Targeting Axl favors an antitumorigenic microenvironment that enhances immunotherapy responses by decreasing Hif-1α levels

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Hypoxia is an important phenomenon in solid tumors that contributes to metastasis, tumor microenvironment (TME) deregulation, and resistance to therapies. The receptor tyrosine kinase Axl is an HIF target, but its roles during hypoxic stress leading to the TME deregulation are not well defined. We report here that the mammary gland–specific deletion of Axl in a HER2 mouse model of breast cancer leads to a normalization of the blood vessels, a proinflammatory TME, and a reduction of lung metastases by dampening the hypoxic response in tumor cells. During hypoxia, interfering with Axl reduces HIF-1α levels altering the hypoxic response leading to a reduction of hypoxia-induced epithelial-to-mesenchymal transition (EMT), invasion, and production of key cytokines for macrophages behaviors. These observations suggest that inhibition of Axl generates a suitable setting to increase immunotherapy. Accordingly, combining pharmacological inhibition of Axl with anti–PD-1 antibody in a preclinical model of HER2 breast cancer reduces the primary tumor and metastatic burdens, suggesting a potential therapeutic approach to manage HER2 patients whose tumors present high hypoxic features.

AXL | HER2 | hypoxia | tumor microenvironment | Immunotherapy

Significance

A significant pool of HER2 breast cancer patients are either unresponsive or become resistant to standards of care. New therapeutic approaches exploiting the tumor microenvironment, including immunotherapies, are attractive. Hypoxia shapes the tumor microenvironment toward therapy resistance and metastasis. Here, we report a role for AXL receptor tyrosine kinase in the hypoxic response by promoting HIF-1α expression. Interfering with Axl in a preclinical model of HER2 breast cancer normalizes the blood vessels and promotes a proinflammatory microenvironment that enhances immunotherapy response to reduce the primary and metastatic tumor burdens. Clinical trials so far suggest that achieving immunotherapy responses in HER2+ cancers might be challenging, and our data might provide an important insight to circumvent a roadblock.

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The authors declare no competing interest.

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immune-suppressive phenotype (CD206+) and also presented an increase in the expression of MHC class II (I-A/I-E), consistent with a proinflammatory activation state in the host, suggesting that this deregulation is a consequence of losing Axl in the cancer cells themselves. Furthermore, when investigating the immune profile of Neu+ Axl knockout (KO) mice were crossed with the HER2+ breast cancer model that expresses an activated form of the HER2 rat ortholog in the mammary gland under the control of mouse mammary tumor virus (MMTV, NeuNLDL2-5). This Neu+ mouse model develops breast tumors that spontaneously metastasize to the lungs, and we previously reported that Neu+ Axl− mice are protected from this metastatic progression without affecting primary tumor growth (20).

To address this, we collected the primary tumors 5 wks after the tumor onset to characterize their TME by immunostaining. Neu+ Axl− tumors showed lower hypoxia levels as indicated by the decrease of the mean fluorescence intensity of two independent hypoxia markers, carbon monoxide and GLUT-1 (Fig. 1D and SI Appendix, Fig. S1A). We next investigated the structural and functional changes in blood vessels and found that Neu+ Axl− tumor blood vessels covered a larger area and had increased diameters when compared to control tumors (Fig. 1E and SI Appendix, Fig. S1B). They were less permeable, as suggested by a reduction of extravascular fibrinogen leakiness, and were better perfused, as demonstrated by intravenous injection of labeled Lectin (Fig. 1 F and G). The vessels also presented an increased coverage by αSMA+ pericytes and Laminin+ basement membrane (BM) (Fig. 1 H and I). Thus, the decrease in intratumoral hypoxia and the vessel normalization observed could contribute to the reduction of metastasis observed in Neu+ Axl− mice.

Moreover, Neu+ Axl− tumors showed a differential immune cells profile compared to control tumors, as assessed by a flow cytometry analysis of a panel of lymphoid and myeloid markers (Fig. J). Interestingly, macrophages were less polarized toward an inflammatory activation state in the Neu+ Axl− tumors (Fig. J). While CD3+ lymphocytes were recruited into the tumor core, as shown by staining of tumor sections, no significant increase of this infiltration in Neu+ Axl− tumors was observed (SI Appendix, Fig. SIC). Nevertheless, the difference of infiltration suggested a possible enhancement of antitumor response with an increase of natural killer (NK) cell activation (as measured by higher CD44 expression) and a significant reduction in the number of regulatory T cells (Treg) in Neu+ Axl− tumors (Fig. J). Collectively, these results suggest that deleting Axl reduced the intratumoral hypoxia, normalized the blood vessels, and mediated a differential immune cells’ representation in tumors.

Conditional Deletion of Axl in the Mammary Epithelial Cells Generates an Antitumorigenic Microenvironment and Reduces the Metastatic Burden. We next asked whether the effects of Axl KO were driven by Axl deletion in the tumor cells intrinsically or by its deletion in cells present in the microenvironment such as immune or endothelial cells. We generated primary tumor cell lines from Neu−/− Axl− and Neu+ Axl− hosts and transplanted them to syngeneic wild-type FVB mice. Notably, RNA sequencing of Neu−/− Axl− tumor cell lines confirmed the gene expression signatures linked to the TME deregulation found initially in the tumors from Neu−/− Axl− mice (SI Appendix, Fig. S2 A and B and Table S2). Neu+ Axl− tumor grafts also presented less hypoxia and produced blood vessels with a decreased permeability, increased perfusion, and increased pericytes and BM coverage (SI Appendix, Fig. S2 C–I). Thus, the normalized blood vessel phenotype could be reproduced when Neu+ Axl− cells were grafted in a wild-type host, suggesting that this deregulation is a consequence of losing Axl in the cancer cells themselves. Furthermore, when investigating the immune profile of Neu+ Axl− tumor grafts by flow cytometry, we found that macrophages were polarized toward a proinflammatory activation state (CD206+, I-A/I-E+), and there was a reduction in neutrophils compared to the controls (SI Appendix, Fig. S2J). These myeloid cells are known to regulate the TME by producing various inflammatory cytokines and proangiogenic factors, suggesting that Axl-expressing cancer cells could educate immune cells to contribute to the TME remodeling (26, 27). Thus, this experiment indicated that the TME changes observed in Neu−/− Axl− mice have been orchestrated by the cancer cells.

To confirm the cancer cell–autonomous role of Axl in the metastatic progression and the deregulation of the TME, Axl conditional KO mice were crossed with MMTV-NIC mice (MMTV-NeuNLDL2-5-IREs-Cre) to obtain a specific deletion in the mammary epithelial cells (SI Appendix, Fig. S3A). Weekly monitoring of cohorts of NIC−/− Axl+/+, NIC−/− Axl+fx/fx, and NIC−/− Axl+fxfx mice by palpation suggested no difference in tumor initiation (Fig. 2A). Moreover, there was no difference in the number of mammary intraepithelial neoplastic lesions 5 wks after tumor onset (SI Appendix, Fig. S3B). The tumor and metastatic burdens were also analyzed 5 wks after the appearance of the first mass. No difference in the total tumor weight per mouse was observed, and there were no significant changes in the proliferative and apoptotic status in the tumors (Ki67 or TUNEL, respectively) (Fig. 2B and SI Appendix, Fig. S3 C and D). Importantly, the loss of Axl altered the lung metastatic frequency and burden, as we observed in total AdKO mice (20), suggesting a strong cancer cell–autonomous role in the phenotype (Fig. 2C and SI Appendix, Fig. S3E). Furthermore, the number of circulating tumor cells in the blood of the mice was reduced in NIC−/− Axl+fxfx and NIC−/− Axl− animals, suggesting that the cancer cells may be poorly invasive, as we have shown in ref. 20, and/or that the TME is unfavorable for intravasation (Fig. 2D). Thus, we decided to investigate whether the TME could also be rewired toward a tumor-suppressive phenotype in those tumors and consequently contribute to limit metastasis. NIC−/− Axl+fxfx tumors displayed reduced hypoxia levels, and the blood vessels looked functionally normalized (Fig. 2 E–I and SI Appendix, Fig. S3 F and G). Similarly, vessels in NIC−/− Axl+fxfx tumors were less permeable, more perfused, and they presented a better pericyte and BM coverage as shown by immunostaining (Fig. 2 E–I). Furthermore, we investigated immune cell infiltration by flow cytometry to assess whether Axl deletion in the tumor cells could also generate a better immune response. Interestingly, macrophages and neutrophils were less present in NIC−/− Axl+fxfx tumors, and again, those macrophages showed a proinflammatory activation...
AXL Is Required to Sustain HIF-1α Levels under Hypoxic Conditions.

We found that removing Axl in the cancer cells in vivo reduced the circulating tumor cells and metastasis. This could be explained by the reduction of hypoxia found in the NIC−/AxlKO tumors that could lead to a decrease of tumor cell invasion, normalized blood vessels, and increased immunosurveillance. Furthermore, we previously reported the interaction and crosstalk between AXL and HER2 to promote metastasis (20). This led us to explore their roles in hypoxic stress using tumor cell lines derived from the NIC−/AxlKO and NIC−/AxlKO mouse model, termed NIC WT (wild type) and Axl KO (knockout). Various receptor tyrosine kinases are known to regulate HIF complex levels and activity via different downstream pathways including PI3K/AKT, JAK/STAT3, and MAPK (28). For instance, HER2 regulates the synthesis of HIF-1α subunits by directly activating the PI3K/AKT/FRAP pathway (29).

**Fig. 1.** Axl contributes to the TME deregulation. (A–C) GSEA of transcriptomic data (RNA-seq) of NeuAxlKO and NeuAxlKO− tumors shows up-regulation of genes related to hypoxia (A) and angiogenesis (B) and a down-regulation of genes related to activation of immune response (C). (D) Pimonidazole staining reveals a reduction of hypoxia in NeuAxlKO tumors compared to control (n = 7 tumors, mean fluorescence intensity [MFI]) (Scale bars, 1 mm; P = 0.0488). (E) NeuAxlKO− tumors present bigger blood vessels (CD31+) with an increased total area (n = 6, * P = 0.0264). (F) Reduced blood vessels permeability is observed in NeuAxlKO− tumors with a decrease of Fibrinogen leakage in tumor tissue (Fibrinogen/CD31 ratio) (n = 6 tumors, *** P < 0.0001). (G) Intravenous injections of Rhodamine-labeled Lectin demonstrate a better perfusion of NeuAxlKO− tumor blood vessels (Colocalization Lectin/CD31) (n = 6 tumors, * P = 0.0168). (H) Staining for CD31 and alpha Smooth Muscle Actin (αSMA) reveals a better pericyte coverage of blood vessels in NeuAxlKO− tumors (Colocalization αSMA/CD31) (n = 6 tumors, ** P = 0.0055). (I) Staining for CD31 and Laminin shows more mature vessels with better BM coverage in NeuAxlKO− tumors (Colocalization Laminin/CD31) (n = 6 tumors, ** P = 0.0048). (J) NeuAxlKO− and NeuAxlKO− tumors present differences in immune cells profile as shown by FACS analyses of a panel of lymphoid and myeloid markers (n = 8 to 12 tumors). NK cells (CD3e−NK1.1+) are more activated (CD44+, *** P = 0.0001) in NeuAxlKO− tumors, and those tumors present a decrease in regulatory T cells (Treg, CD3e−CD4−CD25−FoxP3+, * P = 0.0137), an increase of B cells (CD19+IgM+, ** P = 0.0018) and of macrophages (F4/80+) expressing MHCII (I-A/I-E+, * P = 0.0253), and a decrease of macrophage expressing CD206 (* P = 0.0205) in NeuAxlKO− tumors. Data are presented as mean ± SEM. (Scale bars in E through I, 50 μm.)
Under hypoxic conditions (1% O2 for 24h), Axl KO cells showed drastically reduced Her2 and Hif-1α protein levels when compared to control cells, while Hif-2α was not affected (Fig. 3A). Proteasome inhibition with MG132 rescued Her2 expression, suggesting a role for Axl in Her2 protein stability during hypoxia (SI Appendix, Fig. S4A). Therefore, Axl may modulate Hif-1α levels by regulating Her2 stability or by directly influencing hypoxic signaling pathways. HIF-1α subunit induction by hypoxia was also abrogated upon AXL depletion using small interfering RNA (siRNA) in human MCF10A cells stably expressing HER2, validating in another system the results observed in the mouse-derived cells (SI Appendix, Fig. S4B). Furthermore, in human HER2+ breast cancer cells BT474 that do not express Axl, Axl overexpression potentiated HIF-1α induction and stabilized HER2 under hypoxia (Fig. 3B).

To demonstrate that Axl and Her2 activities are required for this effect, NIC WT cells were treated with Lapatinib (HER2 inhibitor) or R428 (AXL inhibitor). Single Her2 or Axl inhibition significantly decreased HIF-1α expression under hypoxia indicating that Her2 and Axl activity regulates Hif-1α levels under hypoxia in these cells. The combination of both inhibitors caused an additional reduction in Hif-1α levels, suggesting that Her2 and Axl can contribute independently to regulate Hif-1α expression during hypoxia (Fig. 3C). Furthermore, R428 decreased Her2 levels under hypoxia, confirming the role of Axl kinase activity on Her2 stability in hypoxic conditions (Fig. 3C). As expected, Her2 and Axl inhibition reduced pAkt levels, indicating that the PI3k/Akt pathway could be implicated in the signaling of these receptors under hypoxia. We found that PI3k inhibition with LY294002 also reduced Hif-1α levels in NIC WT cells (Fig. 3D). Thus, our results show that both Axl and Her2 can regulate Hif-1α levels under hypoxia potentially via activating the PI3k/Akt pathway. Similar results were obtained in MCF10A-HER2 cells (SI Appendix, Fig. S4 C and D). To further dissect the contribution of AXL independently of HER2 signaling, triple-negative breast cancer cells expressing high levels of AXL, Hs578T, were used. The reduction of AXL levels using an siRNA decreased HIF-1α levels in hypoxia supporting a role for AXL independently of HER2 (Fig. 3E). Furthermore, ectopic expression of Axl in Axl KO cells rescued Her2 and Hif-1α expression (Fig. 3F). However, Hif-1α up-regulation was not completely lost by Lapatinib treatment, suggesting that Axl partially contributes to

Fig. 2. Conditional Axl deletion in the mammary epithelial cells generates an antitumorigenic microenvironment and reduces the metastatic burden. (A and B) NIC-Axloxlox mice develop tumors at the same rate as control animals (A), and there is no difference in tumor mass (B) (n = 20 mice). (C) Axl deletion leads to a decrease of lung metastasis frequency (n = 20 mice, ***P < 0.0001). (D) Less circulating tumor cells are present in the blood of NIC-Axloxlox and NIC-Axloxlox mice compared to controls (n = 10 to 11 mice, *P = 0.0104 and **P = 0.0324). (E) A reduction of hypoxia in NIC-Axloxlox tumors compared to control is seen using pimonidazole staining (n = 5 tumors, *P = 0.0483). (F) Fibrinogen staining in NIC-Axloxlox tumors reveals a diminution of tumor blood vessels permeability (Fibrinogen/CD31 ratio) (n = 6 tumors, ***P = 0.0007). (G) NIC-Axloxlox tumor blood vessels are more perfused by Rhodamine-labeled Lectin (Colocalization Lectin/CD31) (n = 6 tumors, ***P < 0.0001). (H) A better pericycle coverage is observed in NIC-Axloxlox tumors blood vessels via staining with anti-CD31 and anti-iαSMα antibodies (Colocalization iαSMα/CD31) (n = 6 tumors, *P = 0.0048). (I) Staining for CD31 and Laminin reveals more mature blood vessels with better BM coverage in Neu+/Axloxlox tumors (Colocalization Laminin/CD31) (n = 6 tumors, ***P = 0.0001). (J) NIC-Axloxlox and NIC-Axloxlox tumors present differences in immune cells profile as shown by FACS analyses of a panel of lymphoid and myeloid markers (n = 6 to 8 tumors). NIC-Axloxlox tumors present a general increase of CD45+ immune cells (**P = 0.0014), an increase of CD8 and CD4 T cells (CD3e+CD8α, **P = 0.0047 and CD3e+CD4+, **P = 0.0029), and a decrease of macrophages (F4/80+, ***P = 0.0054) and neutrophils (CD11b+Ly6G, ***P = 0.0007). Macrophages were also in a more proinflammatory activation state (CD206, **P = 0.0003, I-A/I-E, *P = 0.0155). Data are represented as mean ± SEM.
Reducing Axl expression in Hs578T cells reduced HIF-1 overexpression in WT and remodeling and metastasis, we sequenced RNA isolated from NIC under hypoxic conditions to understand the role of Axl in hypoxic adaptation required for TME impact on the hypoxic transcriptome. To test this and to better understand HIF-1 regulation, indicating that PI3K/Akt acts downstream of Axl to increase HIF-1α levels under hypoxic conditions (Fig. 3F). Remarkably, AXL expression in breast cancer patient samples from the TCGA-BRCA cohort correlated with HIF-1α gene expression independently of the subtype (SI Appendix, Fig. S4E). Taken together, these results indicate that Axl is required to ensure hypoxic HIF-1α expression by stabilizing Her2 and promoting PI3K/Akt signaling. Hence, Axl can promote the deregulation of the TME and metastatic progression by regulating the HIF-1 complex during hypoxia.

**Axl Is Required for a Complete Hypoxic Response.** Since Axl KO cells reduced HIF-1α levels under hypoxia, Axl deletion could have an impact on the hypoxic transcriptome. To test this and to better understand the role of Axl in hypoxic adaptation required for TME remodeling and metastasis, we sequenced RNA isolated from NIC WT and Axl KO cells under 1% O2. We conducted a gene ontology analysis of hypoxia-up-regulated genes that revealed that NIC WT cells showed an expected hypoxic response that includes the up-regulation of genes involved in migration, angiogenesis, and cytokine secretion. However, this response was reduced in Axl KO cells (Fig. 4A and SI Appendix, Fig. S5A). Overall, there were more up-regulated genes under hypoxia in NIC WT cells (Fig. 4B). The genes specifically up-regulated in Axl KO cells during hypoxia were linked to cell cycle, cellular metabolic process, cellular response to stress, and programmed cell death, suggesting a harder adaptation to hypoxia (SI Appendix, Fig. S5B). On the other hand, the Axl-dependent genes up-regulated under hypoxic conditions were principally associated with the processes mentioned in Fig. 4A but also specifically included the HIF-1α signaling pathway (Fig. 4C).

Indeed, many HIF-1α target genes, mainly related to EMT, invasion, and immune evasion, were less expressed and/or differently modulated in the Axl KO compared to the WT cells (SI Appendix, Fig. S5C). This result suggests that Axl is essential for a complete hypoxic response. Therefore, these findings explain at least in part the changes in the TME and the reduced metastatic burden observed in our mouse models.

To investigate whether these observations can be clinically relevant, we analyzed the genes that correlate with AXL expression in breast cancer samples from METABRIC and TCGA breast cancer cohorts. In general, AXL correlated with the expression of genes linked to cell migration, inflammation, angiogenesis, and hypoxia, among others (SI Appendix, Fig. S5D). Interestingly, a significant proportion of the Axl-dependent genes induced under hypoxia and found in our RNA-seq analyses correlated with AXL expression in breast cancer patients (SI Appendix, Fig. S5E). These candidate genes were also related to processes linked to the TME and metastasis including response to O2 levels, migration, angiogenesis, and inflammation, supporting a role for AXL in the hypoxic response and the TME deregulation in vivo (SI Appendix, Fig. S5F).

**Axl Is Essential for Hypoxia-Induced Changes that Drive Metastasis and TME Remodeling.** Hypoxia can promote metastatic progression by increasing tumor cell EMT and invasion via the up-regulation of EMT transcription factors that are themselves HIF targets (10, 11). In our RNA-seq experiment, Snai2 and Twist1 were found to be up-regulated under hypoxia in an Axl-dependent manner and correlated with AXL expression in human breast cancer samples (SI Appendix, Fig. S6 A–C). This led us to hypothesize that Axl deletion could decrease the ability of cells to become more mesenchymal in a hypoxic environment. Accordingly, the increase in the levels of the signal transduction molecules PI3K and Akt was reduced in the Axl KO cells compared to the WT cells (Fig. 3F). This suggests that Axl is essential for a complete hypoxic response, as Axl deletion could have an impact on the hypoxic transcriptome.
metastasis is impaired in cells to leave the primary site as circulating tumor cells and to form with AXL. This further supports the notion that the ability of cancer hypoxia-induced EMT and invasion/migration when interfering with AXL by KO, siRNA, or R428 treatment reduced invasion assays (Fig. 5A). The same phenomenon was observed in hypoxic NIC WT and Axl KO cells, and this was rescued by re-expression of Axl (Fig. 5A). The phenomenon was also reproduced in wound-healing assays in which AXL was re-quired for hypoxia-induced cell migration (Fig. 5B). We next tested the role of AXL on hypoxia-induced cell motility. We found that hypoxia increased the invasion of NIC cells and MCF10A-HER2 cells in Boyden–Matrigel invasion assays (Fig. 5B and SI Appendix, Fig. S6E). In both cell lines, interferring with AXL by KO, siRNA, or R428 treatment reduced the basal invasion in normoxia and inhibited the promotion of invasion by hypoxia (Fig. 5B and SI Appendix, Fig. S6E). This was also reproduced in wound-healing assays in which AXL was required for hypoxia-induced cell migration (Fig. 5C and D and SI Appendix, Fig. S6 F and G). These results demonstrate defects in hypoxia-induced EMT and invasion/migration when interfering with AXL. This further supports the notion that the ability of cancer cells to leave the primary site as circulating tumor cells and to form metastasis is impaired in NIC Axl KO mice.

Furthermore, cancer cells boost the production of different cytokines to remodel the TME and promote immune suppression under hypoxic conditions (10, 30). Interestingly, our RNA-seq data revealed that Axl removal affects the messenger RNA (mRNA) expression of some chemokines including C-C Motif Chemokine Ligand 2 (Ccl2), Colony-Stimulating Factor 1 (Csf1), C-X-C Motif Chemokine Ligand, and 2 (Cxc1l and Cxc2l) under hypoxia (SI Appendix, Fig. S7A). A cytokine profiling by a multiplex array was done to assess the secretion by WT and Axl KO cells of a variety of chemokines (SI Appendix, Table S3). Indeed, Axl KO cells secreted lower amounts of Ccl2, Csf1, Cxc1l, and Cxc2l when subjected to hypoxic conditions (Fig. 6A and SI Appendix, Fig. S7 B–D and Table S3). Interestingly, among these candidates, CCL2 expression correlated with AXL expression in human breast cancer, suggesting a possible clinical relevance of this finding (SI Appendix, Fig. S7E).

CCL2 is a myeloid and lymphoid cell chemotactant that promotes the recruitment of tumor-associated macrophages and affects their behavior in the TME (26, 31, 32). Tumor-associated macrophages are a major stromal component and can be reprogrammed by environmental signals into specialized subtypes including a spectrum of antitumoral (proinflammarory) or pro-tumoral (immune-suppressive or wound-healing) phenotypes (33–36). Importantly, we observed a reduction in macrophage numbers and polarization toward a protumoral phenotype in our Axl KO breast cancer mouse models (Fig. 7J and SI Appendix, Fig. S2J, and Fig. 2J). This infiltration was also validated by staining in the NIC Axl KO and NIC + Axl tumors (Fig. 6B). Furthermore, these infiltrated macrophages present a decrease of proliferation in Axl-null tumors as demonstrated by fluorescence-activated cell sorting (FACS) analysis of staining against phospho-Histone 3 (Fig. 6C). Therefore, we tested in vitro the requirement of Axl in tumor cells for macrophage proliferation, invasion, and polarization during hypoxia by treating bone marrow–derived macrophages with conditioned media (CM) from normoxic and hypoxic NIC WT and Axl KO cells. First, we observed that CM from hypoxic Axl KO cells was less efficient to promote the proliferation of macrophages (Fig. 6D). The addition of a neutralizing antibody against Ccl2 in WT CM also moderately reduced their proliferation, suggesting a partial implication of Ccl2 in this phenotype (Fig. 6D). Furthermore, CM from hypoxic NIC WT cells increased macrophage invasion compared to normoxic CM (Fig. 6E). However, CM from hypoxic Axl KO cells failed to stimulate invasion to the same extent, suggesting that Axl deletion in tumor cells can also affect the invasion and recruitment of macrophages in the tumor (Fig. 6E). In this case, we revealed a role for Ccl2 as shown by a reduction in invasion when an anti-Ccl2 antibody was added to the hypoxic WT CM (Fig. 6E). Finally, we investigated the effect of the hypoxic NIC CM on the polarization of bone marrow–derived macrophages. First, the immunosuppressive macrophage marker CD206 was less expressed in macrophages treated with hypoxic CM from Axl KO cells compared to control, correlating with in vivo observations (Fig. 6F). This phenotype was also partially recapitulated by the inhibition of Ccl2 (Fig. 6F). Moreover, macrophages treated with hypoxic Axl KO CM secreted less factors known to promote TME deregulation and angiogenesis such as Vegfa, Ccl1, and Ccl2 (Fig. 6G–I). These results suggest that Axl is required for the secretion of factors, such as Ccl2, leading to macrophage proliferation, invasion, and polarization when cancer...
cells are subjected to hypoxia. Consequently, this could affect their recruitment and reprogramming to promote angiogenesis and immune suppression to affect the TME and potentially the metastatic progression in our model of HER2+ breast cancer.

**Axl Inhibition Generates an Antitumorigenic TME and Enhances Anti-PD-1 Immunotherapy Response.** Clinical trials so far suggest that achieving responses to immunotherapy in HER2+ cancers is challenging (3, 4), and a better understanding of the HER2+ TME may help to better classify possible responders. Indeed, there is a growing clinical need to define factors that modulate response to immunotherapies and specifically checkpoint inhibitors (3, 4, 37). The influence of hypoxia in the TME has been suggested to be one of those factors (38). As such, we chose to investigate the landscape of hypoxia among different molecular subtypes of breast cancer. Toward this goal, we performed a single-sample GSEA (ssGSEA). Hypoxia signatures scores, when applied to data from TCGA-BRCA, GSE8644, and METABRIC breast cancer samples (39–41), demonstrated that basal and HER2+ subtypes present higher levels of hypoxia when compared to other breast cancer subtypes (Fig. 7A and B and SI Appendix, Fig. S8A–D). Additionally, HER2+ patients whose tumors display higher hypoxia scores are associated with shorter overall and disease-free survival, suggesting that HER2+ tumors with evidence of elevated signatures of hypoxia are more aggressive (Fig. 7C and SI Appendix, Fig. S8E–K).

In our experiments, hypoxic response elements, identified to be regulated by Axl, EMT, and tumor cell–macrophage interactions, can promote immune evasion and have been established as roadblocks to efficient immunotherapy (26, 42). Moreover, vascular normalization, as observed in Neu−/Axl−/− and Nic−/AxlKO tumors, was demonstrated to enhance cancer immunotherapy (16, 43). Collectively, these observations suggest AXL as a druggable candidate to generate an improved setting for immunotherapy. This could be promising for HER2+ patients that present prominent features of hypoxia.

Therefore, Neu+mice were treated individually and in combination with anti–PD-1 and AXL small-molecule inhibitor R428, as illustrated in SI Appendix, Fig. S9A. We observed that R428 treatment efficiently modulated the TME similarly to what we observed in total and conditional KO models. Indeed, there was a reduction of intratumoral hypoxia and a normalization of the blood vessels characterized by decreased permeability, increased perfusion, and improved pericyte coverage as shown by immunostaining (Fig. 7D–G). Moreover, flow cytometry experiments showed that upon Axl inhibition, macrophages presented a proinflammatory activation state characterized by a reduction of CD206 and an increase of MCH-II (I-A/I-E) expression, confirming the effect of Axl inhibition on macrophage phenotype (Fig. 7H). Furthermore, on analyzing the tumor and metastatic burden of treated mice, we determined that Neu−mice were minimally responsive to anti–PD-1 therapy alone (Fig. 7I–K). Only the combination of the anti–PD-1 immune checkpoint blockade and the Axl inhibitor was able to reduce the total tumor weight per mouse (Fig. 7J). Importantly, R428 treatment alone reduced the metastatic burden and frequency, as we reported previously (20), and the combination with anti–PD-1 potentiated this effect (Fig. 7J and K). Interestingly, tumors treated with the combination of the two therapeutic agents presented an increase of CD8 T cells which, with the NK cells, were more activated (CD44+), possibly explaining the effect of the combined therapy (Fig. 7L). Indeed, an increase in CD3+–infiltrating cells was observed in the core of tumors cotreated with anti–PD-1 and R428 (SI Appendix, Fig. S9B). Furthermore, these tumors showed a significant increase in apoptosis but no difference in proliferation (SI Appendix, Fig. S9 C and D). These results suggest that AXL inhibition could be a powerful approach to generate an antitumorigenic microenvironment to improve immunotherapy and limit the metastatic spreading of breast cancer.

**Fig. 6.** Axl is required for hypoxia-induced secretion of factors that leads to macrophage proliferation, invasion, and polarization. (A) Axl KO cells secrete less Ccl2 in hypoxia compared to WT cells (n = 6, ***P = 0.0001). (B) Staining against F4/80 confirms the decrease of macrophages infiltration in Nic−/AxlKO tumors (n = 6 tumors, *P = 0.0313). (Scale bars, 50 μm.). (C) Levels of phospho-Histone 3 (pH3) by FACS analyses shows less proliferation in infiltrated macrophages (F4/80+) from Nic−/AxlKO tumors compared to Nic−/AxlWT tumors (n = 6 tumors, **P = 0.006). (D) Hypoxic CM from Axl KO cells reduces the proliferation of bone marrow–derived macrophages compared to WT CM, as shown by an increase of cell confluency after 72 h (***P = 0.001). The addition of anti-CCL2 in the WT CM also decreases partially this proliferation. (E) CM from hypoxic Nic cells induces the invasion of bone marrow–derived macrophages in a Boyden–Matrigel invasion assay, and Axl is required for this increase. Neutralizing Ccl2 in the hypoxic WT CM also decreases macrophages invasion (n = 3, ***P < 0.0001, **P = 0.0092, and ***P = 0.0002). (F) The expression of CD206 is reduced in bone marrow–derived macrophages treated with hypoxic CM from Axl KO compared to CM from WT cells. Neutralizing Ccl2 also reduces CD206 expression (n = 3, *P = 0.0202, *P = 0.0142). (G–I) Macrophages treated with Axl KO hypoxic CM secrete less Vegfa, Cxcl1, and Cxcl2 (n = 6, *P = 0.0374, **P = 0.0022, and ***P < 0.0001).
Discussion

We previously reported the requirement of Axl in the metastatic progression of HER2+ breast cancer where a total KO of Axl in the MMTV-Neu model leads to a drastic reduction of the metastatic burden without affecting primary tumor growth (20). Axl was required for the EMT and motility of HER2+ cancer cells, suggesting that the reduction of invasive properties of AXL-depleted cancer cells leads to decreased metastasis. Here, we show that Axl also plays a role in the TME that impacts the metastatic progression. More specifically, Axl deletion in the mammary epithelium decreased the metastasis burden and generated an antitumorigenic TME by altering the hypoxic response in cancer cells. This reduced the metastatic burden without affecting primary tumor growth (20). Axl was re-introduced for the EMT and motility of HER2+ cancer cells, suggesting that the reduction of invasive properties of AXL-depleted cancer cells leads to decreased metastasis. Here, we show that Axl also plays a role in the TME that impacts the metastatic progression. More specifically, Axl deletion in the mammary epithelium decreased the metastasis burden and generated an antitumorigenic TME by altering the hypoxic response in cancer cells. This reduced the metastatic burden without affecting primary tumor growth (20).

It was previously reported that Axl is a direct HIF target and that its expression is up-regulated in clear cell renal cell carcinoma to promote invasion during hypoxia (25). This led to the notion that AXL is a hypoxia-induced protein that drives EMT, invasion, and eventually metastasis. However, Axl was not up-regulated in HER2+ breast cancer cells during hypoxic stress. Interestingly, HIF-1α levels were reduced when Axl expression or activity was decreased, an effect that was further heightened upon hypoxia. During hypoxic stress, Axl was necessary for HIF-1α expression, and interfering with Axl led to an incomplete hypoxic response. This suggests that the Axl effect on HIF-1α expression can be accentuated in vivo in which low oxygen levels generate an adaptive response that has profound effects on the TME and metastasis. We propose a simple model where AXL acts on HIF-1α levels via HER2 stabilization and P3K/AKT pathway, but more studies will be required to completely understand the mechanisms at play. Various RTKs can impinge on HIF levels by affecting its transcription, synthesis, or stability (28). The PI3K/AKT/FRAP pathway was shown to affect HIF-1α synthesis downstream of HER2 (29). Thus, this can also be happening downstream of AXL since we found that interfering with AXL in hypoxia reduced HER2 and AKT phosphorylation. Another possibility would be AXL regulating HIF-1α, either directly or indirectly at the transcriptional level. The latter possibility is supported by our RNAseq data (SI Appendix, Fig. 5S5), where HIF-1α mRNA levels were...
lower in Axl KO cells during hypoxia compared to WT cells. Future investigations aiming at dissecting the AXL–HIF-1α connection would be of great significance.

We previously highlighted the crosstalk of AXL and HER2 leading to invasion, and we showed that the coupling of AXL to HER2 increased AXL stability and localization at the plasma membrane (20). The results in the current report suggest that the latter crosstalk might be extended to hypoxic conditions. Indeed, interfering with AXL in low-O2 conditions reduced HER2 stability, thus possibly affecting the oncogenic signaling generated by HER2, including those leading to HIF-1α expression. To explore the roles of AXL in the hypoxic response, we took advantage of the differential transcriptome between Ad WT and Ad Axl KO cells during hypoxic stress. Gene ontology analyses suggested a role for AXL in cell migration, angiogenesis, and cytokine production among many other processes linked to hypoxia. Consequently, we selected gene candidates whose expression correlated with AXL expression in human breast cancer samples and found that AXL was required for the induction of important EMT transcription factors (Snai2 and Twist1) and key cytokines for macrophage function such as Ccl2. Thus, we showed that AXL was required for hypoxia-induced EMT and invasion. AXL is a well-established context-specific driver of EMT, and we previously showed in our model that AXL is required for TGFB-induced EMT and cell invasion (20). This study showed that AXL is also required for EMT induction and invasion downstream of hypoxic stress, reinforcing the idea that AXL is a major player in these processes in various contexts. The roles of EMT in the metastatic progression are still debated (44, 45). Other than its role in tumor cell invasion, EMT could lead to chemo-resistance and the secretion of cytokines that can affect the TME (42). For example, a positive feedback loop could lead to invasion, and impaired angiogenesis in tumors (46). This suggests that AXL’s role as an EMT driver downstream of hypoxic stress can also be modulating the secretion of factors that affects the TME and thus leading to resistance to treatment and metastasis. Furthermore, the macrophage-related cytokine Ccl2 was found down-regulated in Ad Axl KO cells during hypoxia, and CM from these cells failed to enhance macrophage proliferation, invasion, and polarization. In vivo, macrophages are recruited to hypoxic areas of the tumor where their proangiogenic and anti-inflammatory phenotypes are refined (47, 48). Thus, the significant difference in immune profile and the normalization of the blood vessels observed in NIC−/−AxlKO tumors could be explained by a reduction in macrophage-related cytokines production during hypoxia. Interestingly, the major angiogenic factor, Vegfa, was not differentially mediated in Ad Axl KO cells during hypoxia (SI Appendix, Fig. SSC and Table S3). This further suggested that immunomodulatory roles in the vessel normalization observed in NIC−/−AxlKO tumors. In conclusion, the AXL depletion-mediated changes during the hypoxic response can collectively contribute to an antitumorigenic TME and to reduced metastatic burden in vivo.

Most patients afflicted with HER2+ breast cancer are treated with targeted therapies against HER2 such as Trastuzumab, a drug-conjugated variant of Trastuzumab (T-DM1), or the kinase inhibitor Lapatinib. Unfortunately, some patients do not respond or develop resistance, and different avenues are being explored to overcome these challenges (49, 50). Our laboratory and others previously suggested that combining anti-HER2 and anti-AXL agents could be a good approach to enhance therapy and overcome resistance (20, 51). Furthermore, immunotherapy emerges as a powerful avenue to treat resistant and unresponsive patients, but so far, the PANCEA trial proposing the combination of anti-PD-1 and Trastuzumab offers modest improvements (3). This demonstrates the current clinical struggle of defining the group of patients that would benefit the most from anti-PD-1 therapy (37). The improvement of the TME, including reduced hypoxia, vascular normalization, and increased immune response, is a clinical goal to overcome resistance to various therapies, such as immunotherapy (16, 42, 52). Here, we show that basal and HER2+ tumors present stronger signatures of hypoxia than other breast cancer subtypes, and these are associated with decreased survival in patients with HER2+ tumors. Thus, targeting hypoxia in these tumors could be beneficial, and our experiments support that AXL inhibition could achieve this. AXL is an interesting druggable target because its loss or its inhibition appears to have no harmful effects in mice (20, 53). Thus, numerous clinical trials are ongoing with the AXL inhibitor R428 also known as BGB324 or Bencetinib (54). Some trials are taking place in combination with immunotherapy, but only a few studies in preclinical models were done to support the possible efficiency of this combination (55, 56). Indeed, radiation and checkpoint immunotherapy-resistant PyMT tumors have been shown to overexpress Axl (56). In that study, Axl promotes an immune-suppressive microenvironment by reducing antigen presentation through MHC-I and enhancing immune-suppressive cytokine release. Therefore, low Axl–expressing clones respond better to immune checkpoint blockade (56). Furthermore, other studies link Axl/Pi3k signaling with increased expression of PD-L1 by tumor cells, and Axl inhibition potentiates PD-1 blockade in ID8 graft models (55, 57). Thus, we describe here an underlying mechanism and offer additional proof of concept for a combination of AXL and immune checkpoint inhibitors in a spontaneous pre-clinical cancer model. These notions also raise the question of AXL expression as a reliable predictor of response to immunotherapies in advanced HER2 breast cancer patients or other aggressive highly hypoxic basal breast cancers. Indeed, exploring such avenues might open the gate for potential therapeutic opportunities.

Collectively, our data demonstrate that anti-AXL therapy is sufficient to generate an antitumorigenic microenvironment and reduce metastasis in HER2+ breast cancers by affecting the hypoxic response in cancer cells. This suggests a therapeutic opportunity in which targeting AXL in the context of immunotherapy in HER2+-resistant patients could reduce the primary tumor and metastatic burdens.

Materials and Methods

Animal experiments, tumor progression studies, quantification of circulating tumor cells, and orthoptic grafts are described in SI Appendix, SI Materials and Methods. Furthermore, detailed analysis of immunohistochemistry, immunohistochemistry, and flow cytometry experiments, including the list of antibodies used, are described in SI Appendix, SI Materials and Methods. RNA sequencing, bioinformatics, and human data analyses details are also included in SI Appendix, SI Materials and Methods. Detailed information about cell lines, culture conditions, treatments, cell invasion assay, wound-healing assay, cytokine array, and proliferation assay were also described in SI Appendix, SI Materials and Methods. Statistical analyses were performed using unpaired Student t tests in which P < 0.05 is considered statistically significant, and more details are provided in SI Appendix, SI Materials and Methods.

Data Availability. RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) (GSE158583).

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