IL15RA drives antagonistic mechanisms of cancer development and immune control in lymphocyte-enriched triple-negative breast cancers.

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Abstract

Despite its aggressive nature, triple-negative breast carcinoma (TNBC) often exhibits leucocyte infiltrations which correlate with favorable prognosis. In this study, we offer an explanation for this apparent conundrum by defining TNBC cell subsets which overexpress the IL-15 immune receptor IL15RA. This receptor usually forms a heterotrimer with the IL-2 receptors IL2RB and IL2RG, which regulates the proliferation and differentiation of cytotoxic T cells and NK cells. However, unlike IL15RA, the IL2RB and IL2RG receptors are not upregulated in
basal-like TNBC breast cancer cells that express IL15RA. Mechanistic investigations indicated that IL15RA signaling activated JAK1, STAT1, STAT2, AKT, PRS40 and ERK1/2 in the absence of IL2RB and IL2RG, whereas neither STAT5 nor JAK2 were activated. RNAi-mediated attenuation of IL15RA established its role in cell growth, apoptosis and migration, whereas expression of the IL-15 cytokine in IL15RA-expressing cells stimulated an autocrine signaling cascade that promoted cell proliferation and migration and blocked apoptosis. Notably, co-expression of IL15RA and IL-15 was also sufficient to activate peripheral blood mononuclear cells upon co-culture in a paracrine signaling manner. Overall, our findings offer a mechanistic explanation for the paradoxical association of some high-grade breast tumors with better survival outcomes, due to engagement of the immune stroma.

Introduction

Tumor infiltration by a variety of immune cells including cytotoxic T-cells, regulatory T-cells, NK cells, neutrophils and macrophages, is a common feature of many cancers (1). While tumor infiltration by cytotoxic lymphocytes is
generally correlated with a favorable outcome, substantial evidence exists that other types of leukocytes can instead promote tumorigenesis by supplying cytokines (such as IL-6, TNF, and IL-1β) that stimulate tumor proliferation, tissue invasion and/or angiogenesis (2).

Tumor cells interact with the immune system and have evolved strategies to neutralize immune attack through stimulating immune checkpoint receptors CTLA4 and PD-1, down-regulating MHC class I molecules, interfering with the perforin/granzyme cytotoxicity functions of T cells or TGFβ mediated inactivation of T cell responses (3).

Immune-cell infiltration in breast cancer is, in general, associated with a favorable prognosis (4). However, the mechanisms that underlie this observation and their variation amongst breast cancers, are poorly understood. Breast cancer is recognized as being a heterogeneous disease in both its malignant and stromal cell compartments, with immune-cell infiltration being particularly associated with some subgroups.

Based upon gene-expression profiles, breast cancer can be divided into subgroups commonly referred to as intrinsic subtypes that include a HER2+, a basal and two luminal subtypes (5). Recent evidence (6) has revealed an even greater complexity within breast cancer but has consistently identified a transcriptome belonging to a “basal” group which overlaps substantially, although not entirely, with that of immunohistochemically defined oestrogen (ER), progesterone (PgR) and HER2-receptor negative breast cancers, referred to as triple negative breast cancer (TNBC). In ER-positive breast cancers, expression of proliferative biomarkers is largely associated with a poor prognosis (7). This is not the case in ER-negative breast cancers, which are more
uniformly highly proliferative and in which immune-response signatures have been shown to have a higher prognostic significance (8). Even amongst basal tumors there is still significant histological, immunohistological and prognostic heterogeneity (9). Indeed, some morphologically-recognized groups show excellent long-term prognosis despite a basal expression profile and aggressive histopathological features. One such example is medullary breast carcinoma, that shares many features with the TNBC/basal subtype, including similarity in gene-expression profile, highly proliferative phenotype and TP53 mutations (10). Medullary carcinoma also shows numerous lymphoid cells in the stroma (11) and expression of an immune profile including TH1-associated cytokine receptors such as IL15RA, their associated cytokines including IL15 and genes involved in cytotoxicity (12).

IL15RA usually exists in a heterotrimeric receptor with IL2RB and IL2RG, which also contribute to the trimeric IL2 receptor (13). IL15RA is able to bind IL15 with high affinity in the absence of the beta and gamma subunits. This allows the receptor to function in cis, i.e. the three subunits being on the same cell, or in trans, with the IL15RA subunit bound to IL15 on one cell and the beta and gamma subunits on an adjacent cell. In the latter case, the IL15/IL15RA complex is trans-presented to the beta and gamma subunit with consequent activation of intracellular signaling (14).

IL15-dependent signaling has been implicated in the modulation of adaptive immune responses, (15) the activation and maintenance of distinct lymphocyte populations (16) and the inhibition of apoptosis in multiple systems (17).
Furthermore, IL15-dependent signaling has been shown to play a role in hematological malignancies, where there is evidence that an IL15/IL15RA autocrine growth-stimulation loop plays a role in the progression of human T-cell lymphotrophic virus 1 (HTLV-1)-associated adult T-cell leukemia/lymphoma (14).

There are several reports of IL15 and/or IL15 receptor expression in solid-tumor-derived cell lines or tissues. A functional heterotrimeric IL15 receptor has been described in renal cancer (18) and IL15RA expression, has been found in melanoma models (19).

In the present study we find IL15RA to be frequently amplified and describe elevated expression of both IL15RA and its ligand IL15, in the absence of IL2RB and ILRG, in basal and immune-modulatory forms (20) of TNBC and in BCCLs. We investigate the consequences of this coordinated expression, describing a tumor-promoting IL15 and IL15RA-dependent autocrine signaling mechanism that drives cell growth, colony formation and cell migration and protects from apoptosis. We also show that IL15/IL15RA expression on the plasma membrane of BCCLs drives signaling in peripheral blood mononuclear cells (PBMCs) making BCCLs visible to the immune system and potentially leading to an anti-tumor immune response. In doing so, we provide a potential mechanistic explanation for the paradox in which some basal breast cancers show a high mitotic index, aggressive morphological appearances and yet engage an effective immune-cell response with an associated impact on prognosis.

**Materials and Methods**
Cell lines

BCCLs were obtained from ATCC, HMEC from Life Technologies, and YT cells from Dr S. John (KCL, UK). Growth conditions were as recommended by the suppliers. Cells were authenticated by Short Tandem Repeat analysis and matched to the German Collection of Microorganisms and Cell Cultures (DSMZ)-database.

DNA copy-number and gene-expression analysis

Absolute DNA copy number was obtained for 172 cancers in our TNBC-enriched Guy’s Hospital breast-cancer cohort (21) using Affymetrix SNP6.0 genome profiles and human genome build hg18 mapping. The Tumor Aberration Prediction Suite algorithm (22) was applied to minimize influence of normal tissue. IL15RA and IL15 levels were extracted from the Guy’s Hospital (21), METABRIC (23), TCGA (6) 579 TNBC cohorts (TNBC579_GSE31519; // (24)), as well as two breast-cancer cell line datasets (25) (26). PAM50 classification was as described for the Guy’s (27), METABRIC (23), and TCGA cohorts (6). TNBC subtypes were established using the online TNBCTYPE program (28). IL15RA and IL15 expression was compared using Pearson’s correlation. All analyses were performed with R software (www.r-project.org/).

Quantitative PCR (qPCR) analyses

Methodology for qPCR is described in Supplementary methods.

Western blotting

Western blots were processed as described in Supplementary methods.
**Immuno-fluorescence and confocal microscopy**

Cells were processed as described in Supplementary methods. Confocal images were acquired by an A1R Si Confocal system (Nikon Corp.) equipped with a 100X objective. Optical sections were taken at 1 Airy unit at 1 μm intervals, then reconstructed in a z-stack. Images assembled in Adobe Photoshop CS5.1.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded (FFPE) sections 3 μm thick were processed as described in Supplementary methods then stained with the MAB147 IL15RA antibody (R&D Systems) at 10ng/mL for 2h. Images form digitalized scans of the glass slides specimens were obtained at magnification x20 (0.45 μm/pixel resolution) using a Hamamatsu Nanozoomer 2.0 HT.

**Xenotransplants**

UM13, MC1 and UM2 patient-derived xenotransplants were established from primary breast tumors at University of Michigan as described by Cariati et al (29). Once established in the mouse mammary fat pad, xenotransplants were characterized by IHC: UM13 was ER- positive and negative for PR, HER2, EGFR and cytokeratin 5/6. MC1 and UM2 were ER, PR, HER2- negative but EGFR and CK5/6 – positive.

**Small interfering RNA (siRNA)-mediated silencing**

Human Silencer® Select siRNAs including a non-targeting negative control siRNA were purchased from Ambion (Life Technologies) as detailed in
Supplementary methods and transfected at 50nM by RNAiMax (Life Technologies). Knockdown efficacy was assessed 72 hours post-transfection.

**Small hairpin RNA (shRNA)-mediated gene silencing**

MISSION™ shRNA Library-derived clones (Sigma Aldrich,) were used as detailed in Supplementary methods. Lentiviral particles were produced and titrated as described in Supplementary methods.

**Cell population growth assays**

Cells were seeded at 8 x 10^3 per well in a 96-well plate. Effect on cell growth was assessed using Hoechst 33342 nucleic acid stain (Life Technologies) at the times indicated after seeding, typically 24 h (T₀), 48 h (T₁), 72h (T₂) and 96 h (T₃) using the FLUOstar Omega plate reader (BMG LabTech). Hoechst33342 readings were standardized against T₀ to give a relative growth value.

**Caspase activation analyses**

Caspase 3/7 activity was measured at 24 h after seeding or as indicated, using the Caspase-Glo® 3/7 Assay (Promega.).

**Colony formation assays**

1x10^3 or 2 x 10^3 cells per well were plated in 6-well plates and cultured in their ideal media for 15 days before staining with 0.05% crystal violet in 1% formaldehyde for 20 min. Cells were then washed with PBS and imaged.
proteins expression and phosphorylation

Cells were grown to 50% confluence then serum starved in normal growth medium for 3 h prior to addition of rIL15 (Peprotech) at the indicated concentrations for 15 min at 37 degrees Celsius. The proteins were then harvested by scraping the cells in Laemmli buffer with 10% β-mercaptoethanol, and separated by SDS-PAGE.

Phospho-kinase array

The Human Phospho-Kinase Array (R&D Systems,) was used according to the manufacturer instructions. Briefly, a protein lysate was obtained from approximately 5 x 10^5 control or IL15 stimulated cells in the specific lysis buffered provided. Lysates were then incubated with the filters provided and phosphorylation levels of the individual sites in the array revealed by chemoluminescence on a radiographic film, and quantified by densitometry using ImageJ software.

Data and Statistical analysis

Data and statistical analyses have been performed using Microsoft Excel and GraphPad Prism software.

Cell migration assays

Cell migration was assessed using transwell inserts (96-well microplates, 8 μm pore, Corning-Costar, Amsterdam, The Netherlands) coated with bovine collagen type-1 (Invitrogen) and seeded with 25x10^3 cells resuspended in 90 μl RPMI 0.1% FBS. The microplate inserts were placed on the receiver plate containing...
medium supplemented with 2% FBS and incubated for 4h or overnight at 37°C. Migrated cells were detached from the inserts by trypsin and counted using a plate reader after permeabilisation with 0.01% Triton X-100 and DNA labeling by SYTOX® Green (Invitrogen).

**Cell adhesion assays**

10x10^3 cells were seeded on a thin layer of collagen type-I (Invitrogen) and incubated at 37°C for 60 min. Plates were then washed 3 times with PBS and the remaining attached cells were then permeabilised with 0.01% Triton X-100, DNA labeled by SYTOX® Green (Invitrogen) and counted using a plate reader.

**Flow-cytometry analysis**

Cultured cells were detached with Accutase (PAA Laboratories) and washed twice with PBS before staining. PBMCs were isolated by Ficoll gradient centrifugation of whole blood from healthy volunteers and washed twice with PBS. Cells were first stained with LIVE/DEAD Aqua (Molecular Probes) for 20 minutes at room temperature, then washed twice with FACS buffer (PBS, 2% FCS, 2mM EDTA,) before staining in 1:50 anti-IL15Ra PE (BioLegend clone JM7A4), anti-IL2Rb APC (BioLegend clone TU27) and anti-IL2Rg Brilliant Violet 421 (BD Biosciences clone AG184) or paired isotype controls in FACS buffer for 20 minutes at 4°C. Cells were washed twice with FACS buffer before fixing in CellFIX (BD Biosciences) and acquisition on a BD FACS Canto II.

**ELISA**
2x10^6 cells were seeded in 1 ml of culture medium in 6-well plates and incubated overnight. Supernatants were harvested and centrifuged to remove debris. Cells were detached with Accutase (PAA Laboratories) and counted before lysis in 300 μl of lysis buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 5% glycerol). Cells were incubated on ice with intermittent vortexing every 10 min for 60 min. Lysates and supernatants were assayed for IL15 by sandwich ELISA (R&D Systems Human IL15 DuoSet) as per manufacturer's instructions. IL15 concentration was normalized to 10^6 cells.

**PBMC and breast cancer cell co-culture**

PBMCs were taken from healthy adult donors after IRB approved informed consent. Mononuclear cells were obtained by Ficoll gradient centrifugation (GE Healthcare). 5x10^4 breast cancer cells were plated in a 96-well plate and incubated overnight. The following day, the same number of PBMCs was added. After 30 minutes, PBMCs were harvested and stained with pSTAT5 antibody (BD Biosciences) and analyzed by flow cytometry. When indicated, a blocking anti-IL15 monoclonal mouse antibody (MAB2471, R&D Systems) was added at 10μg/ml to BCCLs 30 minutes prior to co-culture with PBMCs.

**Results**

**Amplification and overexpression of IL15RA in subsets of TNBCs and BCCLs**

Several studies (23) (6), have shown that the most distal region on chromosome 10p is frequently amplified in TNBCs. Investigating the copy number of chromosome 10 in our Guy’s TNBC enriched BC cohort (27) using Affymetrix...
SNP6.0-derived genomic profiles, we confirmed the presence of an amplicon encompassing approximately 10 Mbp of the most distal region of the short arm of chromosome 10 (10:82,824-8,738,800; hg18). Within this amplicon, a number of genes had an absolute copy number of greater than 4 in at least 30% of TNBC cases (Fig. 1A and Suppl. table 1). Analysis of these genes revealed some with features suggestive of a possible role as cancer drivers. Among these, *IL15RA* attracted our interest as it encodes the alpha subunit of the IL15 receptor known to be involved in the signaling cascade controlling proliferation and activation of immune cells. *IL15RA* showed an absolute CN of greater than 4 in 43% (48/111) of TNBC in contrast to 18% (11/61) of non-TNBC (Fisher's exact test; pValue<0.001). To investigate whether the abundance of *IL15RA* expression is enhanced specifically in TNBCs, its expression was extracted from the transcriptomes of the Guy’s TNBC enriched BC collection, the invasive ductal breast carcinomas in METABRIC (23) and the TCGA breast-cancer cohorts (6) (Fig. 1B). Using the PAM50 breast-cancer subtype classification published for each cohort, increased *IL15RA* expression was consistently observed in basal and in normal-like breast cancer subtypes. Given the heterogeneity within TNBCs, we investigated *IL15RA* expression across previously defined TNBC subtypes (20) in the Guy's cohort and a publicly-available dataset of 579 TNBCs (GSE31519) (24) (Fig. 1C). Higher *IL15RA* expression was observed in the immunomodulatory (IM) subtype, characterized by expression of genes involved in several immune-cell signaling pathways that also significantly overlap with gene-signatures for medullary breast cancers (20).

To identify cellular models, we analyzed expression of components of the IL15 receptor in 51 BCCLs (26) finding significantly higher expression of *IL15RA*, but
not IL2RB or IL2RG, in “basal” versus “luminal” BCCLs (t-test pValue < 0.001) (Fig. 1D). To confirm localization and presence of IL15RA and absence of IL2RB and IL2RG on basal-like BCCLs, FACS analyses were performed using antibodies against cell-surface-exposed epitopes on non-permeabilised cells. Plasma membrane IL15 receptor subunit levels were consistent with expression data. Only IL15RA was detected on the plasma membrane of the basal-like BCCLs HCC1143, HCC38 and MDA-MB-231, with all components being absent on the luminal-like BCCLs BT549, HCC1428 and SKBR3, despite all components being detected on positive control YT, NK or peripheral blood mononuclear cells (PBMCs) (Fig. 1E). Immunoblotting of whole cell lysates from BCCLs and non-malignant HMECS confirmed that IL15RA could be detected and was enriched in basal-like BCCLs, whereas neither IL2RB nor II2RG could be detected except in the YT cells (Fig. 1F).

Plasma membrane localization of IL15RA was further confirmed by both confocal microscopy (Fig. 1G) and immunohistochemistry in sections of FFPE embedded HCC1143 cells, where plasma membrane staining was shown to be specific by its substantial diminution by siRNA-mediated IL15RA silencing (Fig. 1H). To examine IL15RA localization in relation to the plasma membrane in patient derived xenotransplant (PDX) tissue in which we had demonstrated presence or absence of IL15RA expression on immunoblot (data not shown), we examined sections of these PDXs stained using the same IL15RA antibodies. This again showed a plasma membrane expression pattern in basal-like PDXs, which was almost absent in a non basal-like ER positive PDX and normal breast tissue (Fig. 1I).
Silencing of IL15RA results in a growth defect in BCCLs

Having confirmed surface expression of an apparently isolated IL15RA receptor component in basal-like BCCLs, we then examined its function. We silenced IL15RA by RNAi in cell lines expressing either high or low levels of IL15RA. The IL15RA shRNAs induced significant impairment of growth in the high-IL15RA expressing MDA-MB-231 but not in the low-expressing SKBR3 cell line (Fig. 2A, B). We also analyzed the effect of IL15RA knockdown on adherent cell colony formation. After a 15-day incubation, MDA-MB-231 cells knocked-down for IL15RA showed a significantly reduced number of colonies compared to control cells (Fig. 2C, D). To test a broader range of cells and an orthogonal RNAi methodology, we transfected siRNAs targeting IL15RA in MDA-MB-231 and HCC1143 cells and confirmed impaired cell-population growth (Fig. 2E, F). No effect was observed in the low-IL15RA expressing SKBR3 and BT549 cell lines (Fig. 2G,H, I).

IL15 and IL15RA have anti-apoptotic effects in BCCLs

IL15 signaling can protect against lethal apoptosis (17). We asked whether the reduction in cell population growth and colony formation, after IL15RA silencing might be in part a consequence of apoptosis. We observed substantial activation of caspase 3/7 upon IL15RA KD in MDA-MB-231 and HCC1143 cells (Fig. 2J). Addition of recombinant IL15 to HCC1143 cells cultured in 1% rather than 10% FBS resulted in dose-dependent inhibition of serum-deprivation induced caspase 3/7 activation (Fig. 2K), suggesting that IL15, via IL15RA, inhibits apoptosis.

IL2RB and IL2RG silencing does not affect BCCLs
The absence of detectable beta and gamma subunits of the hetero-trimeric IL15 receptor in BCCLs suggests that the alpha subunit alone might be sufficient to drive the described effects on cell-population growth. To further exclude the possibility that the IL2RB or IL2RG subunits contribute to IL15RA’s influence on BCCL growth, we silenced IL2RB and IL2RG. This failed to show any significant effect in the growth rate of HCC1143 cells but induced a significant defect in IL2 stimulated proliferation of control YT cells, which express the full hetero-trimeric receptor (Fig. 3).

**An autocrine IL15/IL15RA signal drives cell-population growth in BCCLs**

Previous work has shown that IL15 and IL15RA bind to each other along the secretory pathway, remaining in a complex upon arrival at the plasma membrane (30). Indeed an autocrine IL15/IL15RA-mediated signaling mechanism occurs in T-cell lymphoma (14). We therefore investigated whether TNBCs and BCCLs express IL15, how its expression is correlated with IL15RA expression and to what extent this is required for IL15RA-mediated growth. Within the 579 TNBCs cohort (GSE31519) (24) we found a positive correlation between the mRNA levels of IL15RA and IL15 (Fig. 4A, cor: 0.51; p=2.2e-16). Similarly, in some cell lines, the expression of these two genes was correlated ((26)) (Fig. 4B, cor: 0.36; p=0.007) and on average showed a higher expression of the ligand and receptor in cells of basal-like rather than luminal-like subtypes (Fig. 4B,C).

Since expression of IL15 mRNA does not always correlate with the protein (14), we assessed IL15 protein levels in BCCLs by ELISA and found higher levels in basal-like cell lines that expressed higher levels of IL15RA (Fig. 4D). This led us
to hypothesize that cytokine and receptor expressed by the same cells might signal using an autocrine mechanism promoting proliferation of BCCLs. To test this, we knocked down IL15 expression using shRNAs and observed a significant impairment of growth in MDA-MB-231 and HCC1143 (high IL15RA and IL15) but not in SKBR3 cells (low IL15RA and IL15) (Fig. 4E-G). We examined the effect of knockdown of endogenous IL15 on levels of spontaneous apoptosis in HCC1143 cells and showed significant caspase activation (Fig. 4H).

**IL15 and IL15RA dependent intracellular signaling in BCCLs**

Having observed a reduction in growth and an increase in apoptosis upon IL15RA KD in basal BCCLs, we sought to identify the mechanisms downstream of IL15RA controlling these functions. In T-cells, the IL15 hetero-trimeric receptor signals through JAK1, JAK3 and STAT3 or STAT5 thereby regulating cell proliferation and inhibiting apoptosis (31). We analyzed the activity of a range of intracellular signaling kinases in HCC1143 cells using a validated phospho-antibody array. The stimulation of HCC1143 cells with recombinant IL15 significantly increased phosphorylation of ERK1/2, STAT1, STAT2, AKT and PRAS40 (Fig. 5A, B). ERK1/2 and STAT1 phosphorylation were independently validated in lysates from IL15-stimulated HCC1143 cells using a different set of phospho-protein antibodies and western blots (Fig. 5C).

It has previously been shown that the IL15 receptor signals through JAK1 and JAK3 in T lymphocytes (31) whilst in mast cells, IL15 activates JAK2 and STAT6 through a poorly defined 60-65 kD IL15 receptor sub-unit (IL15RX) (32). We assessed changes in JAK1 and JAK2 phosphorylation upon IL15 stimulation. JAK1
but not JAK2 phosphorylation was induced by IL15 stimulation in HCC1143, MDA-MB-231 and HCC1954, but not in SKBR3, suggesting a dependency upon IL15RA (Fig. 5D). We also observed no change in STAT3, STAT5 and STAT6 phosphorylation, which are known to be downstream of the hetero-trimeric receptor. This finding was validated by western blotting HCC1143 cell lysates using independent antibodies (Fig. 5E).

To confirm IL15RA-dependent activation of JAK1 and ERK1/2, we knocked-down the expression of IL15RA using siRNAs in HCC1954 cells, then stimulated with recombinant IL15 and observed substantial reduction in the levels of IL15-stimulated phospho-JAK1 and ERK1/2 (Fig. 5F).

IL15RA regulates cell adhesion and motility via JAK1/STAT1 independent of its effect on ERK1/2

IL15 signaling has been implicated in T-cell adhesion and motility (14). We hypothesized that interfering with IL15/IL15RA function might impair cell adhesion and/or motility in BCCLs. We tested MDA-MB-231 and HCC1954 cells in a standard transwell 2D-motility assay, performed on a thin layer of collagen type-I. IL15RA KD induced a 35-50% reduction in the number of cells migrating (Fig. 6A). We then investigated whether the migration defect observed was due, at least in part, to a defect in adhesion and confirmed a defect in adhesion of IL15RA silenced MDA-MB-231 cells to the collagen layer (Fig. 6B). Nevertheless, the small degree by which adhesion was reduced suggests that this does not fully account for the reduction migration and that other processes in cell migration are affected by IL15 signaling in these cells.
As the JAK1/STAT1 pathway plays a role in cell motility (33), we investigated the possible influence of the previously-observed IL15-mediated JAK1/STAT1 activation on BCCL motility by silencing JAK1 expression. We observed a significant reduction in HCC1954 cell migration (Fig. 6C) but no concomitant reduction in cell-population growth (Fig. 6D, E). In parallel, we observed abrogation of IL15 dependent STAT1 phosphorylation, whereas no substantial change occurred in the phosphorylation of ERK1/2 (Fig. 6F).

These findings suggest a split signaling cascade downstream of IL15RA in BCCLs, in which IL15-dependent activation of JAK1 leads to phosphorylation of STAT1 and subsequent regulation of cell motility. At the same time ERK1/2 activation occurs by a JAK1/STAT1 independent mechanism and is most likely responsible for promoting cell proliferation.

The presence of an IL15RA/IL15 complex on the surface of BCCLs stimulates a signaling response in immune cells

Given the established role of IL15 and IL15RA as promoters of T cell recruitment and activation (14) we asked whether the levels of IL15 being expressed by basal-like BCCLs, if secreted or exposed by IL15RA on the cell surface were sufficient to activate a response in PBMCs. BCCLs expressing different levels of IL15RA/IL15 were co-cultured with PBMCs and the phosphorylation of STAT5 was measured by flow cytometry in the gated PBMC population. We observed increased STAT5 phosphorylation following exposure of PBMCs to HCC1143 and MDA-MB-231 but not to IL15/IL15RA low-expressing SKBR3 and T47D. The concomitant addition of a blocking anti-IL15 antibody or the silencing of IL15 or
IL15RA through shRNAs, resulted in a significant reduction of STAT5 phosphorylation in PBMCs. This indicates a specific role for IL15/IL15RA produced by the cancer cell leading to STAT5 phosphorylation in the PBMCs (Fig. 7). Furthermore, conditioned medium from MDA-MB-231 cells did not induce STAT5 phosphorylation in PMBCS suggesting trans-presentation, as opposed to secreted IL15, is required for PBMC activation (data not shown).

Discussion

Although expression of IL15RA occurs in several cell types, expression of a functional hetero-trimeric IL15 receptor comprising IL2RB and IL2RG is mostly restricted to immune cells: T lymphocytes, B lymphoblasts and NK cells (34). This is consistent with our finding of undetectable levels of the beta and gamma subunits by flow-cytometry or western-blot in BCCLs. Our demonstration of the high frequency of amplification of IL15RA and of expression IL15RA protein on the surface of sub-groups of TNBCs and BCCLs suggest that this component of the receptor may drive malignant phenotypes, despite isolation from other components of the classical receptor, in a significant proportion of TNBCs. As expected, we did not find a strong correlation between IL15RA copy number and its expression in tumors (data not shown) because of the confounding effect of infiltration of leucocytes that usually express this receptor. IL15RA KD experiments using multiple RNAi methodologies confirmed a role for IL15RA in the regulation of cell growth, migration and apoptosis in basal BCCLs that express it at higher levels.

A positive correlation between IL15 and IL15RA transcript, in both tumors and BCCLs, prompted us to investigate and confirm by ELISA, that IL15 protein was
expressed in basal BCCLs that also express IL15RA. We then confirmed that IL15, known to be capable of binding to IL15RA intracellularly for co-transport to the plasma membrane (30), was also required for growth, suggesting an IL15/IL15RA autocrine driven malignant phenotype in BCCLs. 

To our knowledge, this is the first report of such a role for the IL15/IL15RA complex in the absence of a full hetero-trimeric receptor, although an IL15/IL15RA driven malignant phenotype has been suggested in myeloma and T cell lymphoma (which also express IL2RB and IL2RG) (35, 36) and melanoma (where the expression of IL2RB and IL2RG was neither verified nor disproved) (19).

We then aimed to characterize IL15-dependent signaling in TNBCs. In lymphocytes, the signalling cascade originating from the IL15 hetero-trimeric receptor involves activation of JAK1/3 and STAT3/5 (31). Further data suggested IL15-dependent activation of the PI3K/AKT pathway (37) and the NF-kB pathway in neutrophils (38) or myeloid cells (39). IL15RA also interacts with and signals through SYK in neutrophils (40). Evidence obtained in epithelial cells suggested a signalling function for IL15/IL15RA in the absence of IL2RB (41), while in melanoma, IL15RA can bind and signal through TRAF (19).

Upon stimulation of BCCLs with recombinant IL15, we observed activation of JAK1, STAT1 and STAT2, as well as ERK1/2 and AKT and PRAS40. Intriguingly we did not observe activation of other known effectors such as STAT3, STAT5 or STAT6. We speculate that this is a consequence of the lack of IL2RB and IL2RG that have been reported to specifically mediate the activation of many of these effectors (31)
Our observation of ERK1/2 activation is consistent with an IL15RA dependent stimulation of proliferation in TNBCs. The lack of any evidence of activation of MEK might be explained by our observation of IL15 dependent activation of AKT/PRAS40 which may lead to MEK-independent ERK1/2 activation, as has been suggested in melanoma (42). Interestingly AKT phosphorylates PRAS40 specifically at T246 (43), the same residue that we found phosphorylated in response to IL15 stimulation. AKT activation can inhibit apoptosis (44) and we speculate that this mechanism may be responsible for the inhibition of apoptosis observed in BCCLs upon IL15 stimulation.

JAK1/STAT1 signaling is known to play a role in cell motility (45) (33). This is consistent with our observation of impaired migration of IL15RA/IL15 expressing cells upon JAK1 knockdown in the absence of any significant reduction in growth rate. We also found JAK1 silencing inhibits STAT1 activation but not ERK activation consistent with lack of effect of JAK1 silencing on cell growth.

It is increasingly apparent that the interface between immune cells and tumor cells can induce powerful effects on prognosis. Several reports have indicated positive prognostic correlations with evidence of effective engagement of both humoral and cytotoxic T cell adaptive immune responses (8) while others have shown expression of inflammatory cytokines is associated with a poor prognosis (1).

There is growing evidence suggesting inflammatory cytokines can regulate the growth of breast cancer cells. A co-operative role for IL6 and IL8 in promoting
TNBC growth has recently been suggested and while the expression of IL15RA and IL15 in TNBCs and TNBC-derived cell lines was also noted, it was not further investigated (46). This, together with evidence of IL6 stimulation of IL15 expression observed in HaCaT cells (47), suggests co-operation among pro-inflammatory cytokines to promote cancer cell growth despite known roles in stimulation of the immune response.

We show the endogenous expression of IL15RA and IL15 by basal BCCLs stimulates a pSTAT5 signaling response in freshly isolated PBMCs in co-culture. This phenomenon is abolished by an inhibitory IL15 antibody as well as by silencing of either IL15RA or IL15. Taken together, this evidence points to PBMC activation by IL15 expressed by basal BCCLs co-expressing IL15RA. This most likely occurs via trans-presentation of IL15 by IL15RA, as knockdown of IL15RA inhibited pSTAT5 signaling in PBMCs. IL15 is known to be a potent inducer of the activatory receptor natural killer group 2D (NKG2D) receptor on cytotoxic lymphocytes and is an important survival factor for NK cells (48, 49). Indeed NKG2D mediated anti-tumor immunity is enhanced by IL15 (50).

Our data suggests a dual effect of IL15RA amplification and its consequent high-level expression. IL15RA co-expressed with IL15 in the absence of IL2RB and IL2RG, on the one hand drives cancer proliferation, protection from apoptosis and enhances cell migration while on the other hand is capable of activating signalling in immune cells that, when not edited, suppressed or compromised by other intrinsic tumor or stromal factors, may lead to a relatively better prognosis.

A number of clinical trials (NCT01727076, NCT01885897) are currently
investigating the effects IL15 administration with an aim to stimulate anti-cancer immune responses. Our data adds further insight into the complexities of IL15 signaling in some breast tumors, not only in immune cells in the tumor stroma, but also within malignant cells in which IL15 and IL15RA may also drive hallmarks of malignancy. This may explain why some basal breast carcinomas with aggressive high-grade malignant features are also intensely infiltrated by immune-cells and why these high-grade cancers have a paradoxically good prognosis compared with other basal breast cancers (11). If an IL15 trans-presentation induced activation of immune cells is blocked or edited by immune checkpoints at the immune and cancer cell interface but cancer-cell intrinsic IL15/IL15RA drivers of the malignant phenotype remain intact we speculate there may be adverse affects on prognosis. Our data indicating IL15 dependent cancer cell signaling suggest that IL15 therapies should be investigated cautiously in IL15RA expressing cancers as there may be the potential to drive tumor growth.

A more detailed understanding of the signalling pathway involved in IL15/IL15RA signaling in cancer cells, and the anti-cancer immunological mechanisms that can be enhanced by IL15, would aid identification of cancer cell intrinsic targets downstream of IL15 / IL15RA that might be inhibited to block pro-tumorigenic effects whilst retaining the anti-tumor immunostimulatory effects of an IL15-based therapy.

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References


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Figure Legends:

Legend to Figure 1:

Copy number, mRNA and protein expression of IL15RA in breast cancer and cell models

A, chromosome 10 frequency plot of amplification and deletion across 111 TNBCs and 61 Non-TNBCs profiled with Affymetrix SNP6.0 microarray. The percentage of tumors in which each segment is amplified (defined as more than 4 copies) is plotted in green for TNBCs and yellow for Non-TNBCs, while deletions are shown in red and purple for TNBCs or Non-TNBCs, respectively. The location of IL15RA is indicated by a black line. B, IL15RA gene expression across breast carcinomas of the Guy’s TNBC enriched BC, METABRIC and TCGA cohorts is shown. The classification of the 3 cohorts into molecularly defined breast cancer subtypes was based on their previously published PAM50 centroid correlation (Basal in red, HER2 in pink, Luminal A (LumA) in dark blue, Luminal B (LumB) in light blue, Normal-like (NL) in green). Mean-centered expression values (log2 scale) are shown on the y-axis. C, IL15RA gene expression across Guy’s TNBCs and the 579 TNBCs (GSE31519) is shown using Vanderbilt TNBC subtype classification. (TNBC subtypes are represented pink for basal-like 1 (BL1), blue basal-like 2 (BL2), orange immunomodulatory (IM), grey mesenchymal (M), magenta mesenchymal stem-like (MSL), green luminal androgen receptor (LAR). D, relative expression of IL15RA, IL2RB and IL2RG
across 51 basal-like and luminal-like breast cancer cell lines, each dot represents a cell line. E, FACS-analysis of IL15RA, IL2RB and IL2RG surface expression on HCC38, HCC1143, and MDA-MB-231 (basal-like, high IL15RA, red) or BT549, HCC1428, and SKBR3 (luminal-like, low IL15RA, green) and HMEC (non-malignant, IL15RA-low) with YT NK cells, CD56+ PBMCs and lymphocytes (IL15RA, IL2RB, IL2RG) as positive controls. F, Total protein lysates from non-malignant HMEC, luminal (green) or basal (red) BCCLs, and YT cells (positive control), were probed with anti-IL15RA, IL2RB and IL2RG antibodies and GAPDH as a loading control. G, 3D reconstruction of serial confocal z-axis sections from a non-permeabilized HCC1143 cell stained for IL15RA (red) and DNA (Hoechst 33342, blue). A top view and 2 orthogonal views along the yellow bars are shown. White arrows indicate IL15RA staining which predominantly decorates the contour of the cells.

H, IHC images of HCC1143 cells either untreated (left) or KD for IL15RA (right) by transfection of IL15R siRNA#0 as described in Materials and Methods, and stained with the MAB147 monoclonal antibody against IL15RA (R&D Systems, Minneapolis, USA). I, IHC images of the MC1 and UM2 (ER-, PR- and HER2-negative, CK 5/6 and EGFR+ve) and UM13 (ER-positive, PR- and HER2-negative and K 5/6 and EGFR -ve) PDX models and normal breast tissue, stained with the MAB147 antibody against IL15RA.

Legend to figure 2

**IL15RA silencing impairs cell growth and colony formation and increases**
apoptosis in BCCLs expressing high levels of IL15RA

Analysis of cell population growth of A, MDA-MB-231 (high IL15RA expressing) and B, SKBR3 (low IL15RA expressing) cells transduced with control shRNA or shRNAs targeting IL15RA. C, MDA-MB-231 colony formation assay on plastic. Cells were either untreated (cont) or transduced with lentivirus driving expression of a non-specific shRNA (ns), or shRNAs targeting IL15RA (IL15R sh1, sh2 and sh3) then incubated for 15 days prior to being fixed/stained and imaged. D, quantification of shRNA-mediated IL15RA KD in MDA-MB-231 cells by qPCR (top) and immunoblotting (bottom).

Analysis of cell population growth of MDA-MB-231 (E), HCC1143 (F), SKBR3 (G), and BT549 (H) cells transfected with control siRNA (non-targeting) or siRNAs targeting IL15RA (IL15 siRNA#0, IL15 siRNA#2). MDA-MB-231 and HCC1143 cells express high levels of IL15RA and BT549 and SKBR3, low levels. I, Quantification of siRNA-mediated IL15RA KD in MDA-MB-231 cells by qPCR (top) and immunoblotting (bottom). J, caspase 3/7 activation was assessed in either untreated MDA-MB-231 and HCC1143 cells or cells transduced with a control shRNA (non-targeting) or shRNAs targeting IL15RA, 24h after seeding the cells for the cell population growth assay, equivalent to 7 days post-transduction.

K, the effect of IL15 on caspase 3/7 activation was assessed in HCC1143 cells. Cells were serum starved in the presence of increasing amounts of IL15 as indicated and incubated for 24h prior to assessing caspase activity.

Data representative of at least 2 independent experiments, values are average
±SD, sample size=3, * = p<0.05, **=p<0.01, ***=p<0.001

Legend to figure 3

IL2RB and IL2RG knockdown does not affect growth of BCCLs

Cell population growth assay of HCC1143 cells (A and B) and IL2-stimulated growth of YT NK cells (C and D), following siRNA-mediated knockdown of IL2RB (A and C) and, IL2RG (B and D). YT NK cells were grown in the absence or presence of 20ng/ml IL2 and the raw data obtained from the cells grown without IL2 were subtracted from those of stimulated cells. The difference is referred to as IL-2 dependent growth and shown in the graphs (C and D). IL2 stimulation of HCC1143 did not produce any increase in the cell population growth rate (data not shown). The efficacy of siRNAs targeting, IL2RB (E) and, IL2RG (F) was assessed in YT cells by qPCR.

Data representative of at least 2 independent experiments, values are average ±SD, sample size=3, * = p<0.05, **=p<0.01, ***=p<0.001

Legend to Figure 4

IL15 is expressed by TNBCs and basal BCCLs and its silencing specifically impairs growth in high IL15RA-expressing cell lines

Correlation of gene expression levels between IL15RA and IL15 across A, 579 TNBC (GSE31519) and B, 51 BCCLs. The Pearson r-values and p-values for
significance are shown. C, relative gene expression of IL15 in 51 BCCL dichotomised into the basal-like (triangles) and luminal-like (dots) phenotypes, whereby each symbol represents a cell line. D, ELISA analysis of IL15 levels in a subset of basal or luminal BCCLs. HaCat cells were included as a positive control. IL15 levels were assessed in whole cells (lysate). E,F,G, Cell population growth assays of MDA-MB-231, (E), HCC1143 (F), (high levels of both IL15 and IL15RA) and SKBR3 (G) cells (low levels of both IL15 and IL15RA) following IL15 knockdown by 2 different shRNA species (IL15 sh26 and IL15 sh27). H, caspase 3/7 activation was assessed in HCC1143 cells transduced with a control shRNA (non-targeting) or shRNAs targeting IL15, 24h after seeding the cells for the cell population growth assay, equivalent to 7 days post-transduction. I, Quantification of shRNA-mediated IL15 knockdown in MDA-MB-231 cells by qPCR.

Data representative of at least 2 independent experiments, values are average ±SD, sample size=3, * = p<0.05, **=p<0.01, ***=p<0.001

Legend to figure 5

**IL15 signals through IL15RA to promote phosphorylation of ERK1/2, JAK1, STAT1, STAT2, AKT and PRAS40 in BCCLs.**

A, HCC1143 cells, (high IL15RA), were stimulated with 100 ng/ml of IL15 for 15 min, then lysed and protein phosphorylation assessed using a human phospho-
protein array. Phospho-protein patterns were consistent in 2 independent experiments, for a full description of the phospho-antibodies included in the array please refer to suppl. Fig. 1

**B,** IL15 induced increased ERK1/2, AKT, STAT2, PRAS40, and STAT1 phosphorylation as assessed by densitometry in one representative experiment, error bars represent ±SD of the densitometry readings from the experimental replicates.

**C,** Phospho protein array results were validated by western blotting using a different set of phospho-antibodies and a wider set of BCCLs, either high-IL15RA expressing such as HCC1954 and HCC1143, or low IL15RA-expressing such as SKBR3.

**D,** Western blot analysis of JAK1 and JAK2 phosphorylation in response to IL15 stimulation in HCC1954, HCC1143 and MDA-MB-231 (high IL15RA) or SKBR3 (low-IL15RA) cell lines.

**E,** Western blot analysis of STAT3, STAT5 and STAT6 phosphorylation in response to IL15 stimulation in HCC1143 cells.

**F,** Western blot analysis of JAK1 and ERK1/2 phosphorylation in response to IL15 stimulation following siRNA-mediated IL15RA KD (siRNA #0, #1 and #2) in HCC1954 cells, non-targeting siRNAs used as negative control.
IL15RA knockdown impairs motility of basal BCCLs by a JAK1/STAT1 dependent, ERK1/2-independent mechanism.

A, MDA-MB-231 and HCC1954 cell migration was investigated using a standard transwell assay in cells transfected with a control siRNA (non-targeting) and siRNAs targeting IL15RA (IL15R siRNA#0, IL15R siRNA#2)

B, MDA-MB-231 cells were seeded on a thin layer of collagen type-I and adhesion assessed following transfection with a control siRNA (non-targeting) and siRNAs targeting IL15RA (IL15R siRNA#0, IL15R siRNA#2)

C, western blot showing efficacy of JAK1 knockdown 72h post-transfection

D, MDA-MB-231 and HCC1954 cell migration was investigated using a standard transwell assay in cells transfected with a control siRNA (non-targeting) and siRNAs targeting JAK1 (JAK1 siRNA#47, JAK1 siRNA48).

E, HCC1954 cell population growth was assessed in cells transfected with a control siRNA (non-targeting) and siRNAs targeting JAK1 (JAK1 siRNA#47, JAK1 siRNA48).

F, The impact of JAK1 knockdown on IL15-induced phosphorylation of STAT1 and ERK1/2 was assessed in HCC1954 cells by western blot.

G, Quantitation by densitometry of the IL15-induced phosphorylation of STAT1 as showed in F

H, Quantitation by densitometry of the IL15-induced phosphorylation of
ERK1/2 as showed in F.

Data representative of at least 2 independent experiments, values are average ±SD, sample size=3, * = p<0.05, **=p<0.01, ***=p<0.001

Legend to figure 7

IL15RA/IL15 expressing BCCLs elicit STAT5 phosphorylation in PBMCs upon co-cultivation.

Flow cytometry analysis of STAT5 phosphorylation in PBMCs co-cultured 1:1 with BCCLs or stimulated with recombinant IL15 (rIL15)(100 pg/ml) for 30 min. IL15 blocking antibody (αIL15) effects were assessed by adding 30 min prior to co-culture. The effect of IL15RA knockdown was also assessed following transduction and selection of MDA-MB-231 cells with shRNAs targeting IL15RA (IL15R sh3) or IL15 (IL15 sh26, IL15 sh27) as well as a control shRNA (Non-targeting).

Data representative of at least 2 independent experiments, values are average ±SD, sample size=3, * = p<0.05, **=p<0.01, ***=p<0.001
Figure 3

A

HCC1143

B

HCC1143

C

YT

D

YT

E

IL2RB knockdown

F

IL2RG knockdown

% of control

IL2RB non-targeting

IL2RB siRNA1

IL2RB siRNA3

% of control

IL2RG non-targeting

IL2RG siRNA1

IL2RG siRNA3

Non-targeting

IL2RB siRNA1

IL2RB siRNA3

Non-targeting

IL2RG siRNA1

IL2RG siRNA3

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Figure 5

(A) Control vs IL15 100 ng/ml

(B) Reference

(C) Summary of HCC1143, MDA-MB-231, and SKBR3

(D) Summary of HCC1954, HCC1143, MDA-MB-231, and SKBR3

(E) HCC1143

(F) IL15 targeting

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Figure 7

![Graph showing pSTAT5+ (%) for different treatments](image-url)
IL15RA drives antagonistic mechanisms of cancer development and immune control in lymphocyte-enriched triple-negative breast cancers.

Pierfrancesco Marra, Sumi Mathew, Anita Grigoriadis, et al.

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