AXL Targeting Overcomes Human Lung Cancer Cell Resistance to NK- and CTL-Mediated Cytotoxicity

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Abstract

Immune resistance may arise from both genetic instability and tumor heterogeneity. Microenvironmental stresses such as hypoxia and various resistance mechanisms promote carcinoma cell plasticity. AXL, a member of the TAM (Tyro3, Axl, and Mer) receptor tyrosine kinase family, is widely expressed in human cancers and increasingly recognized for its role in cell plasticity and drug resistance. To investigate mechanisms of immune resistance, we studied multiple human lung cancer clones derived from a model of hypoxia-induced tumor plasticity that exhibited mesenchymal or epithelial features. We demonstrate that AXL expression is increased in mesenchymal lung cancer clones. Expression of AXL in the cells correlated with increased cancer cell–intrinsic resistance to both natural killer (NK)– and cytotoxic T lymphocyte (CTL)–mediated killing. A small-molecule targeting AXL sensitized mesenchymal lung cancer cells to cytotoxic lymphocyte–mediated killing. Mechanistically, we showed that attenuation of AXL-dependent immune resistance involved a molecular network comprising NF-kB activation, increased ICAM1 expression, and upregulation of ULBP1 expression coupled with MAPK inhibition. Higher ICAM1 and ULBP1 tumor expression correlated with improved patient survival in two non–small cell lung cancer (NSCLC) cohorts. These results reveal an AXL-mediated immune-escape regulatory pathway, suggest AXL as a candidate biomarker for tumor resistance to NK and CTL immunity, and support AXL targeting to optimize immune response in NSCLC.

Introduction

Lung cancer is the third most frequent cancer and the first cause of cancer-related death worldwide (1, 2). Approximately 85% of patients present with a disease defined as non–small cell lung cancer (NSCLC), of which lung adenocarcinoma and lung squamous cell carcinoma are the most prevalent histotypes. Like most malignancies, lung carcinoma is heterogeneous and composed of subpopulations of cancer cells, or clones, with distinct molecular and phenotypic features. High intratumoral heterogeneity can pose challenges to the effectiveness of therapies for NSCLC and contributes to the emergence of therapy resistance (3). The underlying biology of tumor heterogeneity and the environmental factors that shape tumor heterogeneity remain unclear. The immune system plays a role during tumor development, with the interplay between cancer cells and their tumor microenvironment. The latter contributes to the development of refractoriness through mechanisms preventing cytotoxic immune effector T cells and natural killer (NK) cells from reaching and destroying their targets (4). Under microenvironmental stresses, carcinoma cells can undergo molecular and phenotypic changes referred to as carcinoma cell plasticity, which contributes to heterogeneity in tumors. Previously, we provided evidence that morphologic changes of carcinoma cells could affect their recognition and killing by CTLs (5). We and others demonstrated that carcinoma cell plasticity results from the activation of epithelial–mesenchymal transition (EMT) programs (6, 7). EMT also confers an immune-resistant phenotype (8–13). Our understanding of the mechanistic links between carcinoma cell plasticity, heterogeneity, and the emergence of tumor immune escape remains poor due to the limited number of preclinical models that recapitulate carcinoma cell phenotypic diversity in the tumor mass.

AXL, a member of the TAM receptor tyrosine kinase family, is overexpressed in various human cancers. Evidence suggests AXL is
associated with carcinoma cell plasticity as well as chemo-, radio-, and drug resistance associated with the mesenchymal phenotype (14–17). AXL may be a promising therapeutic target for overcoming such resistance (16, 18, 19). However, the biological role of AXL as a driver of cancer cell–intrinsic immune resistance has not been investigated.

One hypothesis postulates that microenvironmental stresses such as hypoxia, a hallmark of most solid tumors, promote carcinoma cell plasticity, tumor progression, and immune suppression (20). We used the primary NSCLC IGR-Heu carcinoma cell line subjected to hypoxic stress as a model to generate phenotypic diversity (21). This model recapitulates some of the key features of tumor heterogeneity resulting in the emergence of carcinoma cell clones residing in the epithelial (EPI) or the mesenchymal (MES) states of the EMT spectrum.

Here, we use these clones to explore the mechanisms by which carcinoma cells are positioned in the mesenchymal domain of the EMT spectrum and expressing AXL could affect lysis by CTLs and NK cells. EPI and MES carcinoma cells were shown to differentially express various immune-related genes, which may affect their susceptibility to lysis by cytotoxic effector cells. The present study also showed that MES carcinoma clones with increased expression of AXL were more resistant to cytotoxic immune cell attacks. Inhibition of AXL in MES carcinoma cells resulted in the sensitization of these cells to NK- and CTL-mediated killing through a molecular network involving MAPK and NF-κB activities as well as increased expression of ICAM1 and the NKG2D ligand ULBP1.

Materials and Methods

Cell culture and reagents

Carcinoma clones with EPI or MES phenotypes were isolated as described (21) from primary NSCLC IGR-Heu cells deriving from a resection of a nonmetastatic NSCLC patient (22). Briefly, after cultivating NSCLC IGR-Heu cells for one month and a half under hypoxic conditions (1% PO2 with 5% CO2) in a hypoxia chamber (InVivo2 400 Hypoxia Workstation; Ruskin Technology), cancer clones were obtained by limiting dilution and clonal expansion. For this study, low-passage cells were used (<13 passages) and cultured in DMEM/F12 medium containing 10% heat-inactivated FCS, 1% penicillin–streptomycin, (Thermo Fisher Scientific), 1% Ultroser G (Pall Corporation) at 37°C in a humidified atmosphere with 5% CO2. Cells were authenticated through cell morphology, and expression of TP53 and ACTN4 mutants in parental cells and derivatives. Mycoplasma testing was carried out using the Venor GeM OneStep kit (Minerva Biolabs). The H33 CTL clone originated from the same patient tumor specimen as NSCLC IGR-Heu cells and EPI or MES derivative clones. The CTL clone was cultured and stimulated in complete RPMI media supplemented with 10% human AB serum (Jacques Boy Institute) and rIFN in the presence of irradiated autologous NSCLC IGR-Heu (3 × 105)/well and lymphoblastoid (4 × 105)/well) cell lines. Cells were fed every 3 days with complete media (23). The NSCLC cell lines ADC-Coco, IGR-Pub, ADC-Tor, ADC-Let as well as primary NSCLC cells were derived from tumor biopsies as described previously and used at low passage (24, 25). Human NK cell clones (NK92 and NKL) were maintained in RPMI-1640 media with 10% pooled human serum (Jacques Boy). 5% horse serum (Thermo Fisher Scientific), with or without the presence of IL2 (150 UI/mL) and IL15 (15 ng/mL). TPCA-1 was purchased from Sigma-Aldrich was prepared in DMSO. Bemcentinib (formerly known as BG9324, or R428, BerGenBio ASA) was dissolved in DMSO (10 mmol/L stock solution) and used at the final concentration of 1 μmol/L.

RNA preparation, cDNA synthesis, and quantitative real-time PCR

Total RNA extraction was performed using TRIzol reagent. Reverse transcription was performed using Maxima Reverse Transcriptase followed by qPCR using real-time PCR Master SYBR Green on a StepOnePlus Real-Time PCR system. All products were from Thermo Fisher Scientific. Most oligonucleotide sequences used were designed using Beacon Designer Free Edition and Primer3Plus. The full list is available as Supplementary Table S1. Heat maps were generated using Excel software.

Protein extracts, Western blot analysis, and electrophoretic mobility shift assays

Adherent cells were lysed on ice with lysis buffer (0.25 mol/L Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol) containing a cocktail of protease (Thermo Fisher Scientific) and phosphatase inhibitors (Roche Life Science). Western blotting was performed as previously described (21). Antibodies for Western blots were as follows: antibodies to AXL (C89E7), phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204) D13.14.4E, p44/42 MAPK (Erk1/2; L34F12), phospho-Akt (Ser473; D9E), Akt (pan: 40D4) were from Cell Signaling Technology. Phospho-AXL-y779-antibody was from R&D Systems. Anti-vimentin (Clone V9) was from Dako, anti-ICAM1 (sc-107) was from Santa Cruz Biotechnology, and anti-β-Actin (AC-74) was from Sigma-Aldrich.

For electrophoretic mobility shift assay (EMSA) analysis of NK-/β DNA binding, nuclear extracts were prepared and analyzed for DNA binding activity using the HIV-LTR tandem β oligonucleotide as β probe (26).

Antibodies and flow cytometry

Phenotypic analyses of carcinoma cells, NK, and CTL cells were performed by direct immunostaining. Briefly, 0.2 × 106 cells were collected and resuspended in FACS buffer (PBS with 2% FBS) and stained for 30 minutes in the dark performed at 4°C with the following Abs for extracellular staining: APC-anti-ICAM1, PE-anti-KLRG1, PE-anti-DNAM1 (CD226), APC-anti-CD96, PE-anti-CD16, PE-anti-NKp30, PE-anti-NKp44, PE-anti-NKp46, PE-anti-NKG2C, PE-anti-MICA/MICB, APC-anti-CD137 (4-1BB), PE-anti-human HLA-A,B,C, APC-HLA-A2, APC-anti-TRC α/β, APC-anti-human CD262 (DR5, TRAIL-R2), PE-anti-human CD261 (DR4, TRAIL-R1) and mouse isotopic controls were from BioLegend; APC-anti-CD244 (2B4) APC-anti–LFA-1 (cd11a), PE-anti-NKG2D, PE-anti-NKG2A, PE-anti-KIR2DL1, PE-anti-KIR3DL1, PE-anti-KIR3DH2, and PE-anti-KIR2DL1 from Miltenyi Biotec; PE-anti-TIGIT (FAB7898P), APC-anti-ULBP1 (MAI380) from R&D Systems; APC-anti-CD94, PE-anti-CD3, and APC-anti-CD45 were from BD Biosciences. Acquisitions were performed using a BD Accuri C6 flow cytometer (BD Biosciences), and data were processed using the FlowJo program.
Fluorescence microscopy and conjugate formation

Conjugate formation between carcinoma target cells (T) and immune effector cells (E) was achieved by co-culturing the cells on poly-L-lysine–coated slides in a humidified incubator at 37°C for 30 minutes at a 3:1 E:T ratio, respectively. Cells were then fixed with freshly prepared 3% paraformaldehyde for 10 minutes, incubated in a 50-μmol/L NH4Cl quenching solution (in PBS), permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. Fixed and permeabilized cells were blocked with 10% FCS (v/v) in PBS for 20 minutes and stained using anti-LFA-1 (1:200, CD11a, clone EP1285Y, Abcam) diluted in PBS containing 1 mg/mL BSA for 2 hours or overnight at 4°C followed by 1-hour incubation with secondary anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific) to identify effector cells. Cell nuclei were counterstained and mounted using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Conjugates were imaged on an IX83 microscope using the CellSense Dimension software (Olympus). The efficiency of conjugate formation between the effector and cancer cells was quantified as (the number of effector cells able to form mature conjugates with target cells/target cells × 100). Conjugate formation was evaluated in 10 different visual fields per experimental condition. For dual staining of ICAM1 and F-actin, similar conditions were applied. Immunostaining was performed using anti-ICAM-1 (1:150, SC107, Santa Cruz) in incubation buffer (0.1% BSA/PBS) at 4°C for 16 hours followed by a 1-hour RT incubation with anti-mouse secondary antibody (A11029, Molecular Probes, Molecular Probes). After washing, cells were incubated with 165 nmol/L AF 647-conjugated phalloidin in 1% BSA/PBS (A22287, Molecular Probes). Images were obtained and processed on a Leica TCS SP8 STED 3X confocal microscope (Leica Microsystems) equipped with a 100× objective (HC PL Apo STED white, oil, NA = 1.4, WD = 0.13 mm). Phalloidin (F-actin) staining was also performed on carcinoma cells cultured in poly-L-lysine–coated chamber slides and processed as above to assess morphologic changes of the cells following treatment.

Gene silencing by RNA interference

The functionally validated and predesigned siRNAs used in this study were from Thermo Fisher Scientific (Silencer select) and Qiagen (FlexiTube) and directed against human ULBP1 (S102779847, S102779868), ICAM1 (S1000094347, S10309323), SLAMF7 (S10030004, S102644649), TNSFRF10B (S103038665, S103094063), HIF1A (S102664053), MAPK1 (1027321), ZEB1 (s229970, s229971), ZEB2 (S102664277), TWIST1 (s14523), SNAI2 (s13127, s13128), and SNAI1 (s13185, s13186). siRNAs were transfected using Lipofectamine RNAiMax Transfection reagent (Thermo Fisher Scientific), with appropriate controls.

Cytotoxicity assay

The cytotoxic activity of NK and CTL clones was measured by a conventional 4-h 51Cr release assay as described (21) using 51Cr purchased from Perkin-Elmer. Briefly, after coculturing effector (immune cell):carcinoma target (E:T) cells at various ratios for 4 hours in round-bottomed 96-well plates, the percentage of specific lysis was calculated using the formula [experimental cpm – spontaneous cpm]/total cpm incorporated] × 100, and the results are expressed as the mean of triplicate samples. For some cytotoxic assays, carcinoma cells were incubated with compounds versus DMSO or siRNAs for 48 to 72 hours before their use as targets. Cytotoxicity inhibition with blocking antibodies was performed by preincubating effector or target carcinoma cells for 1 hour with antibodies (low endotoxin-free azide) against CD54 (ICAM1), CD514 (NKG2D, D1D1), and control isotypes obtained from BioLegend, and used at 5 μg/mL concentration, which was maintained during cell lysis.

Microarray analysis

Total RNA was extracted using TRIzol reagent as above except that nucleic acids were resolved in ethanol instead of isoproply alcohol and then transferred onto an RNeasy columns (Qiagen) for the rest of the procedure. Expression profiles of carcinoma cell clones treated or not with bemcentinib were then generated using an Agilent Technologies SurePrint G3 human GE 8 × 60K microarray (G39494) with a single color technology. Data were processed and analyzed as previously described (27). The microarray data and protocols are available at the European Molecular Biology Laboratory European Bioinformatics Institute database (https://www.ebi.ac.uk/arrayexpress/) under accession no. E-MTAB-8294. Gene set enrichment analyses (GSEA) were performed with the GSEA platform of the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) using the Molecular Signature Databases (MSigDB).

Analysis of gene-expression data sets derived from NSCLC patients

The Cancer Genome Atlas (TCGA) RNA-sequencing (RNA-seq) expression data and sample information from human lung adenocarcinoma from TCGA (http://cancergenome.nih.gov/) were accessed on April 28, 2018, from cBioPortal (28) and the TCGA public access data (http://tcga-data.nci.nih.gov/). Cases of the LIIAD TCGA with available expression data and follow-up information (n = 504) were considered and Kaplan–Meier plots generated to assess the prognostic value of ICAM-1, ULBP1, NKG2D, LFA-1, and AXL mRNA expression, as in RSEM normalized counts. Using http://kmplot.com, a similar analysis was performed on a group of surgically managed NSCLC patients who did not receive radiotherapy or chemotherapy (n = 227). Gene-expression data were automatically computed to generate Kaplan–Meier plots as described (29). The Prat and colleagues data set (30) consisting of 35 NSCLC patients treated with anti–PD-1 was investigated to compare mRNA mean expression of ULBP1, PD-1, LFA-1, and ICAM-1 in progression versus non-progression diseases.

Statistical analysis

Data analyses were carried out using GraphPad (GraphPad Prism) and Excel (Microsoft Corp). Statistical tests were performed using a two-tailed P ≤ 0.05 level of significance; ‘’, P ≤ 0.05; ‘’’, P ≤ 0.01; ‘’’’, P ≤ 0.001. Comparisons of the means between groups were performed using the Student t test, Mann–Whitney, or two-way ANOVA as appropriate. Overall survival curves were generated by the Kaplan–Meier method and compared using the Mantel–Cox log-rank test.

Results

Increase in AXL expression associated with resistance to lysis

It has previously been shown that AXL is highly expressed in lung cancers that have undergone EMT (15). We thus investigated the expression of AXL in a panel of EPI and MES NSCLC carcinoma clones derived from IGR-Heu cell cultured under hypoxic stress pressure (Fig. 1A). All the EPI clones, including EPI C2 cells,
lacked detectable expression of AXL, whereas a spectrum of AXL expression from low/undetectable to high was observed in vimentin-positive MES carcinoma clones. MES C30 carcinoma cells displayed the highest AXL expression. The pronounced expression of AXL in this clone may be due to stabilization of hypoxia-inducible factor-1α (HIF-1α) in the cells concomitant with higher expression of the EMT transcription factors (EMT-TF) ZEB-1, SNAI1, and TWIST1 (Supplementary Fig. S1). There were no substantial differences in the morphology of MES carcinoma cells expressing different amounts of AXL (Fig. 1B). We then investigated the susceptibility to lymphocyte-mediated killing of the MES carcinoma clones displaying high (AXLHi), medium (AXLMed), or low (AXLLo) AXL expression. In cytotoxicity assays where carcinoma cells were challenged with either human NK92, NKL cell clones, purified NKd cells or the autologous CTL clone H33, the killing was significantly lower in AXLHi and AXLMed MES cancer cells compared with AXLLo cells (Fig. 1C; Supplementary Fig. S2). These results indicated that AXL expression in MES carcinoma cells may mediate intrinsic resistance to both NK- and CTL-mediated killing.
AXL inhibition increased MES carcinoma cell susceptibility to lysis

AXL is associated with resistance to different BRAF/MEK and EGFR inhibitors (14–16, 31) and AXL inhibitors are in trials for overcoming such resistance. We thus thought to explore the potential of AXL inhibition in sensitizing MES cancer cells to cell-mediated cytotoxicity. Treatment with bemcentinib as an AXL kinase inhibitor increased sensitivity to CTL and NK lysis in MES carcinoma clones expressing medium to high amounts of AXL (Fig. 2A–C). A 2- to 10-fold higher percentage of cancer cell killing was observed for the AXLHI MES and the AXLMed MES cancer cells when assayed in the presence of autologous CTLs or NK clones. A net sensitization was also observed in the presence of purified NKd cells. This contrasted with no significant changes observed for AXLLO MES cells.

To investigate the mechanisms that operate under AXL inhibition in the observed sensitization to lymphocyte-mediated cytotoxicity in the MES carcinoma cells, we examined gene-expression profiles of AXLHI MES C30 cells treated for 72 hours with the AXL inhibitor or vehicle (Fig. 3). Gene-expression analysis from bemcentinib- versus control-treated indicated an average of 700 genes that were significantly changed (499 upregulated and 199 downregulated; Fig. 3A). In contrast, bemcentinib treatment of the AXLNEG EPI C2 or AXLLO MES clones showed a much lower variation (EPI C2: 50 genes upregulated and 6 genes downregulated; MES C29: 39 genes upregulated and 13 genes downregulated; Supplementary Table S2). These results highlight the AXL-targeting specificity of bemcentinib.

GSEA revealed enrichment in the expression of gene sets related to the NK pathway or activation, and antigen processing and presentation (Fig. 3B). An enrichment of regulatory pathways involving NF-κB and MAPK inhibition was also observed. Apoptosis and EMT signatures were not affected by the treatment conditions (Fig. 3B). No obvious changes were observed in the EMT-related gene-expression pattern or in the morphology of the cells (Fig. 3C and D). Nonetheless, staining of cortical actin **

Figure 2.

Treatment of AXLHI MES and AXLLO lung carcinoma clones with an AXL inhibitor results in increased susceptibility to NK- and CTL-mediated lysis. A, CTL H33 cell lysis susceptibility of AXLHI MES (left), AXLMed MES (center), and AXLLO MES (right) clones after 72-hour treatment with the AXL inhibitor bemcentinib (1 μmol/L). Similar experiment evaluating susceptibility to NK92 cell lysis (B) and NKd cells (C). Data are presented as mean ± SEM for three replicate experiments. *, P < 0.05; **, P < 0.01. inhib., inhibitor.
Figure 3.
Treatment of the AXLHI/Mes carcinoma clones with the AXL inhibitor is accompanied by changes in expression of antigen-processing genes and tumor-related factors that mediate NK cell functions. A, Volcano plots generated from expression data depicting the distribution of genes downregulated (green) or upregulated (red) in AXLHI MES C30 and Epi C2 cells treated with 1 μmol/L bemcentinib versus vehicle (DMSO) for 72-hour treatment. Adj., adjusted; inhib., inhibitor. B, Bar graphs generated from GSEA analysis showing the enrichment scores for the top 28 differentially enriched gene sets in AXLHI MES cancer cells. C, Bar graphs from qRT-PCR experiments showing relative mRNA expression of the indicated immune-related genes in inhibitor treated versus vehicle treated in the different MES carcinoma clones. Data are presented as mean ± SEM for three replicate experiments.

Microfilaments in the treatment condition was indicative of changes concomitant with more cell–cell contacts (Fig. 3D; Supplementary Fig. S3). Expression of tumor-related factors that mediate NK cell functions (i.e., ICAM1 (intercellular adhesion molecule 1), ULBP1, the ligand for NKG2D activating NK receptor (UL16 binding protein 1), SLAMF7, also known as CD2-like receptor–activating cytotoxic cell (CRACC), TNFRSF10B (TRAIL-R2)) and antigen processing and presentation (TAP1,
TAPBP, and ERAFP) was upregulated upon AXL inhibition (Fig. 3E). In contrast, no effect on the expression of these genes was observed when EPI C2 carcinoma cells were treated (Supplementary Fig. S4). We further assessed by qRT-PCR the impact of AXL inhibition on these genes upon bemcentinib treatment of AXLIH MES and AXLMED MES clones (Fig. 3F). In complementary experiments, bemcentinib treatment of AXL-expressing NSCLC cell lines (H23, HCC44, H820, and A549), in house generated NSCLC cell lines (ADC-PuB, ADC-TOR, ADC-Coco26, and ADC-let), or primary carcinoma cell cultures freshly isolated from 5 NSCLC patients, resulted in a net upregulation of ULBP1, SLAMF7, and ICAM1 expression in multiple instances (Supplementary Fig. S5). These observations suggested upregulation of ULBP1, SLAMF7, and ICAM1 as primary mechanisms of sensitization to lymphocyte-mediated cytotoxicity upon AXL inhibition.

Increase in ICAM1 and ULBP1 upon AXL inhibition restored cancer cell lysis

Assessment of the MES carcinoma clones for ICAM1 and ULBP1 expression indicated lower amounts in AXLIH and AXLMED MES compared with AXLLO MES clones (Supplementary Fig. S6A and S6B), which could explain immune resistance of these cells (Fig. 1C). Certain AXLIH also appeared to have less MHC class I molecules at the cell surface (Supplementary Fig. S6C). Ligands of NKG2D such as ULBP1 play a role in the recognition of stressed target cells by the immune system, whereas cancer–lymphocyte conjugation and cytotoxic function rely in part on heterophilic interactions between ICAM1 expressed on the target cell and the integrin LFA-1 expressed on immune effector cells. We thus asked whether bemcentinib treatment could affect these interactions. We first validated the increased expression of ICAM1 protein in bemcentinib-treated AXLIH MES and AXLLO MES cancer cells (Fig. 4A). Upregulation of ICAM1 expression was observed at the cell surface of AXLIH MES clones and in various NSCLC cell lines such as NCI-H460 (Fig. 4B and C; Supplementary Fig. S7A–S7C). We then assessed whether bemcentinib treatment could affect target–effector conjugate formation. For this purpose, bemcentinib-treated cancer ‘target’ cells were coincubated for 30 minutes with NK ‘effector’ cells, and conjugate formation was evaluated by imaging. AXL inhibition resulted in an increased number of mature conjugates established between effector and target cells (Fig. 4D). In this respect, effector cells were more distant and had more protrusions toward the vehicle-treated target cells, which may reflect their relative inability to establish stable cell–cell contacts (Fig. 4D; Supplementary Fig. S8). We then examined whether the increased expression of ULBP1 and ICAM1 observed upon AXL inhibition could explain the increased susceptibility to lymphocyte-mediated lysis. To this end, we performed cytolytic assays following AXL inhibition and knockdown of ICAM1, ULBP1, SLAMF7, and TNFRSF10B in AXLIH MES cells. Compared with siRNA control-treated cells, siRNA-ICAM1- and siRNA-ULBP1–treated AXLIH MES cells exhibited lower sensitivity to NK cells (Fig. 4E). siRNAs directed to SLAMF7 showed modest effects, whereas siRNAs to TNFRSF10B had no significant effect on cancer susceptibility to NK-mediated lysis. We verified the expression of various molecules that regulate cell–cell interactions on the surface of NK and CTL clones. Accordingly, the cognate ICAM1 receptor, β2 integrin LFA-1, and the ULBP1 receptor NKG2D were expressed on both CTLs and NK cells consistent with an effect of bemcentinib via ICAM1/LFA-1 and ULBP1/NKG2D interactions (Supplementary Fig. S9).

To confirm the finding that ICAM1 and ULBP1 operate as immune-modulatory factors in the increased cancer cell lysis by cytotoxic effector cells, cytotoxic experiments were carried out with blocking of the ICAM-1/LFA-1 and ULBP1/NKG2D interactions. Treatment with blocking anti-ICAM1 suppressed the increased susceptibility of the carcinoma cells to NK and CTL clones when pretreated with bemcentinib (Fig. 4F). Similar variations in lysis were observed using a blocking antibody directed to NKG2D (Fig. 4G). However, this effect was more apparent in the presence of CTL effector cells compared with those seen in assays using NK effector cells. Together, these data suggest that MES NSCLC clones, which are intrinsically resistant to immune effector cells, can be sensitized by targeted inhibition of AXL through a mechanism involving ICAM1 and ULBP1.

AXL inhibition regulates ICAM1 and ULBP1 expression through NF-κB and MAPK signaling

As ICAM1 is an NF-κB target gene, we examined the status of NF-κB activation by EMSA. Although basal NF-κB DNA binding activity was observed in the vehicle-treated AXLIH MES cancer cells, treatment with bemcentinib led to an increase in NF-κB activity (Fig. 5A). In order to substantiate this finding and examine the contribution of the IKK/NF-κB pathway in the upregulation of ICAM-1, we performed experiments wherein cells were treated with the AXL inhibitor in the presence or absence of the IKK/NF-κB inhibitor TPCA-1. Exposure to TPCA-1 abrogated ICAM1 induction following bemcentinib treatment (Fig. 5B; Supplementary Fig. S10). Similar results were obtained after evaluating three other NF-κB target genes, IL6, IL8, and CCL20 (Fig. 5B).

As AXL inhibition can affect both MAPK/ERK and AKT signaling (14, 18), we examined the phosphorylation status of these proteins. Although a moderate reduction of phospho-AKT was perceived at 24 hours, bemcentinib treatment had no significant effect on phospho-AKT at 48 or 72 hours (Fig. 5C), nor did this treatment significantly change phospho-ERK3-β3 amounts. As expected, phospho-AXL but not total AXL amounts were reduced upon exposure to bemcentinib and phospho-ERK/MAPK amounts decreased (Fig. 5C). This result was consistent with the GSEA analysis showing an enrichment of gene sets associated with MAPK inhibition. To further define the importance of ERK/MAPK in these molecular events, we examined whether MAPK knockdown using siRNAs could directly affect gene expression of the abovementioned deregulated genes in the absence of bemcentinib (Fig. 5D). We observed an increase in ULBP1 and TNFRSF10B expression following MAPK knockdown. Collectively, these data suggested that the AXL kinase inhibition increases the expression immune-modulatory genes, including ICAM-1 and ULBP1 expression, through NF-κB activation and concurrent inhibition of the MAPK signaling, respectively.

Different amounts of ICAM1, ULBP1, and MHC class I found in MES carcinoma cells compared with their EPI counterparts

We have previously demonstrated that hypoxia-induced MES and EPI NSCLC cells have different susceptibility to NK- and CTL-mediated killing (21). However, the molecular basis associated with this differential susceptibility has not been
Figure 4.
AXL inhibition led to increased expression of ICAM-1 and ULBP1 in conjunction with increased NF-κB activity and inhibition of MAPK. A, Immunoblots showing induction of ICAM1 after treatment with 1 μmol/L bemcentinib. Increased ICAM1 is mostly visible in the AXLHi and AXLMed MES cancer cells. B, Increased cell-surface expression of ICAM1 on AXLHi MES cells following 72 hours of treatment as assessed by flow cytometry. C, ICAM1 expression assessed by immunofluorescence staining after coculturing CTLs and AXLHi MES cells pretreated or not with 1 μmol/L bemcentinib for 48 hours. F-actin and counterstaining with DAPI are also shown. Scale bar, 10 μm. Single channel images are provided as Supplementary Fig. S7A. D, Efficiency of conjugate formation between NK92 cells and target cells, AXLHi MES cells, pretreated or not with 1 μmol/L bemcentinib. Immunofluorescence staining for LFA-1 was used to discriminate NK effector cells from target cells. Representative images showing mature conjugates in the bemcentinib-treated condition, which contrasted with vehicle condition, wherein effector cells appeared more distant to the target cells illustrating deficient immunologic synapses. Scale bar, 10 μm. E, Cytotoxicity assay for NK-mediated lysis of the AXLHi MES carcinoma cells after siRNA targeting of ICAM1, ULBP1, SLAMF7, or TNFRSF10B in cells treated with 1 μmol/L bemcentinib for 72 hours. Bars represent the means of three independent experiments ± SEM performed in triplicate. F and G, Cytotoxicity assays for NK-mediated lysis (F) or CTL-mediated lysis (G) of the AXLHi MES cancer cells after antibody neutralization of ICAM1 or the ULBP1 receptor NKG2D. Bars represent the means of three independent experiments ± SEM performed in triplicate. For D-G, *, P < 0.05; **, P < 0.01. inhib., inhibitor.
Targeting AXL to Overcome Immune Resistance of NSCLC Cells

A, Nuclear extracts from the AXL<sup>H</sup> MES carcinoma clone treated with 1 μmol/L bemcentinib were analyzed by EMSA to evaluate NF-κB DNA binding activity. B, mRNA expression data showing that in the AXL<sup>H</sup> MES cancer cells, ICAM1 induction under bemcentinib treatment is blocked when combined with 48-hour treatment with the IKK/NF-κB inhibitor TPCA-1 at the indicated doses. Results obtained for known NF-κB targets IL6, IL8, and CCL20 are also presented. Bars represent the means of three independent experiments ± SEM performed in triplicate. **, P < 0.01; ***, P < 0.001.

C, Immunoblots comparing expression of phosphorylated-AKT, phosphorylated-ERK, and phosphorylated-AXL in AXL<sup>H</sup> MES cancer cells treated for 24, 48, and 72 hours (h) with 1 μmol/L bemcentinib. D, AXL<sup>H</sup> MES cancer cells were pretreated for 72 hours with MAPK1 siRNAs, and effects on the indicated immune-related genes were evaluated by quantitative RT-PCR and flow cytometry. inhib., inhibitor.

ELICIT. We thus asked which mechanisms would also mediate different susceptibility between MES and EPI NSCLC cells. We investigated gene-expression profiles from two (EPI C2 vs. MES C30) representative clones with EPI versus MES phenotypes arising from hypoxic stress. As expected, GSEA revealed enrichment in expression of EMT signature in the MES compared with EPI carcinoma cells (Fig. 6A). Gene sets previously known to be associated with immune-suppressive properties and resistance to cell-mediated killing, including TGFβ, NF-κB, hypoxia, and STAT3/STAT5 signatures were also found to be enriched. We noted expression differences only in a small number of immune-related gene transcritps. MES C30 was found to have downregulated expression of CDH1 (E-cadherin) and upregulated expression of TGFβ1 and TGFβ2, known to be associated with EMT program. Genes encoding for the immune-modulatory molecules such as ICAM1, ULBP1, and MICA (MHC class I polypeptide-related sequence A) were either downregulated or upregulated, respectively (Fig. 6B).

Flow cytometry analysis further indicated that MES carcinoma cells downregulated ICAM1, ULBP1, and MHC class I molecules compared with EPI carcinoma cells (Fig. 6C). This observation indicates that carcinoma cells with EPI (AXL null) or MES (AXL HI/Med) features differ in their expression of ICAM1 and ULBP1, which likely regulates their susceptibility to NK- and CTL-mediated killing.

ULBP1 and ICAM1 tumor expression is associated with better survival in NSCLC patients

To test whether the results obtained are relevant in the clinical setting of immune surveillance of human lung tumors, we examined the gene expression of ICAM1 and ULBP1 in public data sets and investigated their prognostic value in terms of overall survival in NSCLC patients (29, 32). NSCLC patients were stratified into two groups, with high or low expression of these genes, and
Kaplan–Meier plots were generated to compare survival of patients (Fig. 7).

In these NSCLC patients, high expression of ICAM1 and ULBP1 was associated with better survival in the Győrfi and colleagues and TCGA LUAD data sets (Fig. 7A and B; Supplementary Fig. S11A and S11B). AXL mRNA expression was not prognostic in the NSCLC data sets analyzed, despite a more unfavorable trend in the AXL-high group (Supplementary Fig. S11C). We next queried the impact on patient survival of cognate ICAM1 and ULBP1 receptors that are expressed by lymphocytes, LFA-1 and NKG2D, respectively. Akin to the impact observed for ICAM1 and ULBP1, LFA-1 and NKG2D were consistently associated with better survival in these patients (Fig. 7A and B; Supplementary Fig. S11A and S11B). ULBP1 also positively correlated with survival in the TCGA LUSQ data set (Supplementary Fig. S12).

Moreover, in a cohort of NSCLC patients treated with anti–PD-1, LFA-1 and NKG2D were found to be more highly expressed in the nonprogressor patients (Fig. 7C). Thus, the ICAM1/LFA-1 and ULBP1/NKG2D axes regulate the balance between cancer cell susceptibility and tumor progression.

**Figure 6.** Hypoxia-induced EMT is associated with differences in the expression of immune-modulatory genes, including ULBP1 and ICAM1. A, Top 20 differentially enriched gene sets in AXLH⁺ MES C30 versus EPI C2 from GSEA analysis, as classified by enrichment scores. A positive score indicates enrichment in the MES C30 cells, whereas a negative score indicates enrichment in the EPI C2 cells. B, Heat map displaying log₂-transformed expression levels of a panel of immune-related genes in the respective cancer clones from three replicates. C, Fluorescence intensity histograms displaying the surface expression of ICAM1, ULBP1, MICA/B, and HLA-A,B,C in two MES and two EPI carcinoma clones. Isotype control (Ctrl IgG) is shown in gray.

Kaplan–Meier plots were generated to compare survival of patients (Fig. 7).

In these NSCLC patients, high expression of ICAM1 and ULBP1 was associated with better survival in the Győrfi and colleagues and TCGA LUAD data sets (Fig. 7A and B; Supplementary Fig. S11A and S11B). AXL mRNA expression was not prognostic in the NSCLC data sets analyzed, despite a more unfavorable trend in the AXL-high group (Supplementary Fig. S11C). We next queried the impact on patient survival of cognate ICAM1 and ULBP1 receptors that are expressed by lymphocytes, LFA-1 and NKG2D, respectively. Akin to the impact observed for ICAM1 and ULBP1, LFA-1 and NKG2D were consistently associated with better survival in these patients (Fig. 7A and B; Supplementary Fig. S11A and S11B). ULBP1 also positively correlated with survival in the TCGA LUSQ data set (Supplementary Fig. S12). Moreover, in a cohort of NSCLC patients treated with anti–PD-1, LFA-1 and NKG2D were found to be more highly expressed in the nonprogressor patients (Fig. 7C). Thus, the ICAM1/LFA-1 and ULBP1/NKG2D axes regulate the balance between cancer cell susceptibility and tumor progression.
Targeting AXL to Overcome Immune Resistance of NSCLC Cells

Discussion

Although immunotherapy has improved the survival rates for patients with advanced malignancies, the prevalence of nonresponders prompts further investigation of the events underlying the immune resistance of tumors to allow them to be prevented or overcome. Genetic and epigenetic intratumoral heterogeneity contributes to the emergence of resistant clones and immune escape (33). EMT of adenocarcinoma cells promotes the emergence of MES cancer cell populations that exhibit chemo-, radio-, and drug resistance. AXL expression is linked with such resistance and EMT in many cancer types (14, 15, 18, 19, 34). Our survey further showed heterogeneous AXL expression in the different MES cancer clones. How combinatorial expression of various EMT-TF and trans-acting microenvironmental factors such as HIF-1α regulates this heterogeneity remains to be investigated.

AXL mRNA expression was not prognostic in the NSCLC data sets analyzed. Studies suggest AXL activity in both tumor and stromal cells, which complicates interpretation (35). AXL targeting with bemcentinib in transgenic pancreatic cancer mouse models both reversed tumor EMT and repolarized M2 macrophages (35). AXL inhibition has been reported to enhance the sensitivity of cancer cells to chemotherapeutic agents through means including DNA damage response, perturbations of cell death receptors, and increased expression of nucleoside transporters (18, 34, 35). However, how AXL contributes to immune evasion is unknown.

In this study, we found that carcinoma cells positioned in the mesenchymal states of the EMT spectrum, and expressing AXL, can contribute to tumor resistance to NK and CTL immunity. We show that AXL-expressing MES NSCLC clones resist CTL- and NK cell–mediated lysis and that AXL kinase inhibition using bemcentinib, a pharmacologic inhibitor in several clinical trials...
including NSCLC, led to sensitization to NK and CTL-mediated killing.

The mechanism of AXL-mediated intrinsic mesenchymal cell resistance to effector cell cytotoxicity involved decreased ICAM1 and ULBP1 expression and reduced conjugate formation. ICAM-1 on target cells binds to its cognate receptor LFA-1 on effector lymphocytes, strengthening the interaction between the cytotoxic killer cells (CTLs and NK cells) and carcinoma target cells. Ligands of NK2GD such as ULBP1 play a role in the recognition of stressed target cells by the immune system. Previous studies implied ICAM1 was useful for prognosis in patients with colorectal cancer or breast cancer (36–38). Higher expression of ICAM1, ULBP1, and their receptors in NSCLC tumors correlated with better patient survival in two data sets of NSCLC patients, suggesting a contribution of the ICAM-1/LFA-1 and ULBP1/NK2GD axes during immune surveillance.

Antibody-based therapies targeting T-cell inhibitory receptors, mainly PD-1, to reactivate immune cells have led to objective long-term responses for a subset of NSCLC patients (20% to 30%) with a very unfavorable prognosis (2). Other promising approaches are being developed that may widen the spectrum of immunotherapeutic options to include autologous cell therapy, engineered T cells (such as CAR-T cell therapy), or NK cells (39, 40). Nevertheless, some cancer patients fail to respond to these therapeutic interventions and others show disease recurrence after an initial response (2, 41). Future studies should address the role of AXL targeting in these settings.

Although our knowledge of regulation of the NK2GD ligand (ULBP1) expression remains fragmentary, our studies support the notion that AXL and EMT-associated cellular mechanisms may be exploited by carcinoma cells to reduce the expression of stress-induced ligands and escape immune recognition. Among the NSCLC cell lines tested, not all were responsive to the bencemtib treatment. The factors that influence this response are just beginning to be investigated.

Although the NF-κB pathway is best characterized for its protective activity in response to proapoptotic stimuli, it also supports EMT and suppresses cell death in certain cell types. In the course of these studies, we demonstrated that long-term AXL inhibition with bencemtib resulted in slow but persistent NF-κB activation coinciding with an increase in ICAM1 expression and the subsequent increase in target susceptibility to cell-mediated cytotoxicity. Conversely, AXL inhibition resulted in MAPK inhibition leading to ULBP1 upregulation. AXL targeting reduced MAPK signaling and reversed drug resistance in melanoma cells (42). Various drug-related stresses could contribute to a wide range of NF-κB responses including alterations in the DNA-repair machinery identified in the GSEA analysis (43). Gene sets related to DNA repair appeared to be affected upon treatment with bencemtib as shown by negative enrichment scores in the GSEA (Fig. 4). Similar observations were reported by Balaji and colleagues, who used another AXL inhibitor (34). Moreover, it will be essential to determine how AXL signaling interferes with factors such as cytokines that modulate NF-κB and MAPK activation.

In previous studies, we and others have reported that EMT of breast carcinoma cells can confer resistance to CTL-mediated killing through mechanisms such as increased TGFβ signaling or reduced antigen presentation (8, 10, 13). Although AXL was previously shown to suppress antigen-presenting MHC class I molecules in the mouse PyMT model (44), here, AXL inhibition in MES cancer cells had no effect on cell-surface expression of MHC class I molecules such as HLA-A. Nevertheless, AXL-HI MES cancer cells showed lower amounts of MHC class I molecules, and in line with previous studies (8, 10), MES cancer clones frequently exhibited reduced expression of MHC class I molecules compared with more EPI cancer clones. As observed in EMT-like triple-negative breast carcinoma cells, it will be interesting to see if prominent accumulation of actin microfilaments at the immunologic synapse affects cancer cell lysis in this setting (45). In contrast, other reports have raised the possibility that under some circumstances, such as forced expression of SNAIL or following TGFβ exposure, EMT may lead to increased susceptibility to NK killing due to upregulation of MHC class I chain–related molecules A and B (MICA/B; ref. 46), or increased expression of cell adhesion molecule (CADM1; ref. 47), respectively. Our current study has focused on MES lung cancer cell populations exhibiting high resistance to both NK- and CTL-mediated killing. We observed heterogeneity in target cell susceptibility in the MES cancer cell population. However, in contrast to these previous studies, we did not observe variability of MICA/B, and could not find evidence for deregulation of CADM1 in the EPI versus MES carcinoma clones even under bencemtib treatment. However, it would be interesting to test whether AXL signaling or related pathways could regulate MICA/B or CADM1 via various parameters regulating the microenvironmental niche, such as hypoxia.

In this regard, Huergo and colleagues showed that NK cells could confer an EMT-like/NK-protective phenotype to neighboring melanoma cells (48). Studies in mouse models have provided evidence linking AXL to immune-suppressive activities involving various stromal cellular components, including polarization of dendritic cells, myeloid cells, T-cell exclusion, and tumor-associated macrophages (35, 44, 49).

AXL has received much attention for its contribution to invasion, metastasis, and targeted therapy resistance. The current study suggests a link between AXL expression in NSCLC cells and resistance to cytotoxic immune effector cells. Collectively, our results provide insights into how an AXL-based mechanism may control cancer cell susceptibility to immune killer cells and establish AXL as a driver of mesenchymal cells’ resistance to cell-mediated cytotoxicity. Our results provide a rationale to test the ability of AXL inhibitors to overcome EMT-mediated resistance to immunotherapy.

Disclosure of Potential Conflicts of Interest

G. Gaudal has ownership interest (including patents) in BerGenBio ASA. J.B. Lorens is a scientific advisor for, reports receiving a commercial research grant from, and has ownership interest (including patents) in BerGenBio ASA. J.-P. Thiery reports receiving other commercial research support from Biocheetah and is a consultant/advisory board member for Bioysyngen, Aim Biotech, and ACT Genomics. No potential conflicts of interest were disclosed by the other authors.

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References


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