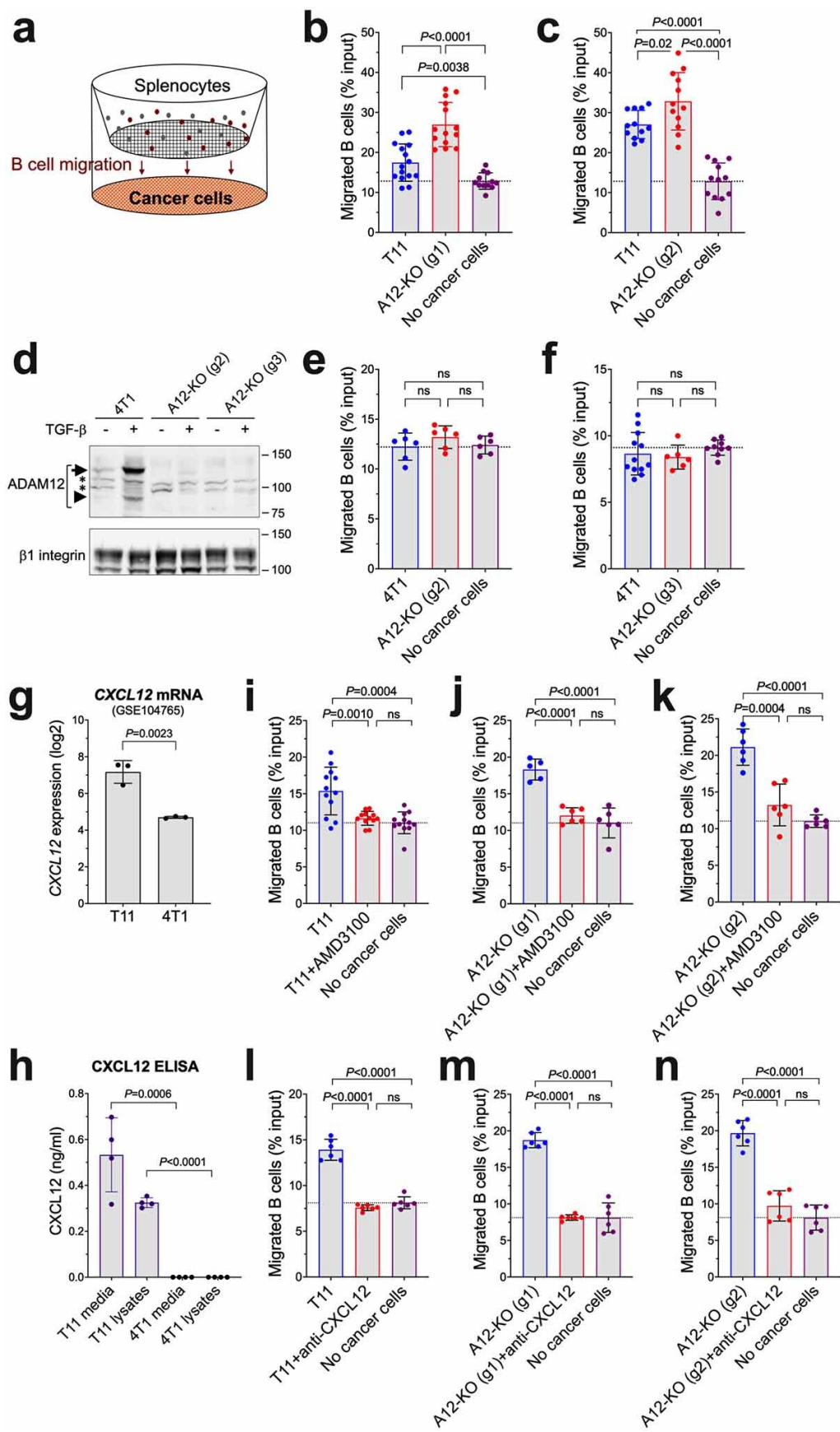


Figure 2.5 The effect of ADAM12 loss in cancer cells on chemotaxis of B cells *in vitro*.

(a) Schematic diagram of transwell B cell migration assays. B cell content among splenocytes seeded in upper chambers and B cells migrated after 4 hours into lower chambers toward cancer cells were measured by flow cytometry. (b, c) B cell migration toward wild-type T11 cells was compared to A12-KO (g1) T11 cells (b) or A12-KO (g2) cells (c). (d) Detection of ADAM12 by Western blotting in control 4T1 cells and in 4T1 cells in which *Adam12* was knocked out using gRNA2 or gRNA3. Glycoprotein enrichment on concanavalin A agarose was as in Figs. 1 and 4. Nascent and mature forms of ADAM12 are indicated with long and short arrows, respectively; non-specific bands are marked with asterisks, β 1 integrin is gel loading control. (e, f) B cell migration toward wild-type 4T1 cells was compared to A12-KO (g2) 4T1 cells (e) or A12-KO (g3) cells (f). (g) *Cxcl12* mRNA levels in T11 and 4T1 cell lines (ref. 18). The data were retrieved from GEO:GSE104765. (h) CXCL12 protein levels in conditioned media and in cell lysates from T11 and 4T1 cells were measured by ELISA. (i-k) Transwell B cell migration assays toward T11 cells (i), A12-KO (g1) (j), or A12-KO (g2) (k) were performed in the absence or presence of 10 μ g/ml AMD3100, an inhibitor of CXCR4, added to the upper chambers. (l-n) Transwell B cell migration assays toward T11 cells (l), A12-KO (g1) (m), or A12-KO (g2) (n) were performed in the absence or presence of 2.5 μ g/ml CXCL12-blocking antibody added to the lower chambers. For each panel, data were pooled from 2 to 5 independent experiments, each experiment included three different transwells per group. Gating strategies for B cell quantification are shown in Supplementary Figure S4.

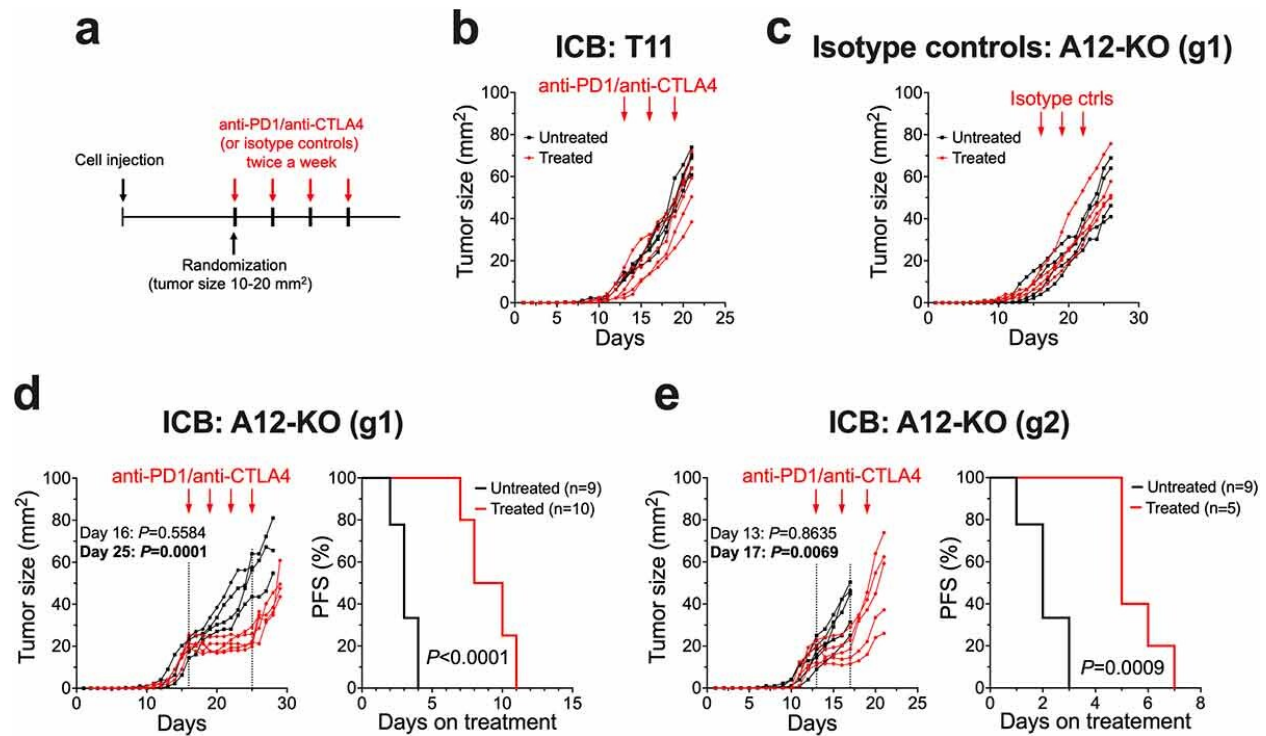
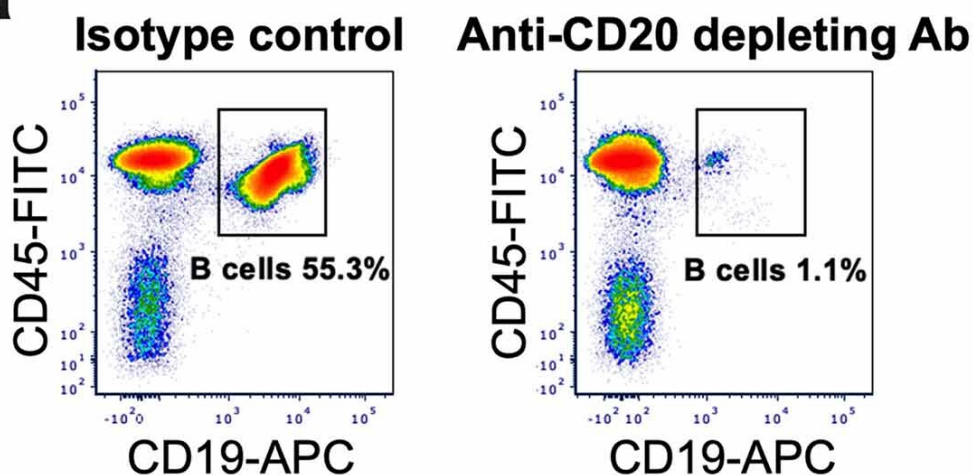
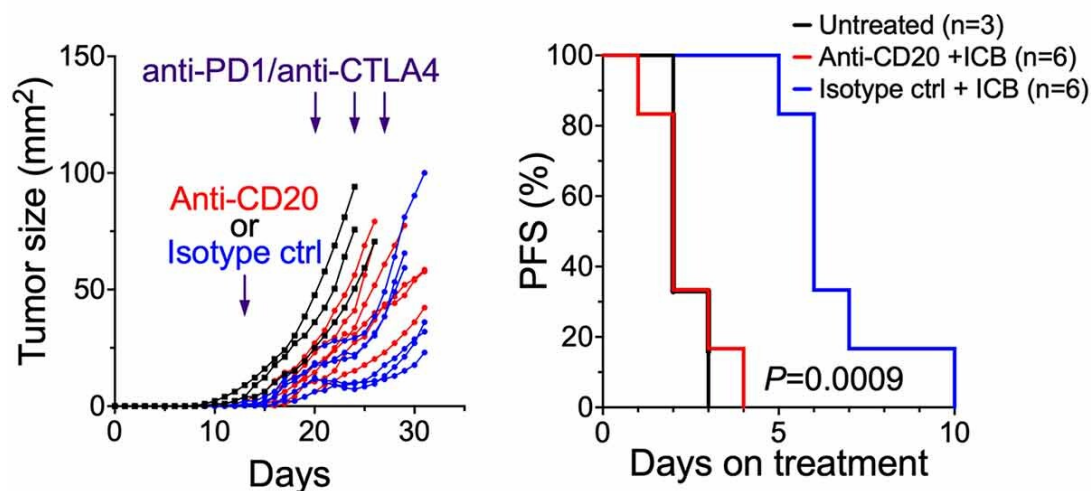


Figure 2.6 Abrogation of ADAM12 sensitizes T11 tumors to anti-PD1/anti-CTLA4 therapy.

(a) Diagram of the treatment. BALB/c mice were orthotopically injected with A12-KO or control T11 cells (2×10^4 cells/per injection). When tumor sizes reached ~ 10 - 20 mm², mice were randomized and injected intraperitoneally with anti-PD1/anti-CTLA4 antibodies or with isotype control antibodies bi-weekly or remained untreated. (b) The effect of anti-PD1/anti-CTLA4 antibodies on T11 tumor growth. (c) The effect of isotype control antibodies on the growth of A12-KO (g1) tumors. (d) Tumor growth rates and progression free survival of A12-KO (g1) tumors treated with anti-PD1/anti-CTLA4 antibodies *versus* untreated tumors. (e) Tumor growth rates and progression free survival of A12-KO (g2) tumors treated with anti-PD1/anti-CTLA4 antibodies *versus* untreated tumors. In d and e, progression was defined as a 20% increase in tumor diameter, or 1.44-fold increase in tumor area.

a**b**

B cell depletion + ICB: A12-KO (g1)

**c**

B cell depletion + ICB: A12-KO (g2)

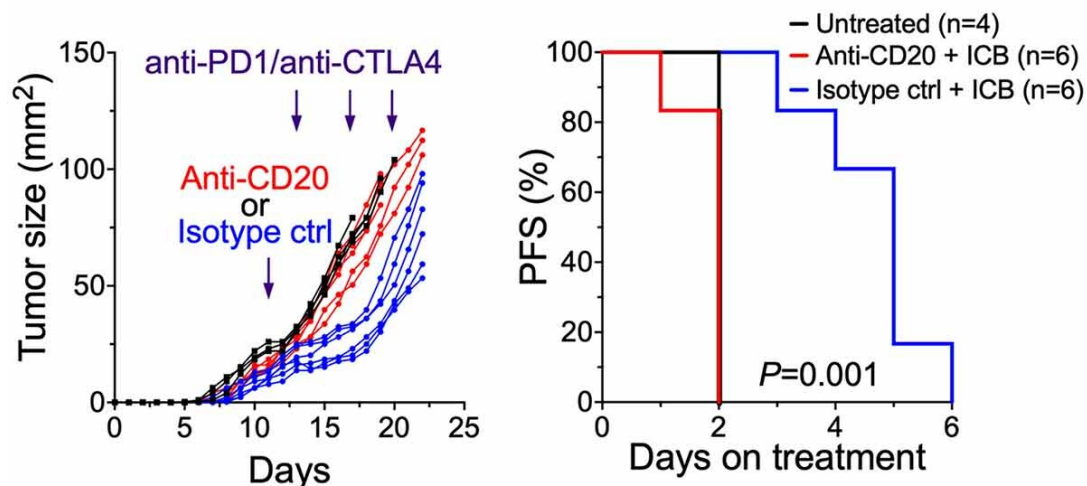


Figure 2.7 Depletion of B cells eliminates the improved response to anti-PD1/anti-CTLA4 therapy in A12-KO T11 tumors.

BALB/c mice were orthotopically injected with A12-KO T11 cells and then either treated with anti-PD1/anti-CTLA4 therapy, as in Fig. 6, or untreated. Three days prior to administration of anti-PD1/anti-CTLA4 treatment, mice received tail vein injections of anti-CD20 depleting or isotope control antibodies. **(a)** Representative FACS analysis of splenic B cells seven days after tail vein injections. **(b, c)** The effect of anti-PD1/anti-CTLA4 antibodies on tumor growth rates (left) and progression-free survival rates (right) in mice with A12-KO (g1) tumors **(b)** and A12-KO (g2) tumors **(c)**. Progression was defined as a 20% increase in tumor diameter, or 1.44-fold increase in tumor area.

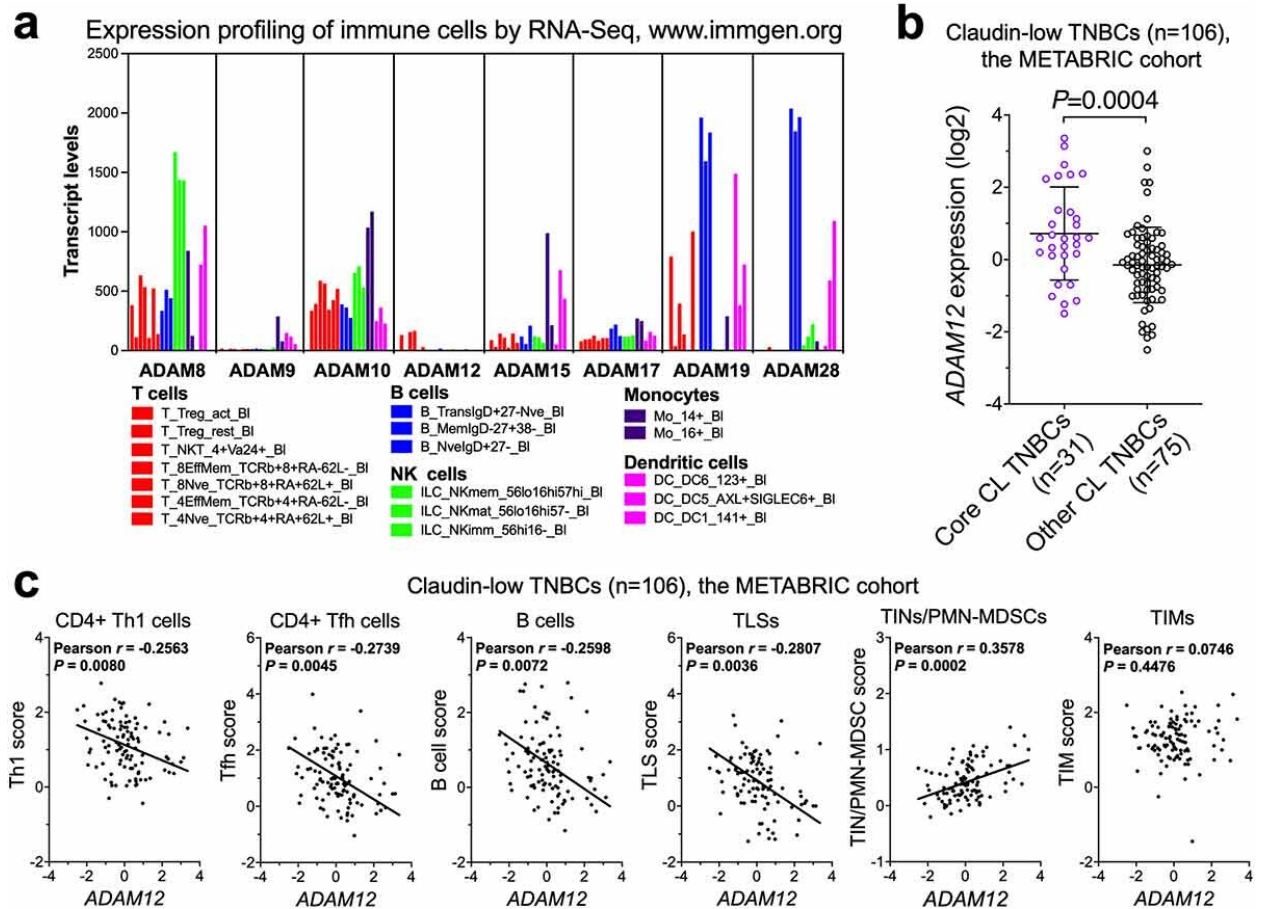


Figure 2.8 . Correlations between human *ADAM12* mRNA levels and immune gene expression signatures in claudin-low TNBCs.

(a) Population-averaged expression of selected *ADAMs* in sorted human immune cells. Sample names of individual cell populations are as described at <http://immunecellatlas.net/>. (b) *ADAM12* expression in core claudin-low (core CL, n=31) TNBC *versus* other types of CL TNBC (n=75). Gene expression data for the METABRIC patient cohort were retrieved from cBioPortal at <https://www.cbioportal.org>. The list of core claudin-low tumors was obtained from ref. 17. (c) Relationships between *ADAM12* mRNA levels and immune cell signature scores in claudin-low TNBC patients (n=106) from the METABRIC cohort. Each signature score was calculated as the average gene expression z-score for individual genes in a gene set (Supplementary Table S3).

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Chapter 3 - The effect of ADAM9 knockout on the tumor immune microenvironment in a mouse transplantation model of breast cancer

3.1 Introduction

Building upon the discussions in Chapter 1, Section 1.1.4 regarding the significant role of ADAM9 in the tumor microenvironment, and in Chapter 1, Section 1.1.3, which explores the innovative immunotherapy approach involving the application of immune checkpoint inhibitors in clinical trials and the associated challenges, the study outlined below aims to uncover the specific involvement of ADAM9 within the tumor microenvironment. This research seeks to provide novel insights that can potentially augment the efficacy of immune checkpoint blockade therapy in the context of breast cancer.

3.2 Materials and methods

Reagents and antibodies

Table 3.1 Reagents and antibodies

Reagent or Resource	Source	Identifier
Antibodies		
APC-conjugated anti-CD19 (clone 6D5)	BioLegend	Cat # 115512
APC-conjugated anti-Ly6C (clone HK1.4)	BioLegend	Cat # 128016
APC-conjugated rat IgG2a, κ isotype control (clone RTK 2758)	BioLegend	Cat # 400511
Brilliant Violet 785-conjugated anti-CD11b (clone M1/70)	BioLegend	Cat # 101243
Brilliant Violet 711-conjugated anti-Ly6G (clone 1A8)	BioLegend	Cat # 127643

FITC-conjugated anti-CD45 (clone 30-F11)	BioLegend	Cat # 103108
FITC-conjugated rat IgG2b,κ isotype control (clone RTK 4530)	BioLegend	Cat # 400605
PE-conjugated anti-Ly6C (clone 1A8)	BioLegend	Cat # 127607
PE-conjugated anti-F4/80 (clone BM8)	BioLegend	Cat # 123110
PE-conjugated rat IgG2a,κ isotype control (clone RTK 2758)	BioLegend	Cat # 400507
PE/Cyanine7-conjugated rat IgG2a,κ isotype control (clone RTK 2758)	BioLegend	Cat # 400521
TruStain FcX Plus anti-CD16/32 (clone S17011E)	BioLegend	Cat # 156604
InVivoMAb anti-mouse CTLA-4 (CD152) (clone 9D9)	BioXcell	Cat # BE0164
InVivoMAb anti-mouse PD-1 (CD279) (clone RMP1-14)	BioXcell	Cat # BE0146
InVivoMAb mouse IgG2b isotype control (clone MPC-11)	BioXcell	Cat # BE0086
InVivoMAb rat IgG2a isotype control (clone 2A3)	BioXcell	Cat # BE0089
Anti-ADAM9 Rabbit mAb (clone D64B5)	Cell Signaling	Cat # 4151
Anti-GAPDH rabbit mAb (clone D16H11)	Cell Signaling	Cat # 5174
Reagents		
ACK Lysing Buffer	Gibco	Cat # A1049201
Alt-R® S.p.HiFi Cas9 Nuclease V3	IDT	Cat # 1081060
Alt-R® Cas9 Electroporation Enhancer	IDT	Cat # 1075915
Bovine Serum Albumin	Amresco	Cat # 9048-46-8
Collagenase type IV	Sigma	Cat # C5138
DNase type IV	Sigma	Cat # D5025

Fetal Bovine Serum	Corning	Cat # MT35015CV
Hyaluronidase type IV-S	Sigma	Cat # H3884
Neon™ Transfection System Kit	Invitrogen	Cat # MPK1096
Precision Count Beads	BioLegend	Cat # 424902
Propidium Iodine Staining Solution	Sigma	Cat # P4864

Cell culture, immunoblotting, orthotopic T11 cell transplantation, isolation of tumor-infiltrating immune cells, flow cytometry, immune checkpoint blockade

These methods were used as described in Chapter 2.3.

3.3 Results

Previous studies have reported that ADAM9 is upregulated in breast cancer cells compared to normal mammary gland epithelial cells [1]. Therefore, we explored the relationship between ADAM9 and changes in the breast tumor immune microenvironment. Moreover, the effect of ADAM9 on response to ICB was analyzed. We first generated *Adam9* gene knockout in T11 cells using the CRISPR/Cas9 gene editing technique. Several single cell clones were selected and expanded. To validate the loss of ADAM9 expression, cell lysates were subjected to immunoblotting analysis. The blot results demonstrated a complete ADAM9 knockout in several single cell clones (Figure 3.1). At the end, two ADAM9 KO clones (g1-7 and g1-9) were selected and combined for further experiments.

Next, we investigated the impact of ADAM9 knockout in T11 tumor cells on the recruitment of immune cells to the tumor microenvironment. In short, mice received an orthotopic injection of 1×10^4 T11 (wildtype or ADAM9 KO) tumor cells. Upon attaining

comparable sizes, tumors were excised, enzymatically digested, and analyzed by flow cytometry to assess the absolute number of leukocytes. Using CD45 as a cell surface marker for leukocytes, we observed a slight but not significant increase in the absolute numbers of leukocytes per gram of tumor tissue in ADAM9 KO tumors compared to T11 control tumors (Figure 3.2A).

Furthermore, we investigated the subpopulation of myeloid CD11b⁺ cells. A small but not significant increase was observed, from $\sim 5 \times 10^6$ to $\sim 6 \times 10^6$ cells/g of tumor tissue (Figure 3.2B).

In this study, we also measured the populations of TIMs (Tumor-Infiltrating Macrophages) and TINs (Tumor-Infiltrating Neutrophils), or PMN-MDSCs (Polymorphonuclear Myeloid-Derived Suppressor Cells), characterized as CD45⁺CD11b⁺Ly6G⁺Ly6C⁺F4/80⁺ and CD45⁺CD11b⁺Ly6G⁺Ly6C^{med/low}, respectively. While the absolute counts of TIMs showed no significant difference between T11 and A9-KO (g1) tumors, there was a noteworthy drop in TIM frequency among all CD45⁺ cells. Specifically, the TIM frequency decreased from approximately 60% in T11 tumors to 40% in A9-KO (g1) tumors, and this reduction was deemed statistically significant (Figure 3.3A). Remarkably, there was an elevation in the absolute count of TINs/PMN-MDSCs, increasing from 7.9×10^5 cells per gram in T11 tumors to 2.7×10^6 cells per gram in A9-KO (g1) tumors. This shift also translated to an increase in the relative TIN numbers among CD45⁺ cells from 20% to 48% in the respective tumor samples (Figure 3.3B).

Next, we examined tumor-infiltrating lymphocytes (TILs), focusing specifically on B cells within both control and A9-KO tumors. A9-KO (g1) tumors exhibited a higher absolute count of CD45⁺CD19⁺ B cells compared to T11 tumors, with counts of 1.08×10^5 and 4.6×10^4 cells/g of tumor tissue, respectively (Figure 3.4A). Our data revealed a significant elevation in the frequency of B cells among all CD45⁺ leukocytes in A9-KO (g1) tumors compared to T11 control tumors, with percentages of 1.56% and 0.8%, respectively (Figure 3.4B). Considering

that the majority of TILs have the CD45⁺CD11b⁻ phenotype, and the CD45⁺CD11b⁻ represents only approximately 10% of all immune cells infiltrating T11 tumors, we next quantified the frequencies of B cells just among the CD45⁺CD11b⁻ cell subset. The analysis revealed a significant increase in B cells, from ~18% in T11 control tumors to ~27% in A9-KO (g1) tumors (Figure 3.4C).

Finally, we investigated whether the changes observed in tumor-infiltrating immune cells in A9-KO (g1) tumors correlated with enhanced responses to immune checkpoint blockade (ICB) therapy. Mice bearing A9-KO (g1) or control T11 tumors underwent treatment with anti-PD1/anti-CTLA4 antibodies. We have already shown the data that T11 control tumors exhibited resistance to ICB therapy (See Chapter 2). Administering equivalent doses of isotype-matched control antibodies had no impact on the growth of A9-KO (g1) tumors (Figure 3.5A). Surprisingly, the growth rate of A9-KO (g1) tumors did not decelerate despite treatment with anti-PD1/anti-CTLA4 antibodies (Figure 3.5A). The median progression-free survival (PFS) data similarly indicated that there was no significant extension of survival observed in mice bearing A9-KO (g1) tumors following ICB treatment (Figure 3.5B). Progression was characterized by either a 20% rise in tumor diameter or a 1.44-fold increase in tumor area.

Collectively, our results highlight the involvement of ADAM9 in reshaping the composition of tumor-infiltrating immune cells, particularly in the accumulation of TINs/PMN-MDSCs and B cells within the tumor immune microenvironment. Despite these alterations, it is noteworthy that these changes did not contribute to an enhancement in the efficacy of ICB therapy.

3.4 Discussion

Our results demonstrate that deletion of ADAM9 in T11 breast cancer cells has a clear influence on the composition of tumor-infiltrating immune cells. Specifically, there was a significant increase in the frequency of B cells in ADAM9-deficient tumors in comparison to control T11 tumors. Nevertheless, ADAM9-KO tumors exhibited distinct patterns in the alterations of TIMs and TINs/PMN-MDSCs compared to ADAM12-KO tumors. ADAM12-KO tumors displayed an increased frequency of TIMs and a decreased frequency of TINs/PMN-MDSCs in comparison to control T11 tumors. In contrast, ADAM9-KO tumors contained less TIMs and more TINs/PMN-MDSCs than control tumors. Additionally, there was no improvement in the response to the ICB therapy in ADAM9-KO tumors. The reason why ADAM12-KO tumors became responsive to ICB while ADAM9-KO tumors were resistant is not clear.

One explanation of the different sensitivity of ADAM12-KO and ADAM9-KO T11 tumors to ICB may be the broader expression of ADAM9 than ADAM12. Although both ADAM12 and ADAM9 expression is upregulated in triple negative breast cancer, ADAM9 has been reported to be upregulated in various cell types within the tumor microenvironment (see chapter 1.4). Specifically, ADAM9 is expressed in monocytes, macrophages, neutrophils, keratinocytes, and fibroblasts [2, 3, 4, 5, 6]. Furthermore, ADAM9 is poorly detected in unstimulated neutrophils, but its level rapidly increases upon neutrophil activation with degranulating agonists [7]. Moreover, the disintegrin domain of ADAM9 has the capability to interact with integrins, leading to the activation of neutrophils and their chemotaxis, generation of reactive oxygen species, formation of neutrophil extracellular traps, and a delay in apoptosis [8]. It has been reported that neutrophils in the tumor microenvironment exhibit

immunosuppressive function through T-cell suppression and innate immune regulation [9].

These findings indicate that neutrophil activation mediated by ADAM9, whether originating from cancer cells or neutrophils themselves, may play a role in tumor progression. Taken together, our data suggest that the absence of ADAM9 expression in cancer cells is insufficient to inhibit the activation and recruitment of neutrophils within the tumor microenvironment.

Therefore, a comprehensive analysis involving additional in vitro studies is imperative to elucidate the multifaceted role of ADAM9 within the tumor immune microenvironment. The focus of future studies should extend beyond the impact of ADAM9 on cancer cells, and it should encompass ADAM9 expression and function in tumor-infiltrating immune cells as well. Such studies will not only enhance our understanding of the role of ADAM9 in cancer but will also provide a rationale for the selection of ADAM12, and not ADAM9, as a potential target to improve the efficacy of the ICB therapy.

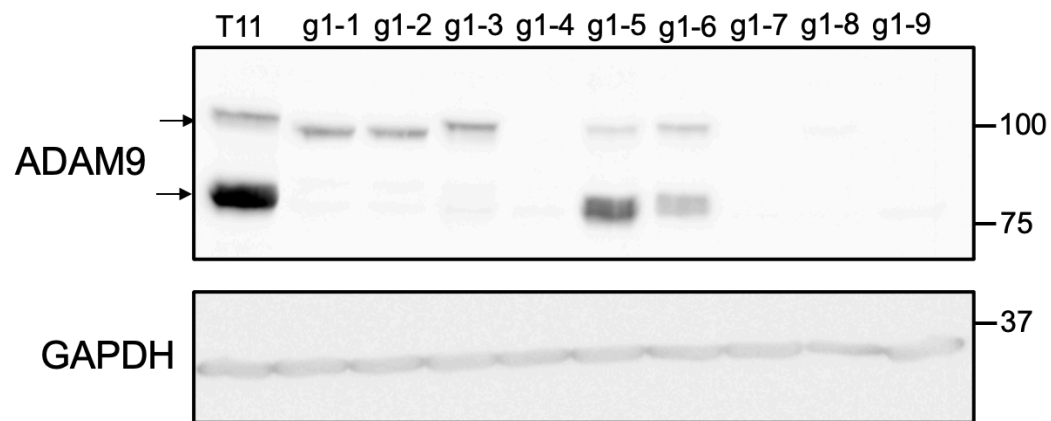


Figure 3.0.1 Detection of ADAM9 in T11 cells by Western blotting.

Adam9 was knocked out using gRNA1 (referred to as A9-KO (g1)). Isolation of single-cell clones (g1-1 to g1-9) from T11 cells was performed using glass cloning cylinders. Total cell lysates were analyzed by immunoblotting using anti-ADAM9 antibody. Top and bottom arrows are used to denote the nascent and the mature forms of ADAM9. GAPDH is a gel-loading control.

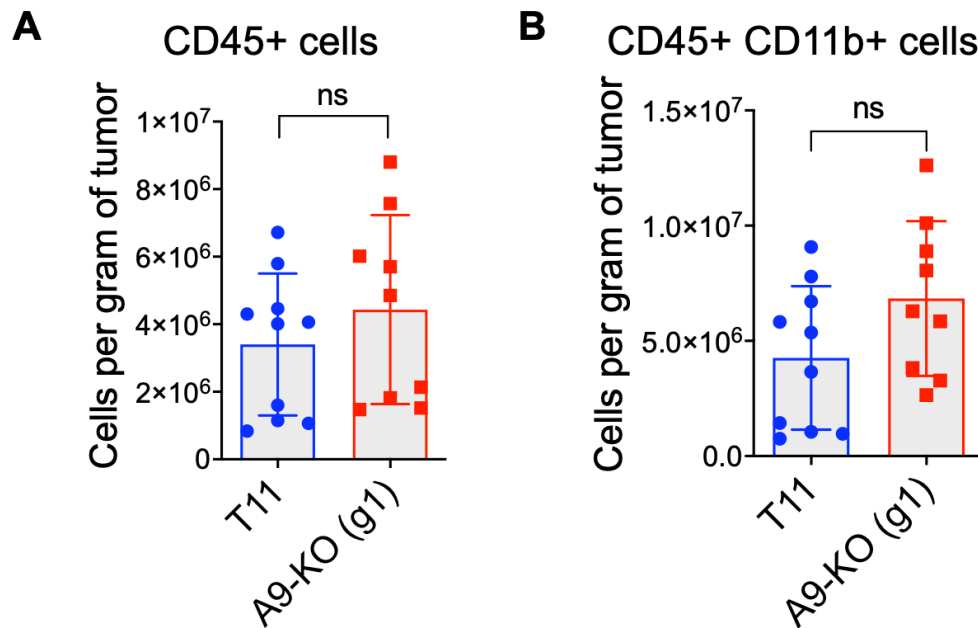


Figure 3.0.2 Quantification of tumor infiltrating CD45+ (A) and CD45+CD11b+ cells (B) in ADAM9-KO (g1) and control T11 tumors.

T11 control or ADAM9-KO cells were orthotopically injected into Balb/cJ mice (T11 n=10, A9-KO n=9). Tumors were harvested and tumor-infiltrating immune cells were isolated, which was followed by staining with anti-CD45+ and anti-CD11b antibodies and subsequent analysis using flow cytometry. Mean values $\pm$ standard deviation are shown, each data point corresponds to an individual tumor from different mice. Statistical significance was assessed using unpaired t-test, where a p-value < 0.05 was considered significant, ns, non-significant.

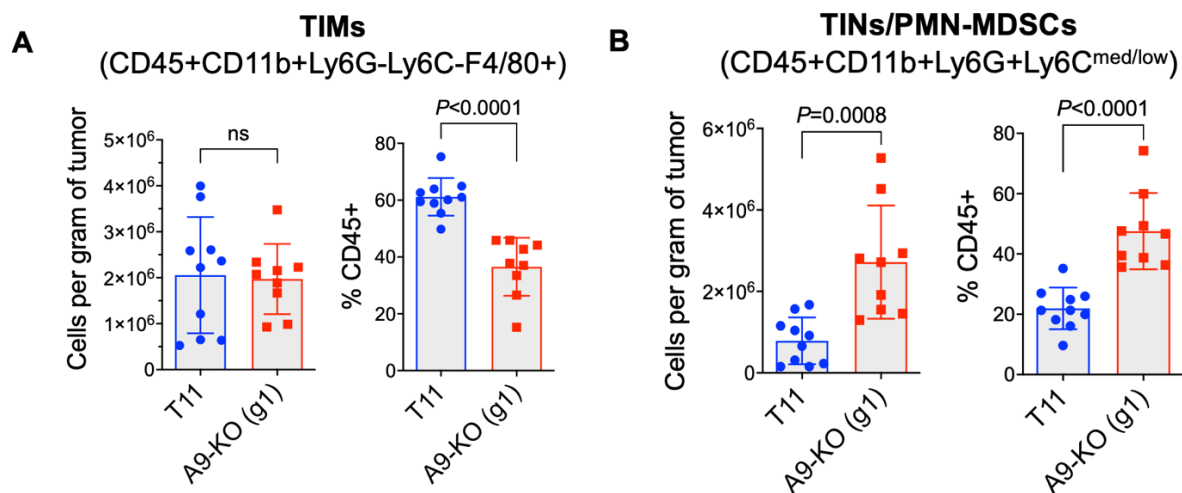


Figure 3.0.3 Quantification of tumor infiltrating immunosuppressive myeloid cells in ADAM9-KO (g1) and control T11 tumors.

T11 control or ADAM9-KO cells were orthotopically injected into Balb/cJ mice (T11 n=10, A9-KO n=9). Tumor-infiltrating immune cells were isolated, labeled with antibodies against the indicated cell surface markers, and analyzed by flow cytometry. Absolute numbers and relative frequencies among CD45+ cells are shown for TIMs (A) and TINs/PMN-MDSCs (B). Statistical significance was assessed using unpaired t-test, where a p-value < 0.05 was considered significant, ns, non-significant.

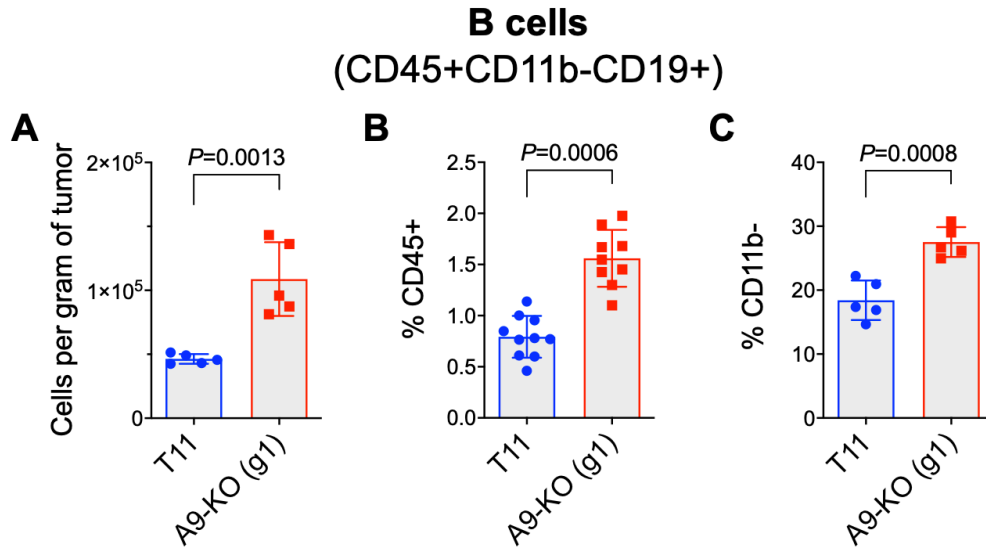


Figure 3.0.4 Quantification of tumor infiltrating lymphocytes (TILs) in ADAM9-KO (g1) and control T11 tumors.

T11 control or ADAM9-KO cells were orthotopically injected into Balb/cJ mice. Tumor-infiltrating immune cells were isolated, labeled with antibodies against the indicated cell surface markers, and analyzed by flow cytometry. (A) Absolute numbers of CD11b-CD19+ B cells (T11 n=5, A9-KO n=5). (B) Percentage of CD11b-CD19+ B cells among CD45+ cells (T11 n=10, A9-KO n=9). (C) Percentage of CD19+ B cells among CD11b- cells (T11 n=10, A9-KO n=9). Statistical significance was assessed using unpaired t-test, where a p-value < 0.05 was considered significant.

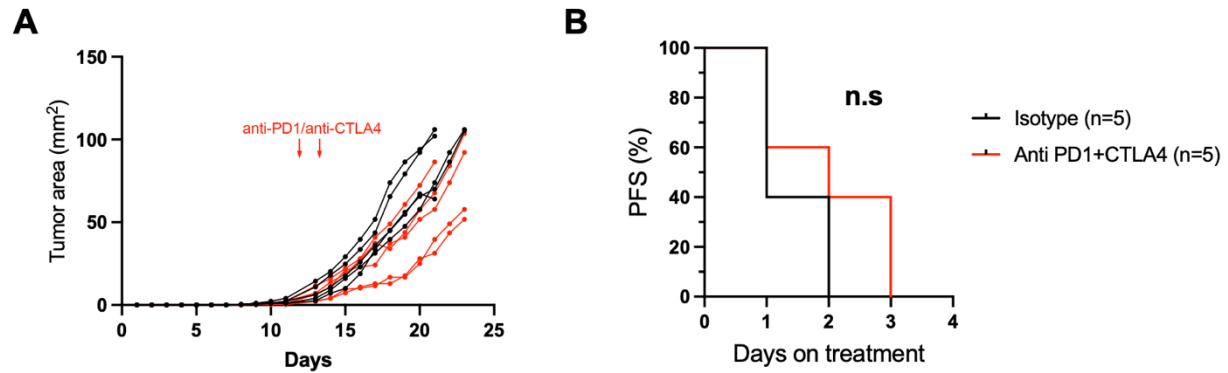


Figure 3.0.5 The effect of anti-PD1/anti-CTLA4 therapy on ADAM9-KO T11 tumors.

ADAM9-KO T11 cells were orthotopically injected into Balb/cJ mice (2×10^4 cells). Upon reaching tumor sizes of approximately 10-20 mm², mice were subjected to bi-weekly intraperitoneal injections of either anti-PD1/anti-CTLA4 antibodies or isotype control antibodies.

(A) Comparison of ADAM9-KO tumor growth rates treated with anti-PD1/anti-CTLA4 antibodies or isotype control antibodies. (B) Progression free survival of ADAM9-KO tumors treated with anti-PD1/anti-CTLA4 antibodies versus isotype control antibodies. Progression was characterized by either a 20% rise in tumor diameter or a 1.44-fold increase in tumor area.

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Chapter 4 - Mechanistic links between the CLPB gene and congenital neutropenia

3.1 Abstract

The human CLPB protein is a widely expressed homolog of microbial caseinolytic peptidase B, which belongs to the ATPases associated with diverse cellular activities (AAA+) family. CLPB resides in the mitochondrial intermembrane space (IMS), but its physiological function is unknown. Mutations resulting in the loss of function of CLPB are linked to several human diseases characterized by neutropenia and neurological disorders. Moreover, loss of CLPB or expression of disease-associated variants of CLPB lead to impaired granulocytic differentiation of human hematopoietic progenitor cells, as well as elevated levels of apoptosis. Thus, the aim of my work was to investigate the role of CLPB during neutrophil differentiation and to verify how CLPB affects the function of mitochondria. We generated CLPB knockout in human and mouse promyelocytic cell lines and evaluated cell differentiation using flow cytometry analysis. We also monitored alterations in the mitochondrial membrane potential (MMP) by staining cells with the MMP-sensitive dye JC-1. Finally, we investigated the consequences of CLPB loss on the expression of OPA1, a cristae-shaping mitochondrial protein. Here, we found a notable reduction in MMP of cells with CLPB knockout. Moreover, we observed that the viability of CLPB-deficient cells was reduced during differentiation. Taken together, our results indicate the importance of CLPB in the physiology of promyelocytic cells.

3.2 Introduction

Neutropenia involves a reduction in the absolute neutrophil count (ANC) in peripheral blood and it can be classified into two categories: congenital and acquired [1]. Severe congenital neutropenia (SCN) is characterized by an extremely low count of circulating neutrophils ($<500/\mu\text{L}$) [1]. A rare set of genetically diverse congenital disorders arrest the differentiation of neutrophilic granulocytes at the promyelocyte or myelocyte stage in the bone marrow [2]. Patients with SCN, especially infants, often experience frequent bacterial infections and carry the risk of developing myeloid malignancy [2]. The primary treatment for patients with SCN is treatment with granulocyte colony stimulating factor (G-CSF), which can stimulate the production of neutrophils [3]. Unfortunately, such treatment cannot completely cure the SCN patients, and the administration of high-doses of G-CSF could also potentially facilitate leukemogenesis [1]. To date, 25 genes associated with congenital neutropenia has been identified. Within these genes, mutations in the *ELANE* gene are the most frequent cause of SCN, accounting for up to 45% of newly diagnosed cases [4]. As the list of genes implicated in SCN continues to expand, it is important to categorize SCN not simply based on the mode of inheritance and clinical features but also by the nature of cellular defects induced by the mutations [4]. Recently, pathogenic heterozygous mutations in the *CLPB* gene have been reported in association with SCN. These newly recognized heterozygous variants in the CLPB protein underscores the notion that CLPB variants represent a common cause of SCN [5].

Mammalian CLPB (caseinolytic peptidase B), also known as SKD3, belongs to the AAA+ ATPase protein family [6]. While mammalian CLPB is considered a homolog of microbial CLPB, there are some important differences between the two proteins. The N-terminus of mammalian CLPB contains a mitochondrial targeting signal (MTS), followed by an ankyrin

repeat (ANK) domain, and concludes with a single C-terminal nucleotide-binding domain (NBD) [7]. Microbial ClpB does not contain an ANK domain and has two NBD domains. Biallelic mutations in the ANK and NBD domains of CLPB are associated with severe mitochondrial disorder known as 3-methylglutaconic aciduria, type VII (MGCA7) [8]. MGCA7 manifests as an autosomal recessive metabolic disorder characterized by high levels of 3-methylglutaconic acid (3-MGA), with clinical features including neurological deterioration and neutropenia [8].

Bacterial *ClpB* has been demonstrated to facilitate protein unfolding and disaggregation, a process dependent on ATP hydrolysis. That in turn enables proper refolding of substrate proteins. Due to the resemblance in the AAA domains of human CLPB and microbial ClpB, it has been further postulated that human CLPB could also function as an ATPase and molecular chaperone/disaggregase [9]. Recently, it has been shown that human CLPB is located in the mitochondrial intermembrane space (IMS) and its loss leads to mitochondrial dysfunction [10]. Moreover, the significance of functional mitochondria in preserving the function of hematopoietic stem cells and cellular differentiation has been suggested [11]. However, although recent studies showed that the expression of dominant-negative variants of CLPB impaired granulocytic differentiation of human hematopoietic progenitor cells [5], the exact function of CLPB during neutrophil differentiation is still not clear.

The goal of this study was to investigate the role of CLPB in neutrophil differentiation and in the maintenance of mitochondrial functions. By measuring the mitochondrial membrane potential, we were able to show that CLPB knockout leads to a partial depolarization of the mitochondria. We demonstrated that downregulation of CLPB affected OPA1, the regulator of cristae morphology. Moreover, we generated cells with exogenous overexpression of dominant-

negative variants of CLPB or CLPB knockout in both human HL60 and mouse 32D cells to investigate the role of CLPB in neutrophil differentiation.

3.3 Materials and Methods

Reagents and antibodies

Neon™ transfection system 100 µL kit and MitoProbe™ JC-1 assay kit (#M34152) were from ThermoFisher Scientific (Waltham, MA). Anti-CLPB rabbit pAb (#HPA039006) and propidium iodine staining solution were from MilliporeSigma (Burlington, MA). Alt-R® S.p.HiFi Cas9 Nuclease V3 and Alt-R® Cas9 electroporation enhancer were from Integrated DNA Technologies (IDT) (Coralville, IA). Fetal bovine serum, heat-inactivated fetal bovine serum, and dimethyl sulfoxide (DMSO) were from Corning (Corning, NY). Interleukin 3 (IL-3), granulocyte-colony stimulating factor (G-CSF), PE-Cyanine7-conjugated anti-mouse/human CD11b antibody (clone M1/70, #101216), FITC-conjugated anti-human FPR1 antibody (clone W15086B, #391603), Zombie Red™ fixable viability kit (#423109), TruStain FcX Plus anti-CD16/32 (clone S17011E, #156604), and human TruStain FcX™ (#422302) were from BioLegend (San Diego, California). Anti-Myc mouse mAb (clone 9B11, #2276), anti-OPA1 rabbit mAb (clone D6U6N, #80471), anti-GAPDH rabbit mAb (clone D16H11, #5174) were from Cell Signaling Technology (Danvers, MA).

Cell culture and differentiation

The human HL60 cell line and the mouse 32D cell line were obtained from American Tissue Culture Collection (ATCC). Cells were cultured in IMDM (HL60 cells) supplemented with 20% fetal bovine serum (FBS) or RPMI-1640 medium (32D cells) supplemented with 10%

heat-inactivated fetal bovine serum (FBS) and 10 ng/ml mouse interleukin-3 (IL-3) at 37°C in a humidified incubator containing 5% CO₂. HL60 cells were induced to differentiate by 10 µM all-trans retinoic acid (ATRA) or 1.25% dimethyl sulfoxide (DMSO). After removing IL-3, granulocyte colony-stimulating factor (G-CSF) (25 ng/ml) was used to induce 32D cells to differentiate.

Generation of human CLPB with SCN-linked mutations

Mutations associated with the severe congenital neutropenia (SCN) disease were introduced into CLPB-Myc in the PIGneo vector (Addgene; #52116) using the Quickchange Lightning Site-Directed mutagenesis kit (Agilent Technologies; #210518). The corresponding mutagenesis primers are provided below:

For the N466K mutation:

FP: 5' CTTCATCATGACCTCCAAAGTGGCCAGCGACGAGATCG 3';

RP: 5' CGATCTCGTCGCTGGCCACTTTGGAGGTCATGATGAAG 3'

For the R531G mutation:

FP: 5' GGGATGAGTTTCTGGGAGGGATCAATGAGATCGTC 3';

RP: 5' GACGATCTCATTGATCCCTCCCAGAACTCATCCC 3'

Following PCR, the products were treated with 2 µl Dpn1 enzyme at 37°C for 2 hours.

Subsequently, 2 µl of the enzyme-treated PCR product was used for the transformation of NEB 5α competent *E. coli* cells (New England Biolabs; #C2987H). Transformants underwent selection on lysogeny broth (LB) agar plates supplemented with 100 µg/mL ampicillin. Single colonies were chosen from the plates and cultured in LB media, supplemented with 100 µg/mL ampicillin, at 37°C overnight. Plasmids were purified using the GeneJET Plasmid Miniprep kit

(ThermoFisher Scientific; #K0502) and subsequently submitted for sequencing (AZENTA). To transfect HL60 cells, plasmids were purified using the PureLink™ Expi Endotoxin-Free Maxi plasmid purification kit (ThermoFisher Scientific; #A31231).

Generation of CLPB knockout in HL60 and 32D cells

To generate the CLPB knockout in HL60 and 32D cells, first a mixture of recombinant *S. pyogenes* HiFi Cas9 Nuclease V3 and sgRNAs was used to form ribonuclear protein complexes. Next, the complexes were nucleofected into HL60 or 32D cells using the Neon electroporation system (Invitrogen). For *CLPB* knockout in HL60 cells, two different sgRNAs were used, *CLPB*-targeting sequences were 5'-CGACTAAAGGAGCACATCAT-3' (g3) and 5'-TTGGATTATGCCCCGAGAAGG-3' (g4). For *Clpb* knockout in 32D cells, we also used two different sgRNAs, 5'-AGGGTTAATCAAATCTCCAT-3' (g1) and 5'-GTCCCCAACAAGACGGGTCT-3' (g2). After 48 hours, cell lysates were collected for immunoblotting analysis.

Transient overexpression of human CLPB in HL60 cells

The PIGneo bicitronic vector encoding GFP and either wildtype CLPB, the N466K mutant, or the R531G mutant (30 µg of plasmid DNA, 2x10⁶ of cells in 100 uL buffer) was nucleofected into HL60 cells using the Neon electroporation system. Cells were seeded into a 6-well plate. Cell lysates were collected for immunoblotting 48 hours after electroporation.

Immunoblotting

Cells were washed twice with DPBS and then treated with lysis buffer (50 mM

Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM AEBSF, 5 µg/mL pepstatin, 5 µg/mL leupeptin, 5 µg/mL aprotinin, and 10 mM 1,10-phenanthroline). Cell lysates were centrifuged for 15 minutes at 17,000xg, supernatants were supplemented with 3xSDS gel-loading buffer. Proteins were resolved through SDS-PAGE and then transferred to a nitrocellulose membrane. After a blocking step with 5% milk and 0.3% Tween-20 in DPBS, the membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated IgG secondary antibodies for 1 hour, and signal detection using the SuperSignal West Pico PLUS chemiluminescence detection kit (ThermoFisher) and the Azure c500 digital imaging system.

Flow cytometry

Cells resuspended in DPBS were stained with the viability dye Zombie Red for 30 min at room temperature. Cells were then washed with the FACS buffer (DPBS containing 3% BSA) and blocked with 2.5 µg/ml TruStain FcX Plus (32D cells) or human TruStain FcX™ (HL60 cells, 5µl/million cells) for 10 minutes on ice. Cells were subsequently stained with fluorochrome-conjugated antibodies in FACS buffer for 30 minutes on ice in the dark. Following a wash with FACS buffer, cells were resuspended in 400 µl FACS buffer. Samples were analyzed with a Cytex® Aurora flow cytometer, and data analysis was performed using FCS Express 7™ flow cytometry analysis software (De Novo Software).

Analysis of the mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was evaluated using the cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide). In healthy cells,

JC-1 enters the energized mitochondria and form aggregates which change the fluorescent property of JC-1 dye. JC-1 monomers exhibit green fluorescence, and the JC-1 dye aggregates show intense red fluorescence. Cells were incubated with the JC-1 dye (working concentration 2 μ M) at 37 °C in a CO₂ incubator for 30 minutes. After washing once with warm DPBS, cells were resuspended in 1 ml DPBS and analyzed by a PerkinElmer fluorometer, using the excitation wavelength 488 nm and the emission at 536 nm (for monomers) and 595 nm (for aggregates). A decreased ratio of red/green fluorescence intensity were interpreted as a decrease in the MMP. Positive controls included cells treated with 50 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP, an uncoupler of oxidative phosphorylation in the mitochondria), for 15 minutes at 37°C. The red/green fluorescence intensity ratio after the CCCP treatment was considered to represent fully depolarized mitochondria. All the other data are expressed as the percentage change relative to the value of the positive controls.

Statistics

Data were analyzed by the GraphPad Prism 9 software. All experiments were repeated at least three times, and representative immunoblots, or flow cytometry plots are shown.

3.4 Results

We investigated the effect of CLPB loss on the function of mitochondria in human and mouse myeloblastic cells. To inactivate the *CLPB* gene in HL60 cells, we used the CRISPR/Cas9 gene editing method and two different gRNAs, g3 and g4. To validate the loss of CLPB protein in HL60 cells, cell lysates were subjected to immunoblotting analysis. The results demonstrated an approximately 80% reduction in CLPB protein levels (Figure 3.1A).

Subsequently, we measured mitochondrial membrane potential (MMP) in control and CLPB KO cells using the JC-1 dye. JC-1 accumulates in mitochondria in a potential-dependent manner. The dye exists as either a green-fluorescent monomer in depolarized mitochondria or as a red-fluorescent aggregate in polarized mitochondria. Thus, a decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization [14]. We established a positive control by treating cells with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which is a potent disruptor of MMP. Cells were examined using the excitation wavelength of 495 nm, and the red/green ratios were calculated using intensity reading at emission wavelength of 535 nm (green fluorescence, monomers) and 595 nm (red fluorescence, aggregates). The representative JC-1 fluorescence spectra obtained for control and CLPB KO cells are shown in Figure 3.1B. As shown in Figure 3.1C, the red/green fluorescence intensity ratios indicated a modest but significant reduction in MMP in CLPB KO cells compared to control cells.

OPA1 is located in the inner mitochondrial membrane and serves as one of the key proteins regulating the mitochondrial fusion and cristae morphology [16]. Several different mRNA splice variants exist, giving rise to several OPA1 protein isoforms [17]. All OPA1 isoforms contain an N-terminal mitochondrial transit sequence (MTS), a transmembrane domain (TM), and a GTPase domain. In the mitochondria, the MTS is cleaved off to generate the long form (L-OPA1). Proteolytic cleavage of the long isoform by OMA1 or YME1L further gives rise to short isoforms (S-OPA1) lacking the TM domain (Figure 3.2A). In agreement with the literature, we observed five OPA1 protein bands in control HL60 cells, marked by a, b, c, d, and e. Intriguingly, we found the accumulation of the 'e' form S-OPA1 (bottom band) in CLPB knockout cells (Figure 3.2B). A similar effect - loss of long 'a' and 'b' forms of OPA1 and

accumulation of the short 'e' form - was achieved when HL60 cells were treated with CCCP, confirming the identity of the 'e' isoform (Figure 3.2C).

Next, we assessed the role of CLPB in neutrophil differentiation. HL60 cells have the capability to undergo differentiation into neutrophil-like cells upon treatment with all-trans retinoic acid (ATRA) or polar-planar compounds (e.g., dimethyl sulfoxide (DMSO) and dimethylformamide (DMF)), or dibutyryl cyclic AMP (dbcAMP) [18]. In this study, HL60 control and CLPB-KO cells were cultured in the presence of 1.25% DMSO or 10 μ M ATRA for three days to induce cell differentiation. Cell survival rates were determined on day 0 and day 3 of treatment by flow cytometry. While both ATRA and DMSO treatment induced some cell death, there was no difference in cell viability between control and CLPB KO cells (Figure 3.3A). To determine the extent of cell differentiation, cells were stained with antibodies specific for two cell surface markers of differentiation, CD11b and formyl peptide receptor 1 (FPR1) [19] [20]. As expected, treatment with either DMSO or ATRA caused increased expression of CD11b and FPR1 markers (Figure 3.3B, C). CD11b expression was similar in DMSO-treated HL60 cells and ATRA-treated cells, while FPR1 was induced more potently in DMSO-treated cells than in ATRA-treated cells (Figure 3.3B, C). Importantly, CLPB-deficient cells showed similar expression level of CD11b and FPR1 compared to control HL60 cells (Figure 3.3B, C). Collectively, these data indicate that CLPB loss does not impair the differentiation process in HL60 cells.

To investigate whether dominant-negative mutations in CLPB affect neutrophil differentiation, we transiently overexpressed the wild-type CLPB (WT) or two different mutants, N466K or R531G, in HL60 cells (Figure 3.4). HL60 cells were electroporated with a bicistronic expression vector encoding GFP and CLPB with a C-terminal Myc tag. We included empty

vector (EV) electroporated HL60 cells as negative control. Of note, the efficiency of WT CLPB expression was reproducibly lower than the expression of the mutants (Figure 3.5A). Flow cytometry analysis showed that cells in all groups - EV, WT CLPB, or mutant CLPB - contained approximately 30% of GFP⁺ cells. This suggests that the electroporation efficiency was around 30% for all constructs. Representative flow cytometry results for cells overexpressing WT CLPB are shown in Figure 3.5B. Treatment with 1.25% of DMSO for three days resulted in the effective induction of CD11b expression in HL60 cells (Figure 3.4C). Importantly, there was no apparent difference in anti-CD11b staining between GFP⁺ HL60 cells transfected with EV, WT CLPB or CLPB mutants (Figure 3.5C). The overall expression of the CD11b marker in GFP⁺ and GFP⁻ cell populations was similar (Figures 3.5C and 3.5D).

3.5 Discussion

Although homologs of CLPB have been extensively studied in bacteria and yeast, where they function as protein chaperones responsible for disaggregation of misfolded proteins, [22], the function of mammalian CLPB remains poorly understood. Mammalian CLPB has a mitochondrial localization sequence and resides in the mitochondrial intermembrane space [6]. Therefore, in this study, we examined the effect of CLPB loss on several aspects of the mitochondrial function. Our data demonstrate that knockout of CLPB results in a reduction of mitochondrial membrane potential in human HL60. The magnitude of MMP serves as an indicator of the mitochondrial energy status, as the generation of MMP occurs through the H⁺ pumping from the mitochondrial matrix to the intermembrane space and is coupled to the electron transport by four respiratory complexes located in the inner mitochondrial membrane [23]. Although mature granulocytes possess a limited number of mitochondria, these organelles

play an important role in governing diverse effector functions of granulocytes, and a reduction in MMP results in mitochondrial fragmentation [24]. Mitochondria also participate in metabolic reprogramming during neutrophil differentiation [25]. A recent study showed that overexpression of SCN-linked CLPB dominant-negative mutants in human myeloid progenitor cell line (MOLM-13) also leads to a decrease in MMP compared to control cells [5].

The accumulation of the short “e” form of OPA1 protein in CLPB KO cells further confirms that the presence of CLPB is important for the mitochondrial health. OPA1 is associated with mitochondrial fusion, mitochondrial DNA maintenance, bioenergetics, and cristae integrity [26]. So far, it remains unclear how long and short forms of OPA1 contribute to the processes of mitochondrial fusion and fission. At present, multiple studies indicate that long forms of OPA1 by themselves possess the fusion competency [27]. In contrast, it has been proposed that short forms of OPA1 display fission activity, since overexpression of short OPA1 forms results in mitochondrial fragmentation [28]. Nevertheless, the equilibrium between fusion and fission depends on the relative proportions of long and short forms of OPA1. Consequently, the reduction of long forms of OPA1 and the accumulation of short forms during stress-induced activation of OMA1 leads to mitochondrial fragmentation [29]. Our results agree with the previous studies that disruption of MMP by treatment with CCCP also augment the cleavage of long form of OPA1, resulting in the accumulation of short form of OPA1 [30]. Together, we found that loss of CLPB leads to abnormal mitochondrial function, characterized by processing of long-forms of OPA1 into its short-forms. Since OMA1 has been identified as a stress-activated protease that is responsible for the rapid cleavage of long OPA1 forms, further investigations are needed. to determine whether the loss of CLPB may lead to the activation of OMA1.

Although CLPB knockout clearly resulted in a mitochondrial dysfunction (decreased MMP and increased accumulation of short OPA1), loss of CLPB or overexpression of SCN-linked disease mutants in HL60 cells did not compromise cell viability and did not inhibit cell differentiation. The HL60 cell line is a widely used model for neutrophil differentiation, as these cells can acquire some features of neutrophil-like cells after treatment with ATRA or DMSO. However, the exact mechanisms underlying these changes are not clear. In addition, HL60 cells have more limitations as a model for studying neutrophil differentiation. HL60 cell line was derived from a patient with leukemia, which means that these cells carry abnormalities inherent to cancer cells [31]. Moreover, HL60 cells do not fully differentiate into mature neutrophils, and they do not exhibit all the features which are typical for mature neutrophils, such as the production of secondary and tertiary granules containing anti-microbial enzymes. Thus, application of primary cells or induced pluripotent stem cells [32] might be more appropriate to address the role of CLPB in neutrophil differentiation.

As an expansion of this project, our lab has recently initiated studies on the role of murine CLPB in mouse promyeloblastic 32D cells. The 32D cell line was derived from murine bone marrow, 32D cells are non-cancerous, and they differentiate along the neutrophil lineage into mature neutrophils after granulocytic colony stimulating factor (G-CSF) treatment [33]. Therefore, 32D cells offer a more suitable model for the evaluation of the physiological role of CLPB in neutrophil differentiation. Interestingly, early studies suggest that CLPB knockout in 32D cells significantly increases apoptotic cell death after induction of cell differentiation.

Collectively, these results suggest that the absence of CLPB might influence the regulation of granulopoiesis by impairing mitochondrial function. Together, these findings may contribute to our understanding of how CLPB mutations are linked to neutropenia.

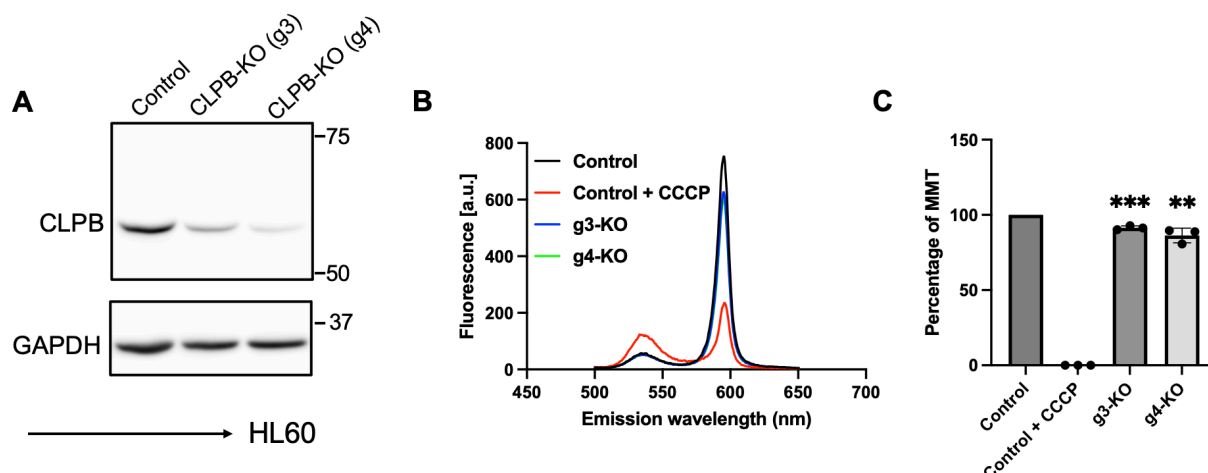


Figure 4.1 CLPB knockout leads to a decreased mitochondrial membrane potential in HL60 cells.

(A) HL60 cells were electroporated with two different guide RNAs targeting CLPB (CLPB-KO g3 or CLPB-KO g4) complexed together with recombinant Cas9 protein. After 48 hours, total cell lysates were subjected to Western blot analysis using an anti-CLPB antibody. (B) HL60 control, g3-KO, and g4-KO cells were stained with the JC-1 dye to measure mitochondrial membrane potential (MMP). The excitation wavelength was 495 nm, and representative emission spectra are shown. Cells treated with 50 μ M CCCP were used as positive control. (C) The ratios of red ($\lambda_{em} = 595$ nm, JC-1 aggregates) to green ($\lambda_{em} = 535$ nm, JC-1 monomers) fluorescence intensities were determined to measure the changes in MMP.

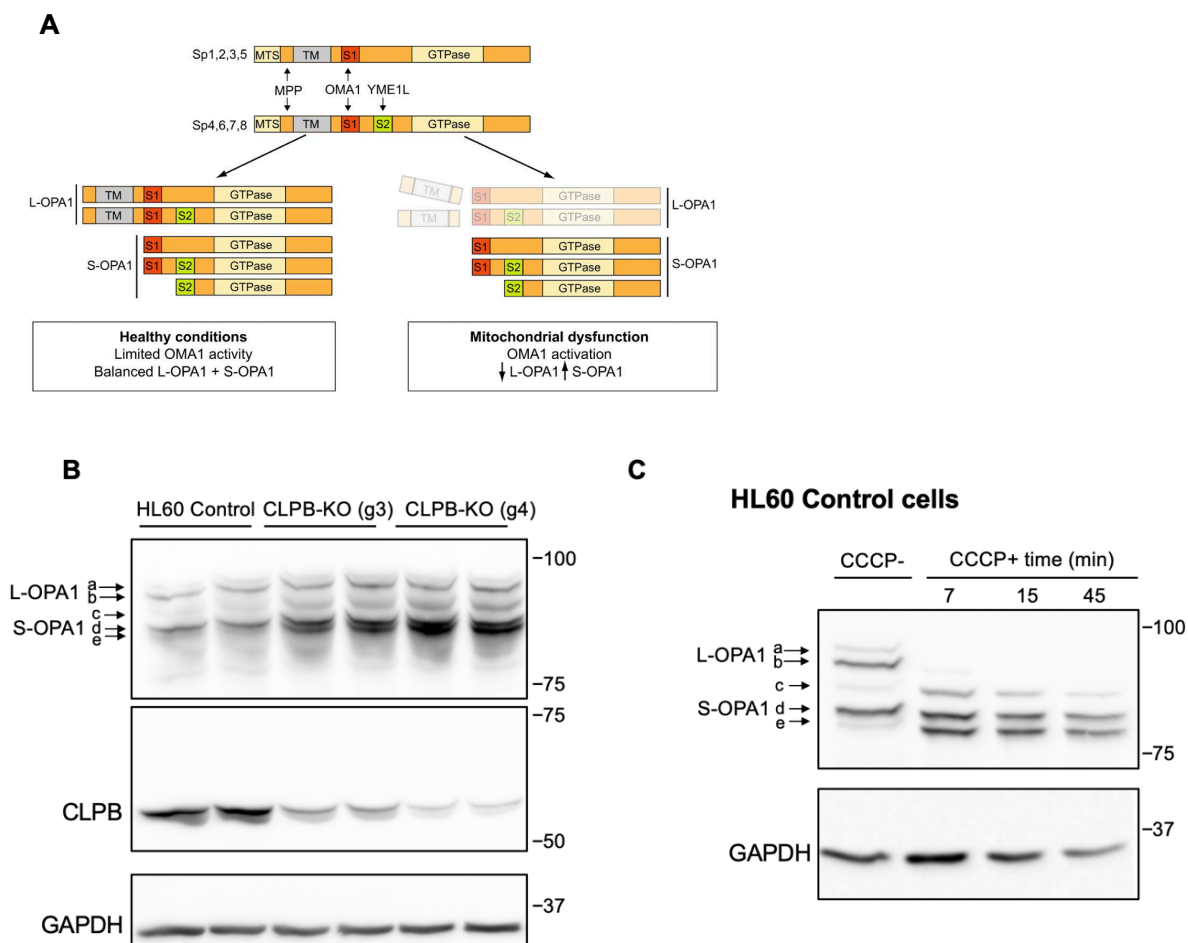


Figure 4.2 CLPB loss in HL60 cells leads to the accumulation of S-OPA1 isoform e.

(A) Adapted from MacVicar *et al.* 2016. Schematic diagram of OPA1 protein isoforms.

Mitochondrial processing peptidase (MPP) mediates cleavage of the MTS, resulting in the generation of long isoforms. Further cleavage at either site S1 or S2 site generates short

isoforms. (B) Total cell lysates of control, CLPB-KO (g3), and CLPB-KO (g4) HL60 cells were

analyzed by Western blotting using anti-CLPB and anti-OPA1 antibodies. (C) Confirmation of

the identity of OPA1 isoform e. HL60 cells were treated with 50 μ M CCCP to induce

mitochondrial depolarization, which led to the accumulation of isoform e. GAPDH is a gel-loading control.

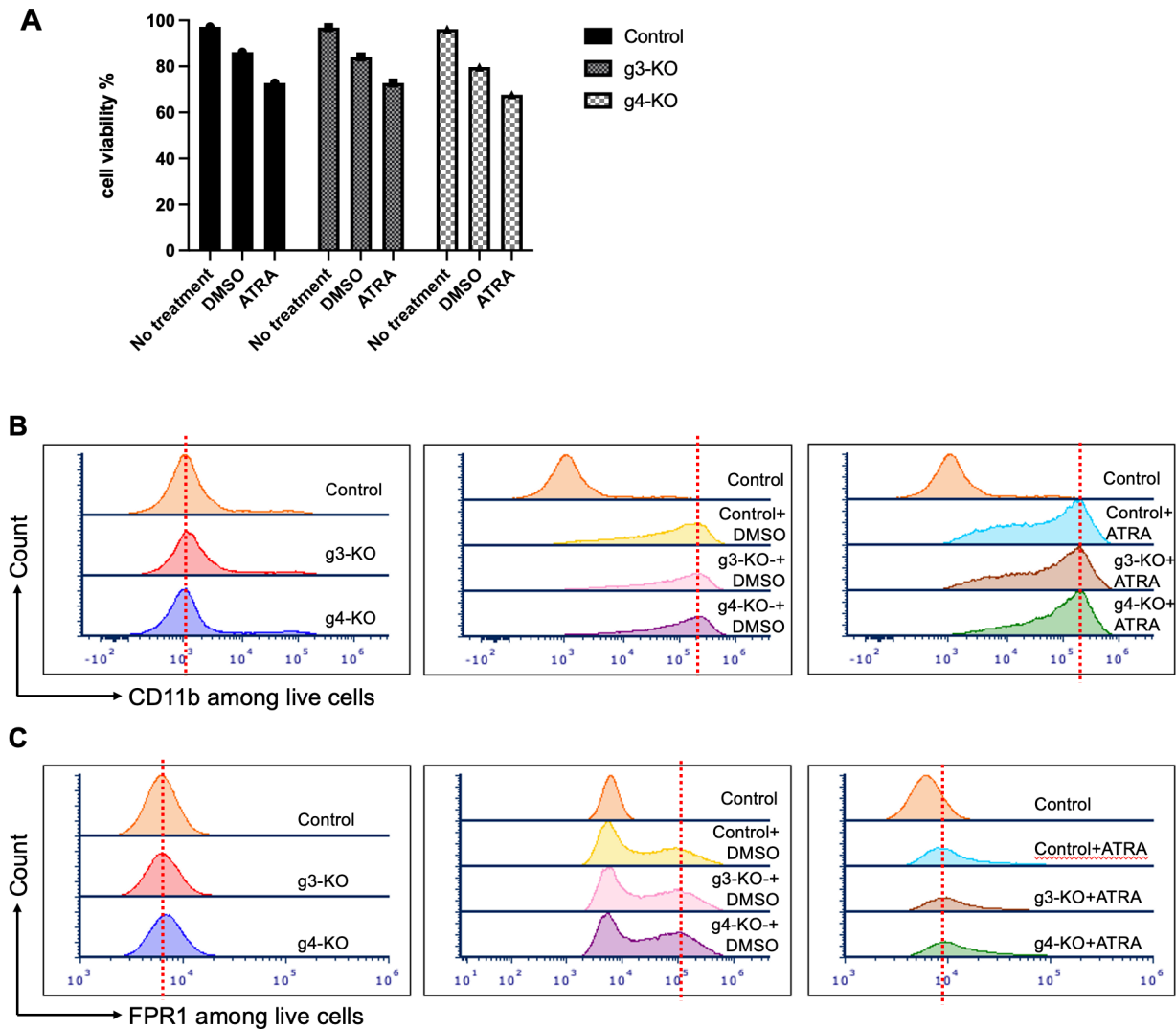


Figure 4.3.

(A) Control and CLPB KO HL60 cells (g3-KO and g4-KO) were differentiated into a neutrophil-like state by incubation with all-trans retinoic acid (ATRA) (10 μ M) or dimethyl sulfoxide (DMSO) (1.25%). The percentage of viable cells was compared after three days of treatment after cell staining with a viability dye and analysis by flow cytometry. (B) Histograms of the CD11b marker protein expression in control cells, g3-KO, and g4-KO HL60 cells treated with DMSO or ATRA. (D) Histograms of FPR1 marker protein expression in control cells, g3-KO, and g4-KO HL60 cells treated with DMSO or ATRA.

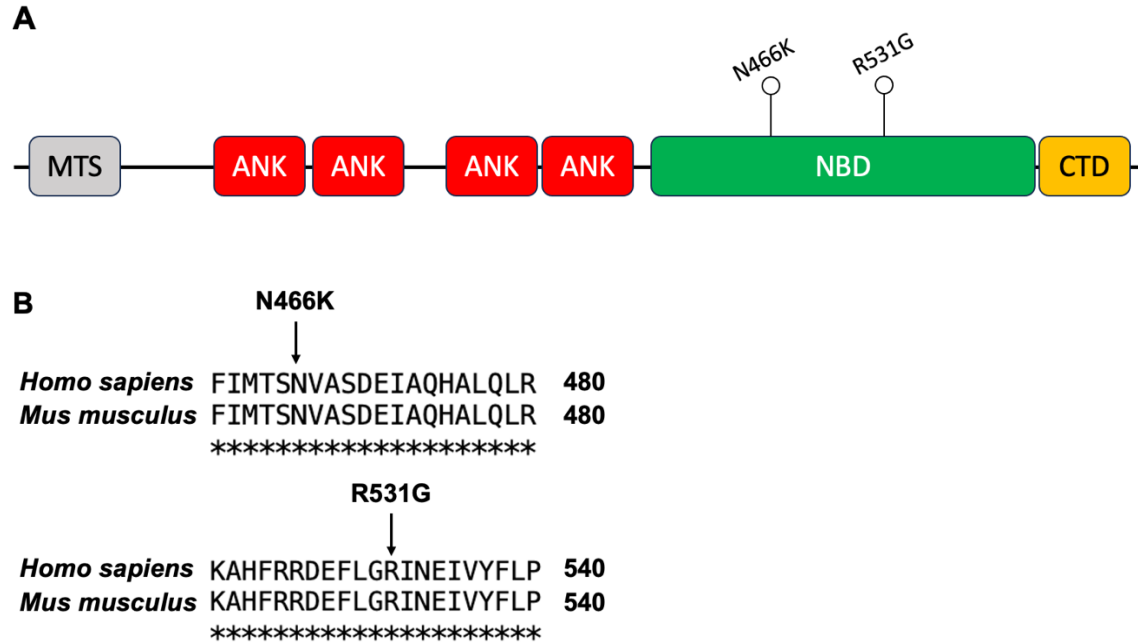


Figure 4.4 Selected dominant-negative mutations in CLPB linked to severe congenital neutropenia.

(A) The domain organization of human CLPB is depicted, with mitochondrial targeting sequence (MTS), ankyrin-rich repeats (ANK), nucleotide binding domain (NBD), C-terminal domain (CTD). Two SCN pathogenic variants, N466K and R531G, are located in the NBD domain. (B) Alignment of the amino acid sequences surrounding asparagine 466 and arginine 531 in human and mouse CLPB.

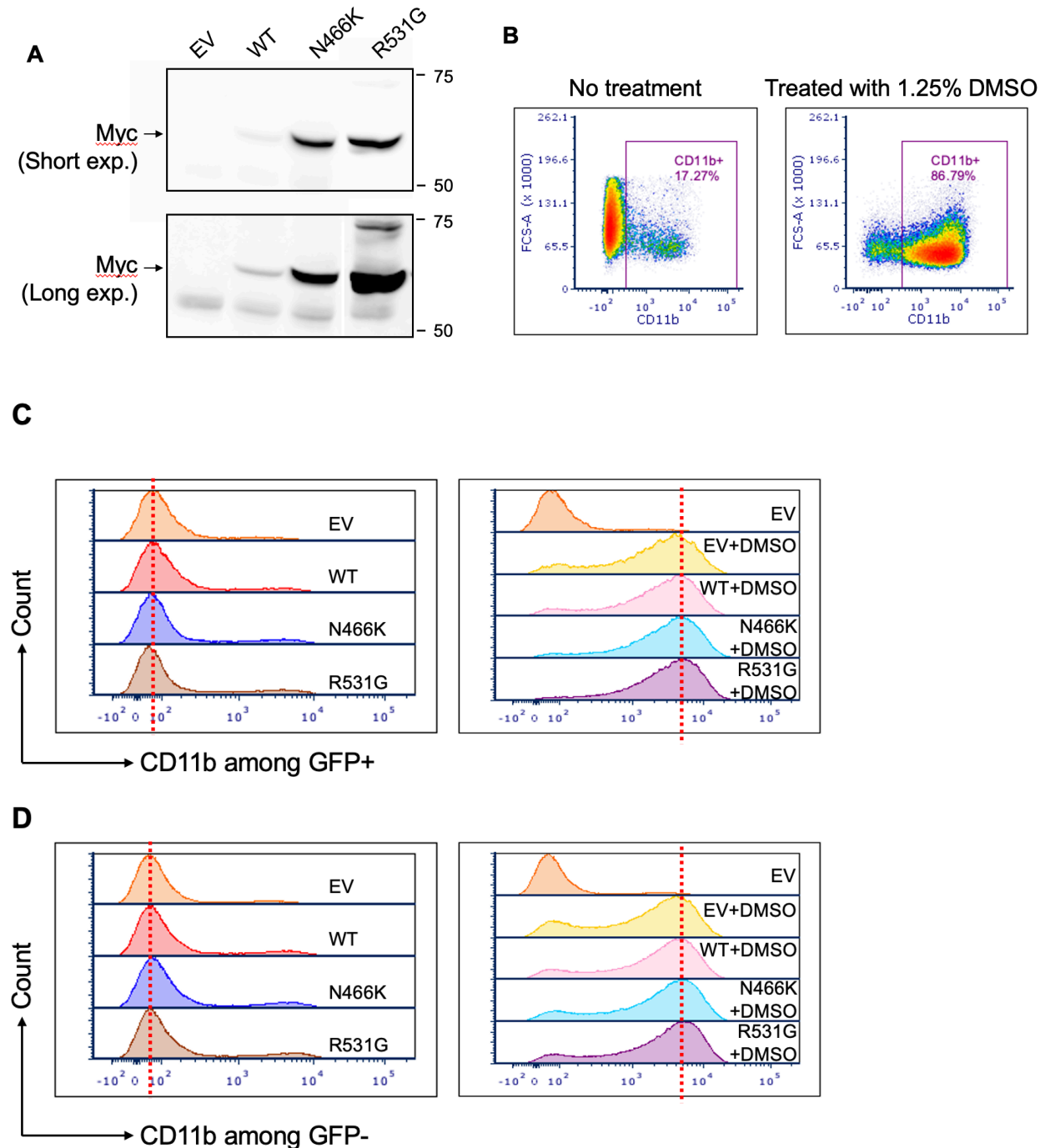


Figure 4.5 Transient overexpression of dominant-negative CLPB mutants does not affect differentiation of HL60 cells.

(A) Transient overexpression of WT CLPB-Myc/GFP, N466K-CLPB-Myc/GFP, or R531G-CLPB-Myc/GFP in HL60 cells. Total cell lysates were analyzed by Western blotting 48 hours after electroporation using anti-Myc antibody. (B) HL-60 cells underwent differentiation into a

neutrophil-like state by a three-day stimulation with dimethyl sulfoxide (DMSO) (1.25%). Representative FACS plots showing untreated HL60 cells (left) and DMSO-treated HL60 cells (right). CD11b was used as a neutrophil-like state cell surface marker to validate the differentiation. (C) Comparison of CD11b expression histograms in the GFP⁺ population of HL60 cells transfected with empty vector (EV), WT CLPB, the N466K mutant, or the R531G mutant. (D) Comparison of CD11b expression histograms in the GFP⁻ population of HL60 cells transfected with empty vector (EV), WT CLPB, the N466K mutant, or the R531G mutant.

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Chapter 5 - Conclusions

This dissertation centers on elucidation of the intricate interplay between immune cells and various diseases. The exploration delves into the crucial roles of two metalloprotease proteins, namely ADAM12 and ADAM9, with the emphasis on their contribution to modifying the tumor immune microenvironment. This involves a detailed examination of alterations in the composition of immune cell populations. Additionally, the study explores the impact of human CLPB mutations associated with severe congenital neutropenia or the loss of CLPB expression on several properties of human HL60 leukemia cells.

In Chapter 2, I investigated the role of ADAM12 in the tumor microenvironment using the T11 mouse model of claudin-low breast cancer. Our results showed that the knockout of ADAM12 in T11 breast cancer cells exerted a notable effect on the composition of immune cells infiltrating tumors originated from transplanted cancer cells. This suggests a consequential alteration in the immune cell landscape within the tumor microenvironment, underscoring the potential role of ADAM12 in modulating the immune response to claudin-low breast cancer cells.

In order to investigate the functional role of ADAM12 in T11 cells, we used the CRISPR/Cas9 gene-editing approach to specifically target the *Adam12* gene. Upon injecting ADAM12-null T11 cells into the mammary glands of Balb/cJ mice, we conducted a comparative analysis to assess both the quantity and composition of immune cells infiltrating tumors in the context of ADAM12 knockout (A12-KO) versus control T11 tumors. We observed a reduction in the total count of tumor-infiltrating CD45⁺ immune cells in A12-KO tumors compared to T11 tumors. This finding primarily stemmed from the diminished CD11b⁺ myeloid cell population, in particular tumor-infiltrating neutrophils (TINs)/polymorphonuclear myeloid-derived

suppressor cells (PMN-MDSCs). Interestingly, both the absolute numbers and frequencies of TIMs/PMN-MDSCs exhibited a significant decrease in A12-KO tumors. In contrast, the absolute numbers of tumor-infiltrating macrophages (TIMs), the second major population of myeloid cells, were similar in A12-KO and control tumors. The frequency of TIMs among all CD45⁺ cells was even slightly increased.

Subsequently, when comparing tumor-infiltrating lymphocytes (TILs) between control and A12-deficient T11 tumors, we found that A12-KO tumors exhibited elevated percentages of CD3⁺ T cells and CD19⁺ B cells compared to T11 tumors. We next aimed to investigate whether the absence of ADAM12 in cancer cells directly affected B cell chemotaxis. To assess that, we conducted transwell cell migration assays. Our observations indicated that the presence of T11 cells in the bottom transwell chambers led to an elevated B cell migration. Notably, B cell migration towards A12-KO T11 cells showed a significant increase compared to migration towards control T11 cells. The addition of AMD3100, a CXCR4 inhibitor, or the use of an anti-CXCL12 blocking antibody effectively suppressed B cell migration towards T11 cells, indicating that the chemokine T11 cell-derived CXCL12 was recognized as a chemoattractant for B cells.

Next, we explored whether the observed alterations in tumor-infiltrating immune cells in A12-KO tumors corresponded to enhanced responses of the tumors to the immune checkpoint blockade (ICB) therapy. Mice bearing A12-KO or control T11 tumors were administered anti-PD1/anti-CTLA4 antibodies. Predictably, T11 tumors exhibited resistance to ICB therapy, whereas A12-KO tumors stabilized and showed no signs of progression for several days post-antibody administration. To further delineate the contribution of B cells to the improved tumor responses seen in A12-KO tumors, we conducted experiments involving B cell depletion. The rationale behind these experiments was that the increased frequencies of tumor-infiltrating B

cells observed in ADAM12-deficient tumors might be responsible for the improved response to ICB. Importantly, A12-deficient tumors became resistant to anti-PD1/anti-CTLA4 treatment upon B cell depletion, underscoring the pivotal role of B cells in mediating the enhanced response to ICB therapy in the context of ADAM12 deficiency. Future studies should focus on the comprehensive analysis of alterations in immune cell composition throughout the time course during the ICB treatment. It is essential to investigate the underlying factors contributing to the initial responsiveness of A12-KO tumors to ICB therapy, followed by the subsequent development of the therapy resistance.

In chapter 3, I investigated the role of ADAM9 in the tumor microenvironment using the same T11 mouse model of breast cancer. Our findings demonstrated that deletion of ADAM9, similar to deletion of ADAM12, in T11 cells significantly affected the composition of infiltrating immune cells within the tumor. However, I found several important differences between ADAM12-deficient and ADAM9-deficient tumors. Most notably, there was a drop in TIM frequencies among all CD45⁺ cells, accompanied by a significant elevation in TIM/PMN-MDSC frequencies. In contrast, the frequency of B cells was increased in ADAM9 KO tumors compared to control T11 tumors, an effect also seen in ADAM12 KO tumors. Finally, mice with ADAM9 KO tumors did not demonstrate an enhanced response to ICB therapy. These results indicate that ADAM12 and ADAM9 play distinct and non-redundant roles in the regulation of tumor immune microenvironment.

In chapter 4, my research focused on elucidating the function of CLPB in neutrophil differentiation. This exploration aimed to get a better insight into the regulation of mitochondrial functions by CLPB. We first employed CRISPR/Cas9 gene editing to block CLPB expression in HL60 neutrophil-like cells. Next, we explored the effect of CLPB deletion on the

mitochondrial membrane potential (MMP), employing the JC-1 fluorescent dye. A modest but significant reduction in the MMP was detected in HL60 cells following CLPB knockout, in comparison to HL60 control cells. Next, I demonstrated an impact of CLPB loss on OPA1, a key regulator of mitochondrial fusion and cristae morphology. I showed that CLPB loss leads to accumulation of the 'e' short form of OPA1. Unexpectedly, CLPB-deficient cells exhibited comparable levels of neutrophil differentiation markers to control cells after treatment with DMSO or ATRA, indicating that the loss of CLPB does not hinder the differentiation process in HL60 cells. We then extended our investigations to dominant-negative CLPB variants. In agreement with the results obtained for CLPB KO cells, HL60 cells overexpressing dominant-negative CLPB mutants did not exhibit any discernible differences in cell differentiation or cell viability when compared to HL60 cells overexpressing the wildtype CLPB. Thus, despite of the diminished function of mitochondria (decreased MMP and increased accumulation of short form of OPA1), the differentiation program and the survival of HL-60 cells were not impaired. One explanation of these results could be that HL60 cells are leukemia cells, in which many processes are dysregulated. The treatments typically used to induce HL60 to differentiate to a “neutrophil-like” state, DMSO and ATRA, are non-physiological. Future studies to examine the role of CLPB in mitochondria during neutrophil differentiation should use better and more physiologically-relevant models, such as primary progenitor cells or non-cancer cell lines.

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**Appendix B - Supplementary Information for ADAM12 abrogation
alters immune cell infiltration and improves response to checkpoint
blockade therapy in the T11 murine model of triple-negative breast
cancer**

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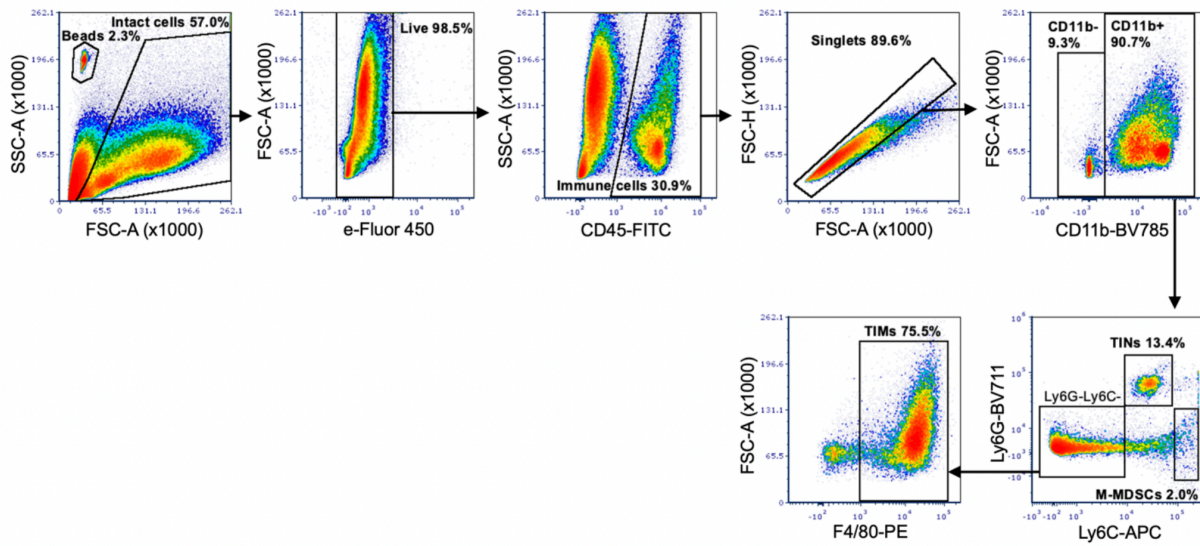


Figure B. S1. Flow cytometry gating strategy for tumor-infiltrating macrophages (TIMs), tumor-infiltrating neutrophils (TINs)/polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), and monocytic myeloid-derived suppressor cells (M-MDSCs).

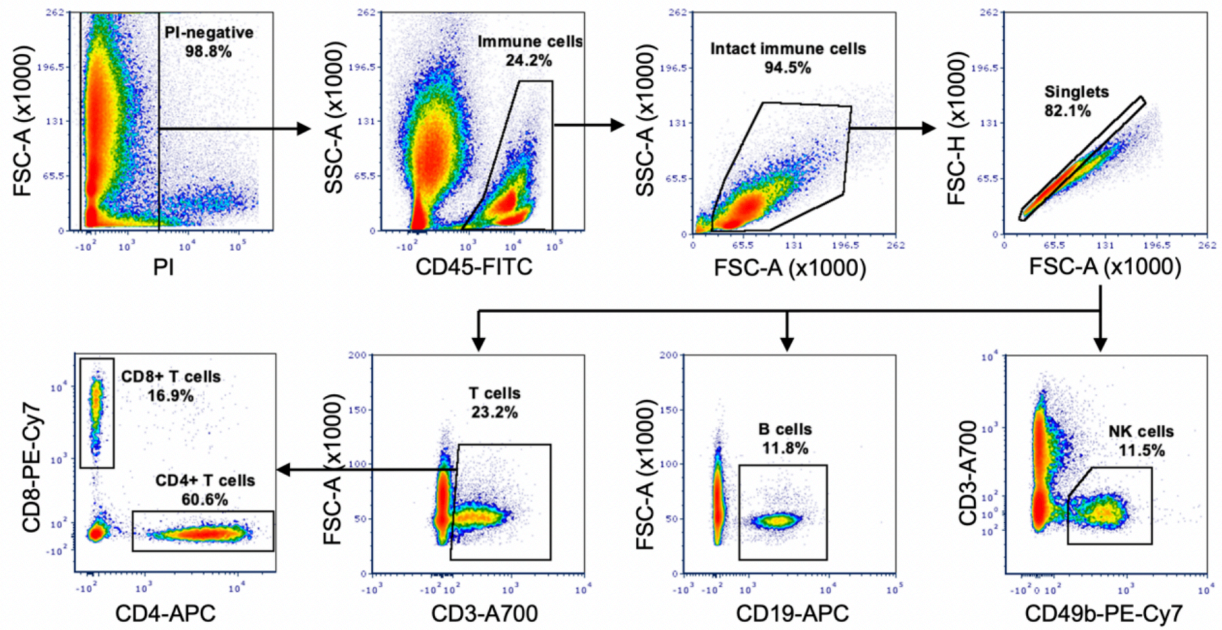


Figure B. S2. Flow cytometry gating strategy for tumor-infiltrating T cells (CD4+ and CD8+), B cells, and NK cells.

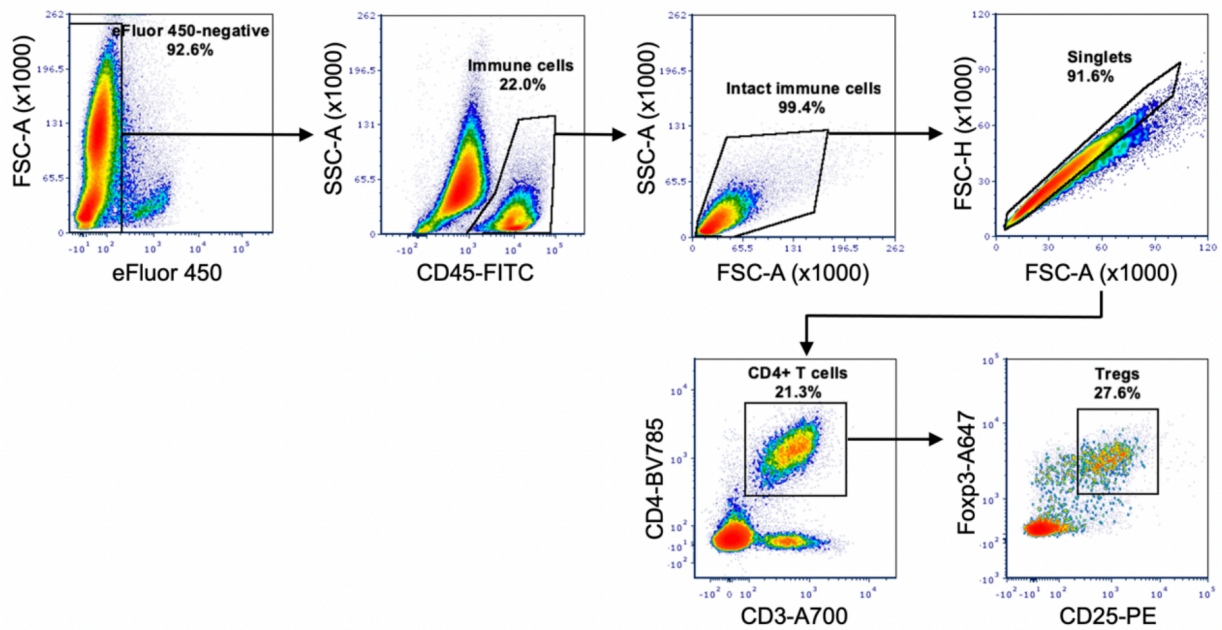


Figure B. S3. Flow cytometry gating strategy for tumor-infiltrating regulatory T cells (Tregs).

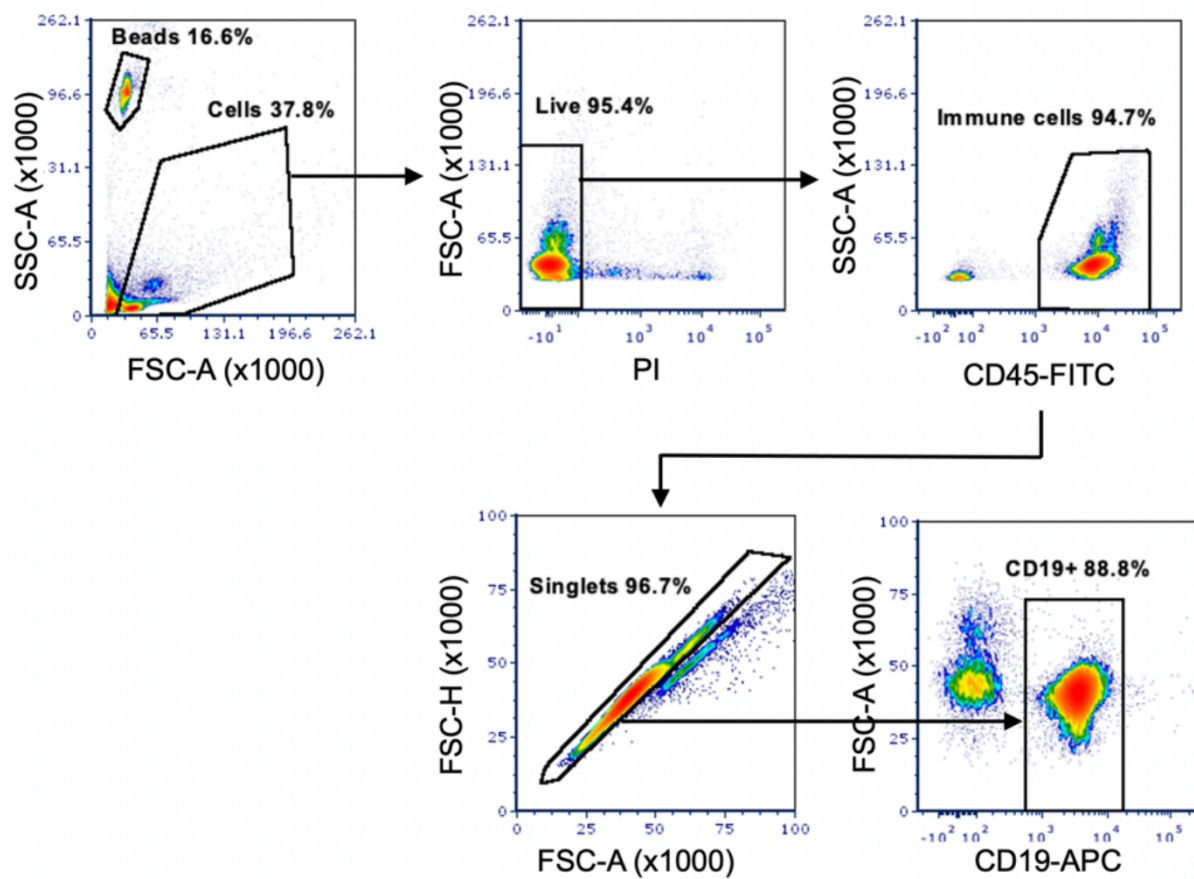


Figure B. S4. Flow cytometry gating strategy for splenic B cells.

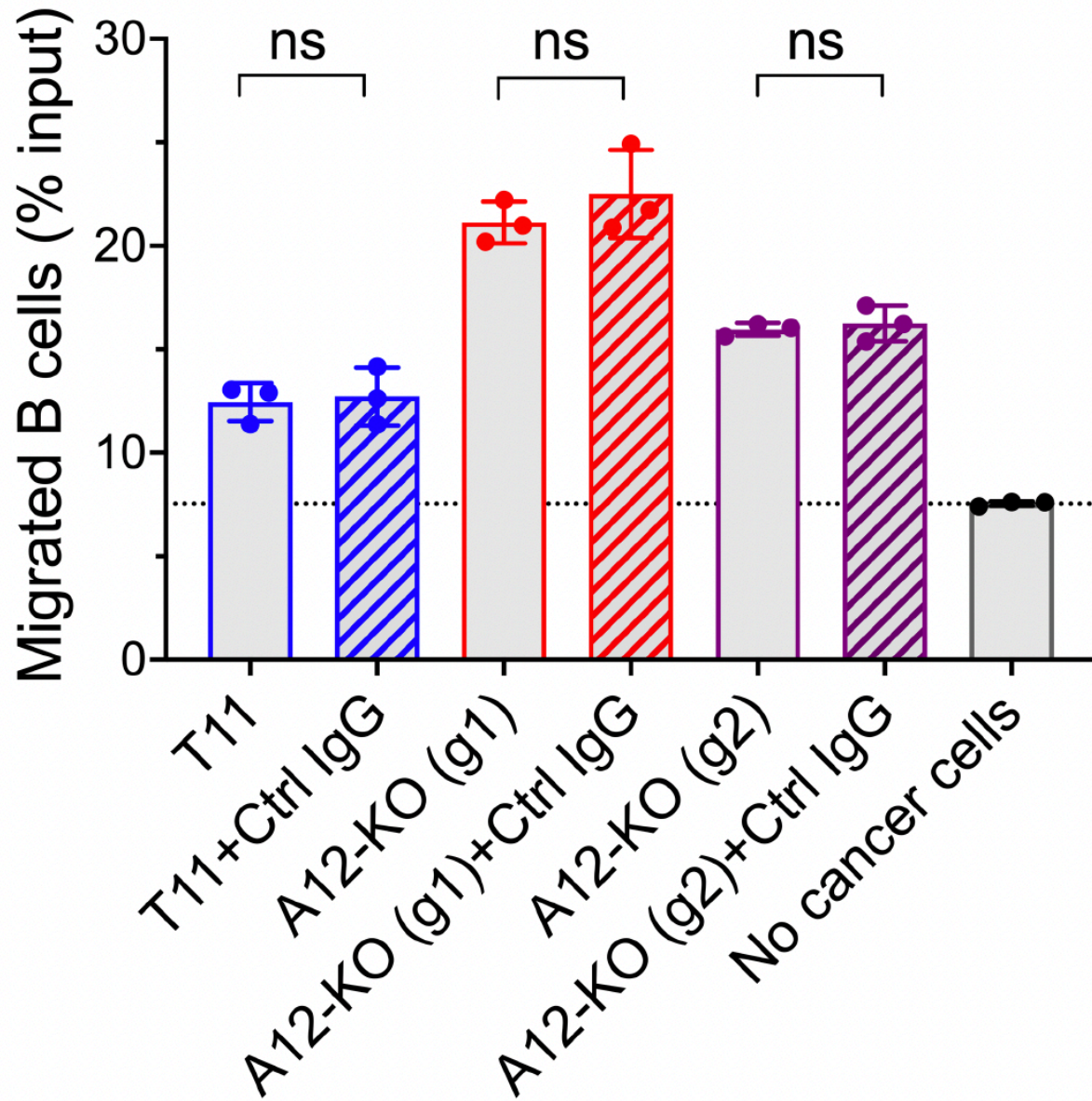


Figure B. S5. B cell migration toward T11, A12-KO (g1), or A12-KO (g2) cells, in the absence or presence of 2.5 $\mu\text{g/ml}$ rat IgG2a, κ isotype control antibody added to the lower transwell chambers.

Table B. S1. Reagents and resources.

Reagent or Resource	Source	Identifier
Antibodies		
Alexa Fluor 700-conjugated anti-CD3 (clone 17A2)	BioLegend	Cat # 100216
Alexa Fluor 700-conjugated rat IgG2b, κ isotype control (clone RTK 4530)	BioLegend	Cat # 400628
Alexa Fluor 647-conjugated anti-Foxp3 (clone MF-14)	BioLegend	Cat # 126408
Alexa Fluor 647-conjugated rat IgG2b, κ isotype control (clone RTK 4530)	BioLegend	Cat # 400626
APC-conjugated anti-CD4 (clone RM4-5)	BioLegend	Cat # 100516
APC-conjugated anti-CD19 (clone 6D5)	BioLegend	Cat # 115512
APC-conjugated anti-Ly6C (clone HK1.4)	BioLegend	Cat # 128016
APC-conjugated anti-CD11c (clone N418)	BioLegend	Cat # 117309
APC-conjugated rat IgG2a, κ isotype control (clone RTK 2758)	BioLegend	Cat # 400511
APC-conjugated Armenian hamster IgG isotype control (clone HTK888)	BioLegend	Cat # 400911
Brilliant Violet 785-conjugated anti-CD4 (clone RM4-5)	BioLegend	Cat # 100552
Brilliant Violet 785-conjugated anti-CD11b (clone M1/70)	BioLegend	Cat # 101243
Brilliant Violet 711-conjugated anti-Ly6G (clone 1A8)	BioLegend	Cat # 127643
Brilliant Violet 785-conjugated rat IgG2a, κ isotype control (clone RTK 2758)	BioLegend	Cat # 400546
FITC-conjugated anti-CD45 (clone 30-F11)	BioLegend	Cat # 103108
FITC-conjugated rat IgG2b, κ isotype control (clone RTK 4530)	BioLegend	Cat # 400605
PE-conjugated anti-CD8a (clone 53-6.7)	BioLegend	Cat # 100707
PE-conjugated anti-Ly6C (clone 1A8)	BioLegend	Cat # 127607
PE-conjugated anti-F4/80 (clone BM8)	BioLegend	Cat # 123110
PE-conjugated rat IgG2a, κ isotype control (clone RTK 2758)	BioLegend	Cat # 400507
PE/Cyanine7-conjugated anti-CD8a (clone 53-6.7)	BioLegend	Cat # 100721
PE/Cyanine7-conjugated anti-CD49b (clone DX5)	BioLegend	Cat # 108921
PE/Cyanine7-conjugated rat IgG2a, κ isotype control (clone RTK 2758)	BioLegend	Cat # 400521
TruStain FcX Plus anti-CD16/32 (clone S17011E)	BioLegend	Cat # 156604
<i>InVivo</i> MAb anti-mouse CTLA-4 (CD152) (clone 9D9)	BioXcell	Cat # BE0164
<i>InVivo</i> MAb anti-mouse PD-1 (CD279) (clone RMP1-14)	BioXcell	Cat # BE0146
<i>InVivo</i> MAb mouse IgG2b isotype control (clone MPC-11)	BioXcell	Cat # BE0086
<i>InVivo</i> MAb rat IgG2a isotype control (clone 2A3)	BioXcell	Cat # BE0089
Ultra-LEAF™ purified anti-mouse CD20 (clone SA271G2)	BioLegend	Cat # 152115
Ultra-LEAF™ purified rat IgG2b, κ isotype control (clone RTK4530)	BioLegend	Cat # 400671
Ultra-LEAF™ purified anti-CXCL12 (SDF-1 β) (clone W15149B)	BioLegend	Cat # 939903
Purified rat IgG2a, κ isotype control (clone RTK2758)	BioLegend	Cat # 400502
Anti-CD29 (clone 18/CD29)	BD Biosciences	Cat # 610467
Rabbit polyclonal anti-ADAM12 antibody raised against amino acid 774-791 of the cytoplasmic tail of mouse ADAM12	Bethyl Laboratories	Custom-made
Reagents		
ACK Lysing Buffer	Gibco	Cat # A1049201
AMD 3100 octahydrochloride	TOCRIS	Cat # 3299
Alt-R® S.p.HiFi Cas9 Nuclease V3	IDT	Cat # 1081060
Alt-R® Cas9 Electroporation Enhancer	IDT	Cat # 1075915
BCA Protein Assay Kit	ThermoFisher	Cat # 23225
Bovine Serum Albumin	Amresco	Cat # 9048-46-8
Collagenase type IV	Sigma	Cat # C5138
Concanavalin A agarose	Sigma	Cat # C7555
Cyto-Last Buffer	BioLegend	Cat # 422501
DNase type IV	Sigma	Cat # D5025
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	Cat # DY008
eFluor 450 Fixable Viability Dye	Invitrogen	Cat # 65086314
EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific	Cat # 21335
Fetal Bovine Serum	Corning	Cat # MT35015CV
Hyaluronidase type IV-S	Sigma	Cat # H3884
KN2.0, non-homology mediated CRISPR knockout kit	OriGene	Cat # KN508813
Mouse CXCL12/SDF-1 α Quantikine ELISA kit	R&D Systems	Cat # MCX120
Neon™ Transfection System Kit	Invitrogen	Cat # MPK1096
NeutrAvidin agarose	Thermo Scientific	Cat # 29200
Percoll	Cytiva	Cat # 17544502
Phusion Green Hot Start II High-fidelity PCR master mix	ThermoFisher	Cat # F566S
Precision Count Beads	BioLegend	Cat # 424902
Propidium Iodine Staining Solution	Sigma	Cat # P4864
Quick Change II Site-Directed mutagenesis Kit	Agilent Technologies	Cat # 200523-5
Transforming growth factor β	R&D Systems	Cat # 7666-MB-005
True Nuclear transcription factor buffer set	BioLegend	Cat # 424401
Turbodectin 8.0	OriGene	Cat # TF81001
UltraComp eBeads Compensation Beads	Invitrogen	Cat # 01-2222-42

Table B. S2. Claudin-low ER-PR-HER2- tumors from the METABRIC study.

Retrieved from cBioPortal at <https://www.cbioportal.org>.

Classification into "core claudin-low" and "other claudin-low" is according to Fougner C, Bergholtz H, Norum JH, Sorlie T. "Re-definition of claudin-low as a breast cancer phenotype". *Nat Commun* 2020;11:1787.

Core claudin-low	Other claudin-low
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Patient_ID	Patient_ID
------------	------------

MB-0634	MB-0157
MB-2724	MB-0164
MB-0489	MB-0206
MB-7008	MB-0211
MB-2993	MB-0238
MB-0617	MB-0303
MB-3752	MB-0352
MB-0284	MB-0354
MB-7078	MB-0400
MB-0494	MB-0401
MB-3395	MB-0414
MB-2963	MB-0420
MB-5392	MB-0446
MB-0893	MB-0558
MB-0220	MB-0608
MB-7023	MB-0613
MB-0495	MB-0627
MB-5162	MB-0658
MB-5408	MB-0906
MB-2643	MB-2833
MB-0470	MB-2850
MB-0289	MB-2857
MB-0540	MB-2904
MB-2556	MB-2957
MB-0424	MB-3001
MB-0265	MB-3062
MB-7025	MB-3067
MB-0269	MB-3271
MB-0174	MB-3292
MB-0502	MB-3383
MB-0179	MB-3476

MB-3567
MB-3702
MB-4416
MB-4622
MB-4696
MB-4733
MB-4859
MB-4865
MB-4888
MB-4893
MB-4904
MB-4931
MB-4942
MB-5041
MB-5052
MB-5065
MB-5100
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MB-6144
MB-6188
MB-6272
MB-6305
MB-7017
MB-7052
MB-7081
MB-7089
MB-7121
MB-7158

Table B. S3. Immune gene signatures analyzed in the study.

CD4+ Th1 cell signature (Gu-Trantien et al., J Clin Invest 2013;123:2873-2892)						
CCND2	CTLA4	RAB33A	STAT1			
CD38	RAB27A	SH2D2A	TNFRSF4			

CD4+ Tfh cell signature (Gu-Trantien et al., J Clin Invest 2013;123:2873-2892, and Hollern et al., Cell 2019;179:1191-1206)			
CXCL13	CD40LG	MAF	ICOS
CXCR5	PDCD1	IL21	

B cell signature (Hollern et al., Cell 2019;179:1191-1206, Danaher et al., J Immunother Cancer 2017;5:18, and Iglesia et al., Clin Cancer Res 2014;20:3818-3829)						
BANK1	CD22	CR2	IGHG1	JCHAIN	TNFRSF17	IRF4
BLK	CD79A	EBF1	IGKC	MS4A1	SDC1	
CD19	CD79B	FFAR1	IL9R	VPREB3	XBP1	

TLS signature (Crotty, Nat Rev Immunol 2015;15:185-189)		
CCL19	CCL21	CXCL13

TIM signature (Cassetta et al., Cancer Cell 2019;35:588-602)						
AIF1	CCL4	DOK2	GBP5	LAIR1	PSMB9	VSIG4
C1QA	CCL8	FCN1	GIMAP6	LAP3	SIGLEC1	
C1QB	CD4	FGD2	HCST	LILRB4	TCN2	
C1QC	CD40	FOLR2	HLA-DOA	LST1	TLR7	
CCL2	CD83	GBP1	IRF8	NCF1C	TNFAIP3	
CCL3	CLEC7A	GBP4	ITGB2	PLTP	VAV1	

TIN/PMN-MDSC signature (Kim et al., Nature Cell Biol 2019;21:1113-1126)						
AMBP	CCL5	CISH	EGR1	HLA-DQB1	NFKBIZ	SOCS3
ARG1	CCL7	CLEC6A	EGR2	HSPA1A	NUPR1	TNFAIP3
ATF3	CCL8	CSF1	FOSB	IER3	P2RY6	
CCL2	CCRL2	CXCL1	GADD45B	IGFBP6	PLK3	
CCL3	CD14	CXCL2	GPR35	MMP12	RGS1	
CCL4	CDKN1A	DUSP16	HLA-DMB	MS4A6A	SOAT2	

Table B. S4. Murine tumors and normal mammary gland samples.

Retrieved from Gene Expression Omnibus (GEO) at <https://www.ncbi.nlm.nih.gov/geo/>, datasets GSE3165, GSE8516, GSE9343, GSE14457, GSE15263, GSE17916, GSE27101, and GSE42640. Classification into "claudin-low" and "other" tumor types is according to Pfeifferle AD, Herschkowitz JL, Usary J, Harrell JC, Spike BT, Adams JR, Torres-Arzuay MI, Brown M, Egan SE, Wahl GM, Rosen JM, Perou CM. "Transcriptomic classification of genetically engineered mouse models of breast cancer identifies human subtype counterparts". *Genome Biol* 2013;14:R125.

Claudin low tumors		Other tumors		Normal mammary gland tissue	
GEO number	Sample name	GEO number	Sample name	GEO number	Sample name
GSM1046309	BALB/c_p53null_2247_transplant_134654	GSM1046307	BALB/c_p53null_2153_transplant_137324	GSM1046325	C57B6_Mammary_Norm_131546_GMURROW
GSM1046323	Balbc_p53het_18_C1201-4_spontaneous_GMURROW	GSM1046308	BALB/c_p53null_2224_transplant_135434	GSM1046467	C57B6_Mammary_Norm_131547_GMURROW
GSM1046324	Balbc_p53null_T11-CL-X1_128333_PEROU	GSM1046310	BALB/c_p53null_2250_transplant_135434	GSM1046468	C57B6_Mammary_Norm_134742_GMURROW
GSM1046474	FVB_C3(1)-Tag_117733_untreated_GMURROW	GSM1046311	BALB/c_p53null_2250_transplant_147753	GSM1046469	C57B6_Mammary_Norm_437_1_FURTH
GSM1046480	FVB_MMTV_Ab1_1348_MBROWN	GSM1046312	BALB/c_p53null_2304_transplant_137326	GSM1046470	C57B6_Mammary_Norm_439_2_FURTH
GSM211519	MIXED_Brg1het_T176_BULTMAN	GSM1046313	BALB/c_p53null_9263-3_transplant_135908	GSM1046471	C57B6_Mammary_Norm_443_1_FURTH
GSM381097	MMTV_LPA1-T4_GMills_repeat	GSM1046314	BALB/c_p18null_133657	GSM1046927	FVB_NJ_NORMAL_CA02_451A_Lactating
GSM381098	MMTV_LPA1-T4_Mills	GSM1046315	BALB/c_p18null_200_XIONG	GSM1046928	FVB_NJ_NORMAL_CA03_603A_Lactating
GSM381108	MMTV_LPA3-T4_GMills	GSM1046316	BALB/c_p18null_205_XIONG	GSM69568	BALBc_NORMAL_MAMMARYGLAND_100989_SIMIN
GSM381109	MMTV_LPA3-T5_GMills	GSM1046317	BALB/c_p18null_228_XIONG	GSM69572	BALBc_NORMAL_MAMMARYGLAND_100990_SIMIN
GSM661693	BALB/c_p53null_spontaneous_47061_MEDINA	GSM1046318	BALB/c_p18null_250_XIONG	GSM69573	BALBc_NORMAL_MAMMARYGLAND_100991_SIMIN
GSM661705	Balbc_p53null_22478_transplant_GMURROW	GSM1046319	BALB/c_p18null_411_bRep_XIONG	GSM69574	BALBc_NORMAL_MAMMARYGLAND_100992_SIMIN
GSM662191	Balbc_p53null_transplant_21518_IH	GSM1046320	BALB/c_p18null_426_XIONG	GSM69575	BALBc_NORMAL_MAMMARYGLAND_100993_SIMIN
GSM662235	Balbc_p53null_transplant_753R_IH	GSM1046321	BALB/c_p18null_474_XIONG	GSM69631	FVB_NJ_NORMAL_MAMMARYGLAND_CA02_450A
GSM662244	FVB_C3(1)-Tag_123049_untreated_GMURROW	GSM1046322	BALB/c_p18null_854_XIONG	GSM69632	FVB_NJ_NORMAL_MAMMARYGLAND_CA02_459A
GSM69587	B6DZF1_Wap_T121_580_SIMIN	GSM1046472	FVB_C3(1)-Tag_116966_untreated_GMURROW	GSM69633	FVB_NJ_NORMAL_MAMMARYGLAND_CA04_677A
GSM69598	FVB_DMBA_11_Spindee_GLAZER	GSM1046473	FVB_C3(1)-Tag_117485_untreated_GMURROW	GSM69634	FVB_NJ_NORMAL_MAMMARYGLAND_CA04_678A
GSM69599	FVB_DMBA_12_Spindee_GLAZER	GSM1046475	FVB_C3(1)-Tag_120209_no_treatment_PEROU	GSM69635	FVB_NJ_NORMAL_MAMMARYGLAND_CA04_679A
GSM69600	FVB_DMBA_13_Spindee_GLAZER	GSM1046476	FVB_C57B6_C3(1)_Tag_117122_HERSCH		
GSM69610	FVB_MMTV_Neu_404_GREEN	GSM1046477	FVB_MMTV_Ab1_H323_MBROWN		
GSM800430	C57B6_MMTV_Cre_Brc1LoCo_p53het_108b_FURTH	GSM1046478	FVB_MMTV_Ab1_4225_MBROWN		
GSM800436	C57B6_MMTV_Cre_Brc1LoCo_p53het_88c1_FURTH	GSM1046479	FVB_MMTV_Ab1_4246_MBROWN		
GSM800437	C57B6_MMTV_Cre_Brc1LoCo_p53het_88a2_FURTH	GSM1046481	FVB_MMTV_Ab1_4355_MBROWN		
GSM800453	BALB/c_p53null_26578_PEROU	GSM1046482	FVB_MMTV_Ab1_459_MBROWN		
GSM800456	BALB/c_p53het_18_C1301_4_KOLLER	GSM1046483	FVB_MMTV_Ab1_T34_MBROWN		
		GSM1046484	FVB_MMTV_Ab1_T36_MBROWN		
		GSM1046485	FVB_MMTV_Ab1_T8_MBROWN		
		GSM1046486	FVB_NR_MMTV_FGF3_465_VARMUS		
		GSM1046487	FVB_NR_MMTV_FGF3_466_VARMUS		
		GSM1046488	FVB_NR_MMTV_FGF3_467_VARMUS		
		GSM1046489	FVB_NR_MMTV_FGF3_468_VARMUS		
		GSM1046490	FVB_NR_MMTV_FGF3_469_VARMUS		
		GSM1046491	NA_MMTV_HRAS_29_VARMUS		
		GSM1046492	NA_MMTV_HRAS_43_VARMUS		
		GSM1046493	NA_MMTV_HRAS_4379-1_Li		
		GSM1046494	NA_MMTV_HRAS_44_VARMUS		
		GSM1046495	NA_MMTV_HRAS_4492_Li		
		GSM1046496	NA_MMTV_HRAS_4633_Li		
		GSM1046497	NA_MMTV_HRAS_4861_Li		
		GSM1046498	NA_MMTV_HRAS_4863_Li		
		GSM1046700	FVB_MMTV_Myc_128491_untreated_GMURROW		
		GSM1046702	FVB_MMTV_Myc_128492_untreated_GMURROW		
		GSM1046704	FVB_MMTV_Myc_128620_untreated_GMURROW		
		GSM1046707	FVB_MMTV_Myc_128818_untreated_GMURROW		
		GSM1046710	FVB_MMTV_Myc_128990_untreated_GMURROW		
		GSM1046714	FVB_MMTV_Neu_116843-2_untreated_GMURROW		
		GSM1046717	FVB_MMTV_Neu_641971_PEROU		
		GSM1046720	FVB_MMTV_PyMT_103571_untreated_GMURROW		
		GSM1046723	FVB_MMTV_PyMT_103572-1_untreated_GMURROW		
		GSM1046725	FVB_MMTV_PyMT_2097_SHAW		
		GSM1046728	FVB_MMTV_PyMT_2577_SHAW		
		GSM1046730	FVB_MMTV_PyMT_3117_SHAW		
		GSM1046733	FVB_MMTV_PyMT_3937_SHAW		
		GSM1046736	FVB_MMTV_PyMT_4237_SHAW		
		GSM1046738	FVB_MMTV_PyMT_574_PEROU		
		GSM1046741	FVB_MMTV_Wnt1_119265-2_cn_Usary		
		GSM1046744	FVB_MMTV_Wnt1_227.8T_COWIN		
		GSM1046747	FVB_MMTV_Wnt1_28T.4T_COWIN		
		GSM1046749	FVB_MMTV_Wnt1_531_USARY		
		GSM1046752	FVB_MMTV_Wnt1_9T.8T_COWIN		
		GSM1046755	FVB_MMTV_Wnt1_CA03_613A_CHURCHILL		
		GSM1046757	FVB_MMTV_Neu_121304-2 Non-treated		
		GSM1046761	FVB_MMTV_Neu_121575 Non-treated		
		GSM1046766	FVB_MMTV_Neu_121576 Non-treated		
		GSM1046769	FVB_MMTV_Neu_123299 Non-treated		
		GSM1046774	FVB_MMTV_PyMT_117704_Non-treated		
		GSM1046778	FVB_MMTV_PyMT_118021_Non-treated		
		GSM1046781	FVB_MMTV_Wnt1_124607_Non-treated		
		GSM1046784	FVB_MMTV_Wnt1_125799_Non-treated		
		GSM1046929	FVB_Wap_Myc_116885_untreated_GMURROW		
		GSM1046930	FVB_Wap_Myc_117166_PEROU		
		GSM1046931	FVB_Wap_Myc_117250_PEROU		
		GSM1046932	FVB_Wap_Myc_117252_PEROU		
		GSM1046933	FVB_Wap_Myc_117254_PEROU		
		GSM1046934	FVB_Wap_Myc_117318_bRep_PEROU		
		GSM1046935	FVB_Wap_Myc_CA04_812A_CHURCHILL		
		GSM1046936	FVB_Wap_Myc_CA05_868A_CHURCHILL		
		GSM1046937	Mixed_Rb_7 Oct22.07_UHNC		
		GSM1046938	PIK3CA_Egan_3093		
		GSM1046939	PIK3CA_Egan_7048A		
		GSM1046940	PIK3CA_Egan_A0853		
		GSM1046941	PIK3CA_Egan_3073		
		GSM1046942	PIK3CA_Egan_4714		
		GSM1046943	MMTV-Cre_R26-Pk3caH107R_7281A_2R-Egan		
		GSM1046944	MMTV-Cre_R26-Pk3caH107R_7286A_5L-Egan		
		GSM1046945	PIK3CA_Egan_9529		
		GSM1046946	MMTV-Cre_R26-Pk3caH107R_9529_4R-Egan		
		GSM1046947	MMTV-Cre_R26-Pk3caH107R_9529_5L-Egan		
		GSM1046948	PIK3CA_Egan_A0255		
		GSM1046949	MMTV-Cre_R26-Pk3caH107R_A0835_3L-Egan		
		GSM1046950	STAT1-J_Schreiber_11253		
		GSM1046951	STAT1-J_Schreiber_4524		
		GSM1046952	STAT1-J_Schreiber_9444		
		GSM1046953	STAT1-J_Schreiber_TAC59		
		GSM1046954	STAT1-J_Schreiber_TAC77		
		GSM1046955	STAT1-J_Schreiber_TAC78		
		GSM1046956	STAT1-J_Schreiber_TAC80		
		GSM211515	Brg1+/- murine mammary tumor (MIXED_Brg1het_T135_BULTMAN)		
		GSM211516	Brg1+/- murine mammary tumor (MIXED_Brg1het_T160_BULTMAN)		
		GSM211517	Brg1+/- murine mammary tumor (MIXED_Brg1het_T172_BULTMAN)		
		GSM211518	Brg1+/- murine mammary tumor (MIXED_Brg1het_T172_BULTMAN_biorep)		
		GSM211520	Brg1+/- murine mammary tumor (MIXED_Brg1het_T2023_BULTMAN)		

GSM211521	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T2134_BULTMAN)
GSM211522	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T221_BULTMAN)
GSM211523	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T284_BULTMAN)
GSM211524	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T284_BULTMAN_biorep)
GSM211525	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T372_BULTMAN)
GSM211526	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T55_BULTMAN)
GSM211527	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T646_BULTMAN)
GSM211528	Brg1 ^{+/+} -murine mammary tumor (MIXED_Brg1het_T920_BULTMAN)
GSM237856	MIXED_Wap_Cre_ETV6_NTRK3_C117T1_ORKIN
GSM237857	MIXED_Wap_Cre_ETV6_NTRK3_C383T1_ORKIN
GSM237858	MIXED_Wap_Cre_ETV6_NTRK3_C395T5_ORKIN
GSM237859	MIXED_Wap_Cre_ETV6_NTRK3_CR115_ORKIN
GSM237860	MIXED_Wap_Cre_ETV6_NTRK3_CR90-1_ORKIN
GSM237861	MIXED_Wap_Cre_ETV6_NTRK3_D1471_ORKIN
GSM237862	MIXED_Wap_Cre_ETV6_NTRK3_D1472_ORKIN
GSM237863	MIXED_Wap_Cre_ETV6_NTRK3_D203T1_ORKIN
GSM237864	MIXED_Wap_Cre_ETV6_NTRK3_D254T1_ORKIN
GSM237865	MIXED_Wap_Cre_ETV6_NTRK3_D279T1_ORKIN
GSM237866	MIXED_Wap_Cre_ETV6_NTRK3_D481_ORKIN
GSM237867	MIXED_Wap_Cre_ETV6_NTRK3_E93T1_ORKIN
GSM381089	Agilent-8440_4
GSM381090	Agilent-8440_3
GSM381091	Agilent-8436_4
GSM381092	Agilent-8439_1
GSM381093	Agilent-8439_3
GSM381094	Agilent-8440_4
GSM381095	Agilent-9441_1
GSM381096	Agilent-8440_2
GSM381099	Agilent-9440_1
GSM381100	Agilent-9441_2
GSM381101	Agilent-9441_3
GSM381102	Agilent-8439_2
GSM381103	Agilent-8437_1
GSM381104	Agilent-9440_2
GSM381105	Agilent-8437_4
GSM381106	Agilent-8439_4
GSM381107	Agilent-8437_2
GSM447004	FVB_MMTV_Wnt1_2406_4R_Rosen
GSM447559	FVB_MMTV_Wnt1_2494_2L_Rosen
GSM447560	FVB_MMTV_Wnt1_2495_1L_Rosen
GSM447561	FVB_MMTV_Wnt1_2543_4L_Rosen
GSM447562	FVB_MMTV_Wnt1_3065_Rosen
GSM447563	FVB_MMTV_Wnt1_3718_3R_Rosen
GSM447564	FVB_MMTV_Wnt1_JFGR1_2549_4L_ROSEN
GSM447565	FVB_MMTV_Wnt1_JFGR1_3851_5L_ROSEN
GSM447566	FVB_MMTV_Wnt1_JFGR1_4004_3L_ROSEN
GSM447567	FVB_MMTV_Wnt1_JFGR1_4013_3L_ROSEN
GSM447568	FVB_MMTV_Wnt1_JFGR2_2960_5L_ROSEN
GSM447569	FVB_MMTV_Wnt1_JFGR2_3068_2L_ROSEN
GSM447570	FVB_MMTV_Wnt1_JFGR2_3855_3R_ROSEN
GSM447571	FVB_MMTV_Wnt1_JFGR2_3861_3L_ROSEN
GSM447572	FVB_MMTV_Wnt1_JR2_5101_3L_Rosen
GSM447573	FVB_MMTV_Wnt1_JR2_5103_3L_Rosen
GSM447574	FVB_MMTV_Wnt1_JR2_5105_3R_Rosen
GSM447575	FVB_MMTV_Wnt1_JR2_5106_3L_Rosen
GSM660763	Balbc_p53null_transplant_T1-3
GSM661689	Balbc_p53het_IR_A2989-7_KOLLER_44K
GSM661692	Balbc_p53het_IR_C0323-4_KOLLER_44K
GSM661694	BALBc_p53null_spontaneous_4100R_MEDINA
GSM661695	BALBc_p53null_spontaneous_4127R_MEDINA
GSM661696	BALBc_p53null_spontaneous_4702L_MEDINA
GSM661697	BALBc_p53null_spontaneous_4729L_MEDINA
GSM661698	BALBc_p53null_spontaneous_2297R_MEDINA
GSM661699	Balbc_p53null_2153R_transplant_JIH
GSM661700	Balbc_p53null_2208L_transplant_JIH
GSM661702	Balbc_p53null_2225L_transplant_JIH
GSM661703	Balbc_p53null_2225R_transplant_JIH
GSM661704	Balbc_p53null_2245R_transplant_JIH
GSM661706	Balbc_p53null_2250L_transplant_JIH
GSM661707	Balbc_p53null_2334L_transplant_JIH
GSM661708	Balbc_p53null_2333R_transplant_JIH
GSM661709	Balbc_p53null_T2-336L_transplant_JIH
GSM661710	Balbc_p53null_T7_transplant_JIH
GSM661711	Balbc_p53null_transplant_2151L_JIH
GSM661712	Balbc_p53null_transplant_2151L_JIH_repeat
GSM661712	Balbc_p53null_transplant_2153L_JIH
GSM661713	Balbc_p53null_transplant_2154L_JIH
GSM661714	Balbc_p53null_transplant_2209R_JIH
GSM661715	Balbc_p53null_transplant_2210L_JIH
GSM661716	Balbc_p53null_transplant_2211L_JIH
GSM661717	Balbc_p53null_transplant_2211R_JIH
GSM661718	Balbc_p53null_transplant_2224L_JIH
GSM661719	Balbc_p53null_transplant_2228R_JIH
GSM661720	Balbc_p53null_transplant_2243L_JIH
GSM661721	Balbc_p53null_transplant_2249L_JIH
GSM661722	Balbc_p53null_transplant_2331L_JIH
GSM661723	Balbc_p53null_transplant_2336R_JIH
GSM661724	Balbc_p53null_transplant_2349L_JIH
GSM661725	Balbc_p53null_transplant_2349R_JIH
GSM661726	Balbc_p53null_transplant_2350R_JIH
GSM661727	Balbc_p53null_transplant_2356R_JIH
GSM661728	Balbc_p53null_transplant_2374L_JIH
GSM661729	Balbc_p53null_transplant_2374R_JIH
GSM661730	Balbc_p53null_transplant_2377R_JIH
GSM661731	Balbc_p53null_transplant_2393R_JIH
GSM661732	Balbc_p53null_transplant_2396R_JIH
GSM661733	Balbc_p53null_transplant_2397L_JIH
GSM661734	Balbc_p53null_transplant_2412R_JIH
GSM661735	Balbc_p53null_transplant_2530R_JIH
GSM661736	Balbc_p53null_transplant_T1-355-3_JIH
GSM662237	FVB_C3(1)-Tag-120555_PEROU
GSM662238	FVB_C3(1)-Tag-121415_PEROU
GSM662239	FVB_C3(1)-Tag-116409C_PEROU
GSM662240	FVB_C3(1)-Tag-116410B_PEROU
GSM662241	FVB_C3(1)-Tag-117338-1_PEROU
GSM662242	FVB_C3(1)-Tag-117517_PEROU
GSM662243	FVB_C3(1)-Tag-120157_PEROU
GSM662245	FVB_MMTV_Neu_110277_PEROU
GSM662246	FVB_MMTV_Neu_110286_PEROU
GSM662247	FVB_MMTV_Neu_116913_PEROU

GM662248	FVB_MMTV_Neu_116916_PEROU
GM662249	FVB_MMTV_Neu_116922_PEROU
GM662250	FVB_MMTV_Neu_117391_PEROU
GM69576	BALB_BRCA1het_p53het_IR_B1129_4_KOLLER
GM69577	BALB_BRCA1het_p53het_IR_B9964_6_KOLLER
GM69578	BALB_BRCA1het_p53het_IR_B9965_1_KOLLER
GM69579	BALB_BRCA1het_p53het_IR_C0379_5_KOLLER
GM69580	BALB_p53het_IR_10915_7_KOLLER
GM69581	BALB_p53het_IR_A1446_1_KOLLER_repeat
GM69582	BALB_p53het_IR_A2989_7_KOLLER
GM69583	BALB_p53het_IR_A5824_7_KOLLER
GM69584	BALB_TgWAPT121_KS555
GM69585	BALB_TgWAPT121_KS556
GM69586	BOF1_TgWAPT121p53het_KS581_Solid
GM69588	BOF1_TgWAPT121_KS643_Solid
GM69589	BOF1_TgWAPT121_KS644_Solid+Gland
GM69590	FVB_C3(1)_Tag_#72_GREEN
GM69591	FVB_C3(1)_Tag_#74_GREEN
GM69592	FVB_C3(1)_Tag_#76_GREEN
GM69593	FVB_C3(1)_Tag_#84_GREEN
GM69594	FVB_C3(1)_Tag_#86_GREEN
GM69595	FVB_C3(1)_Tag_E29_1A_614_BROWN
GM69596	FVB_C3(1)_Tag_E29_2A_632_BROWN
GM69597	FVB_C3(1)_Tag_E29_5A_645_BROWN
GM69601	FVB_DMB_A_1_Adeno_GLAZER_repeat
GM69602	FVB_DMB_A_2_Adeno_GLAZER
GM69603	FVB_DMB_A_3_Adeno_GLAZER_repeat
GM69604	FVB_DMB_A_4_Adeno_GLAZER
GM69605	FVB_DMB_A_5_Squa_GLAZER
GM69606	FVB_DMB_A_6_Squa_GLAZER
GM69607	FVB_DMB_A_8_Squa_GLAZER
GM69608	FVB_DMB_A_9_Squa_GLAZER
GM69609	FVB_DMB_A_9_Squa_GLAZER_repeat
GM69611	FVB_MMTV_Neu_7-6-99_GREEN
GM69612	FVB_MMTV_Neu_8-2-99_GREEN
GM69613	FVB_MMTV_Neu_CA01_416A_CHURCHILL
GM69614	FVB_MMTV_Neu_CA01_416C_repeat_CHURCHILL
GM69615	FVB_MMTV_Neu_CA01_431A_CHURCHILL
GM69616	FVB_MMTV_Neu_CA01_432A_CHURCHILL
GM69617	FVB_MMTV_PyVT_31_SIMIN
GM69618	FVB_MMTV_PyVT_89_SIMIN
GM69619	FVB_MMTV_PyVT_91#2_SIMIN
GM69620	FVB_MMTV_PyVT_91#3_SIMIN
GM69621	FVB_MMTV_PyVT_91_SIMIN
GM69622	FVB_MMTV_Wnt1_CA02_467A_CHURCHILL
GM69623	FVB_MMTV_Wnt1_CA02_478A_CHURCHILL
GM69624	FVB_MMTV_Wnt1_CA02_486A_CHURCHILL
GM69625	FVB_MMTV_Wnt1_CA02_493A_CHURCHILL
GM69626	FVB_MMTV_Wnt1_CA02_506A_CHURCHILL
GM69627	FVB_MMTV_Wnt1_CA02_570A_CHURCHILL
GM69628	FVB_MMTV_Wnt1_CA02_570B_CHURCHILL
GM69629	FVB_MMTV_Wnt1_CA03_587A_CHURCHILL
GM69630	FVB_MMTV_Wnt1_CA03_634A_CHURCHILL
GM69636	FVB_Wap_Int3_CA01_426A_CHURCHILL_repeat
GM69637	FVB_Wap_Int3_CA01_433A_CHURCHILL
GM69638	FVB_Wap_Int3_CA01_433A_dup2_CHURCHILL
GM69639	FVB_Wap_Int3_CA01_433A_dup_CHURCHILL
GM69640	FVB_Wap_Int3_CA01_434A_CHURCHILL
GM69641	FVB_Wap_Int3_CA01_434B_CHURCHILL_repeat
GM69642	FVB_Wap_Int3_CA02_437A_CHURCHILL
GM69643	FVB_Wap_Int3_CA02_566A_CHURCHILL
GM69644	FVB_Wap_Int3_CA02_575A_CHURCHILL
GM69645	FVB_Wap_Myc_CA02_540A_CHURCHILL
GM69646	FVB_Wap_Myc_CA02_540B_spindloid_CHURCHILL
GM69647	FVB_Wap_Myc_CA02_540B_spindloid_dup_CHURCHILL
GM69648	FVB_Wap_Myc_CA02_544A_dup_CHURCHILL
GM69649	FVB_Wap_Myc_CA02_545A_CHURCHILL
GM69650	FVB_Wap_Myc_CA02_548A_CHURCHILL
GM69651	FVB_Wap_Myc_CA02_549A_CHURCHILL
GM69652	FVB_Wap_Myc_CA02_550A_spindloid_CHURCHILL
GM69653	FVB_Wap_Myc_CA02_567C_CHURCHILL
GM69654	FVB_Wap_Myc_CA02_569A_CHURCHILL
GM69655	FVB_Wap_Myc_CA02_579C_CHURCHILL
GM69656	FVB_Wap_Myc_CA02_579F_CHURCHILL
GM90424	FVB_MMTV_PyVT_575_Usary
GM90425	FVB_MMTV_PyVT_430_Usary
GM90426	FVB_MMTV_Neu_CA05_861A_CHURCHILL
GM90427	FVB_MMTV_Neu_CA05_875A_CHURCHILL
GM90428	FVB_Wap_Myc_CA05_869A_CHURCHILL
GM90429	FVB_MMTV_Wnt1_CA04_676A_CHURCHILL
GM90431	C57BL_6J_Wap_Tag_CA_224A_CHURCHILL
GM90432	FVB_Wap_Myc_CA05_867A_CHURCHILL
GM90433	C57BL_6J_Wap_Tag_CA_215A_CHURCHILL
GM90434	C57BL_6J_Wap_Tag_CA_213A_CHURCHILL
GM90435	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_106c1_FURTH
GM90436	BALB_BRCA1het_p53het_IR_C0912_13_KOLLER
GM90439	BALB_BRCA1het_p53het_IR_C0917_4_KOLLER
GM90440	BALB_BRCA1het_p53het_IR_C0912_12_KOLLER
GM90441	BALB_p53het_IR_C0323_4_KOLLER
GM90442	BALB_p53het_IR_C1301_1_KOLLER
GM90443	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_129_FURTH
GM90444	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_172d_FURTH
GM90445	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_145a2_FURTH
GM90446	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_96b_FURTH
GM90447	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_113a_FURTH
GM90448	C57BL6_MMTV_Cre_BRCA1CoCo_p53het_100a_FURTH
GM90449	BOF1_WAP_T121_KS150_SIMIN
GM90450	FVB_MMTV_Neu_59331_USARY
GM90451	PB_p53null_3941R
GM90452	PB_p53null_3939R
GM90454	FVB_MMTV_Wnt1_CA04_683A_CHURCHILL
GM90455	C57BL_6J_Wap_Tag_CA_226A_CHURCHILL
GM90457	C57BL_6J_Wap_Tag_CA_226B_CHURCHILL
GM90458	PB_p53null_1634R
GM90459	PB_p53null_4304R