IL4 Receptor ILR4α Regulates Metastatic Colonization by Mammary Tumors through Multiple Signaling Pathways

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Abstract

IL4, a cytokine produced mainly by immune cells, may promote the growth of epithelial tumors by mediating increased proliferation and survival. Here, we show that the type II IL4 receptor (IL4R) is expressed and activated in human breast cancer and mouse models of breast cancer. In metastatic mouse breast cancer cells, RNAi-mediated silencing of IL4Rα, a component of the IL4R, was sufficient to attenuate growth at metastatic sites. Similar results were obtained with control tumor cells in IL4-deficient mice. Decreased metastatic capacity of IL4Rα” knockdown” cells was attributed, in part, to reductions in proliferation and survival of breast cancer cells. In addition, we observed an overall increase in immune infiltrates within IL4Rα knockdown tumors, indicating that enhanced clearance of knockdown tumor cells could also contribute to the reduction in knockdown tumor size. Pharmacologic investigations suggested that IL4-induced cancer cell colonization was mediated, in part, by activation of Erk1/2, Akt, and mTOR. Reduced levels of pAkt and pErk1/2 in IL4Rα knockdown tumor metastases were associated with limited outgrowth, supporting roles for Akt and Erk activation in mediating the tumor-promoting effects of IL4Rα. Collectively, our results offer a preclinical proof-of-concept for targeting IL4/IL4Rα signaling as a therapeutic strategy to limit breast cancer metastasis. Cancer Res; 74(16): 4329–40. ©2014 AACR.

Introduction

Breast cancer is still the second leading cause of cancer-related deaths among women in the United States (1). This is largely due to the high mortality rate in metastatic disease. Cytokines and chemokines in the tumor microenvironment are known to promote breast cancer progression and metastasis (2). Significantly, IL4, a Th2 cytokine, is upregulated in the microenvironment of breast carcinomas (3), and the IL4 receptor (IL4R) is overexpressed by breast cancer cells themselves (4). In the immune system, IL4 is produced predominantly by activated T cells, mast cells, basophils, and eosinophils, and is known to regulate the survival, proliferation, and differentiation of B and T lymphocytes through IL4R (5). Therefore, it is plausible that upregulated epithelial IL4R could promote breast tumor growth.

The IL4/IL4R interaction has been shown to enhance the proliferation and survival of breast cancer cells in vitro (6, 7), and the survival of other epithelial cancer cell types in vivo (4).

Still, there are no data about the influence of IL4R expression on the proliferation and survival of breast cancer cells in vivo, or on metastatic tumor growth in general. Furthermore, activation of signaling pathways downstream of the IL4/IL4R interaction that mediate enhanced tumor growth remains largely unresolved in breast cancer cells that overexpress a different form of the IL4R than immune cells.

Lymphoid cells express the type I IL4R composed of the common γ-chain (γc) and the IL4Rα chain (5). Heterodimerization is necessary for receptor functionality (8). Nonlymphoid cells, including breast cancer cells, express the type II IL4R, composed of the IL4Rα and IL13 receptor alpha 1 (IL13Rα1) chains. IL13 is the only other cytokine known to bind and activate type II IL4Rs. However, IL4 binds with higher affinity and is the prototypical ligand for the IL4R (2).

In lymphoid cells, the IL4α/type I IL4R interaction can result in the activation of two primary pathways: (i) the insulin receptor substrate protein (IRS)/PI3K/Akt pathway and (ii) the JAK/Stat6 pathway (5). Activated Akt (9) and Stat6 (5, 10, 11) are known regulators of proliferation and survival. However, IL4Rα signaling from the type II IL4R may differ from the type I receptor. Demonstrative of this concept, the activation of Akt in response to IL4 seems to be context and cell-type dependent (5, 12), and IL4 has also been shown to activate Erk in several nonhematopoietic cell types (5). Here, we investigate whether IL4Rα, a key component of the IL4R, can promote the metastatic colonization and outgrowth of mammary tumor cells, and explore mechanisms of IL4/IL4R-induced colonization ability in vivo.
Materials and Methods

Cell lines and culture
The 4T1 cell line was acquired from Dr. Albert Reynolds at Vanderbilt University (Nashville, TN). PyVT-R221a cells were isolated from a polyoma middle T oncprotein (PyMT) tumor as previously described (13). The R221a line was authenticated by expression of the polyoma antigen, and the morphology and growth rates of both cell lines were consistently monitored. Cell lines were maintained in DMEM (Invitrogen) containing 10% FBS (Atlanta Biologicals) at 37°C with 5% CO2. Mission lentiviral shRNA particles (Sigma) targeting two different regions of murine IL4Rα (NM_010557) along with control nontarget particles were used to infect 4T1 cells. Control nontarget and specific murine IL4Ra-targeted shRNA lentiviral particles (Santa Cruz Biotechnologies) were used to infect R221a cells. Postinfection, shRNA-expressing cells were selected with puromycin dihydrochloride (Sigma). The percentage of IL4Rα knockdown was calculated by densitometry in Adobe Photoshop following Western blot analysis.

Reagents and antibodies
Murine IL4 was purchased from BD Biosciences. The small-molecule inhibitors, U1026 (EMD Millipore), Ly294002, and rapamycin (both Sigma) were dissolved in DMSO (Sigma) and diluted with culture medium to the appropriate concentration for Western blot analyses and clonogenic assays. The following antibodies were used to probe Western blots: murine IL4Ra, Stat6, Erk2, and β-actin from Santa Cruz Biotechnologies, anti-pStat6 (pY641) from Invitrogen, pErk1/2 (Thr202/Tyr204), Akt, pAkt (ser473), pAkt (Thr308), mTOR, pmTOR (ser2481), pmTOR (ser2448), IRS1, and pIRS1 (ser612) from Cell Signaling Technology. An IL4Ra-Fc chimeric protein (R&D Systems) and isotype human IgG1 control antibody (SouthernBiotech) were used in clonogenic assays.

Commercial arrays
A human tissue array containing 16 stage IIIa/IIIb and 34 stage IIA/IIB primary breast carcinomas from patients with lymph node metastases, cat#BR10010a, was purchased from U. S. Biomax. The array was probed with antibodies for human IL4Ra (Santa Cruz Biotechnologies) and pStat6 (pY641; Sigma). Samples were scored categorically as positive or negative for IL4Ra and pStat6. Conditioned media (serum free) from R221a or 4T1 combined sh-control or IL4Ra knockdown clones were added to an antibody array to detect 62 murine secreted factors (RayBiotech Inc. #AAM-CYT-3-4) per manufacturer’s instructions. Protein expression was quantified by densitometry in ImageJ relative to positive controls.

Analysis of murine tumor models
Animal procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals following approval by the Institutional Animal Care and Use Committee. Female age-matched BALB/c, FVB/N, and BALB/c IL4−/− mice (BALB/c-JlBR10010a; Jackson Laboratories) were used. Two individual clones for each cell line (R221a or 4T1) were mixed to generate combined sh-control or IL4Ra knockdown cells preinjection. Additional assays with 4T1 cells were performed using one knockdown clone, which recapitulated findings with combined clones. At necropsy, lungs were inflated with Bouin's solution (Ricca Chemical Company) to facilitate surface tumor counts. Formalin-fixed, paraffin-embedded tissue sections from lungs and livers were stained with hematoxylin and eosin (H&E) to assess tumor burden. Tissue sections were also stained with primary antibodies for IL4Rα (Santa Cruz Biotechnologies), Ki67 (Abcam), cleaved caspase-3 (Cell Signaling Technology), Ly6B.2, CD3-e, or F4/80 (AbD Serotec). In addition, lung sections were probed for pStat6 (pY641; Sigma), pErk1/2 (Santa Cruz Biotechnologies), and pAkt (ser473; Cell Signaling Technology). Archival paraffin-embedded PyVT and orthotopic 4T1 and R221a sh-control mammary tumors were stained for IL4Ra and pStat6 and analyzed similarly to the human tissue array. Biotinylated secondary antibodies were obtained from Vector Laboratories. Images were white balanced for presentation using Adobe Photoshop.

Statistical analysis
All statistical analyses were performed using GraphPad Prism software. For comparison of two conditions, data were analyzed using the parametric unpaired Student t test, or the nonparametric Mann–Whitney. Otherwise, ANOVA or Kruskal–Wallis with posttest was used. In graphs, error bars represent the SD, and P values are indicated by asterisks (*, ≤0.05; **, ≤0.01; and ***, ≤0.001).

Results
IL4Ra expression and activation in human breast cancer and murine mammary cancer models
To examine the relative abundance and activation of IL4Ra in human breast cancers, we first immunostained a human tissue array consisting of 50 primary node-positive breast cancer samples for IL4Ra and downstream phosphorylation of STAT6 (pSTAT6). A total of 82% of the samples were IL4Ra positive, and 42% were positive for both IL4Ra and pSTAT6 (Fig. 1A). The majority of carcinomas were classifiable by HER2 status (38 HER2 positive and 12 HER2 negative, of which five were triple negative). However, sample numbers were too low to make statistically significant correlations between subtype and IL4Ra or pSTAT6 positivity. Mammary tumors from the PyMT oncogene transgenic mouse, and tumors resulting from orthotopic injection of R221a and 4T1 murine mammary cancer cells, were also positive for IL4Ra and pStat6 (Fig. 1B). Thus, human breast cancers and our murine models both show IL4R expression and activation.

IL4Ra promotes the growth of murine mammary tumor metastases in the lung and liver
Because the IL4/IL4R interaction is species specific, only murine mammary tumor cell lines could be used to study functional roles of IL4R signaling in mouse models. The 4T1 line was derived from a spontaneous mammary cancer in a BALB/c mouse (14). The R221A line came from a mammary tumor in an MMTV-PyVT transgenic mouse on the FVB/N background. (Fig. 1C). The 4T1 cell line was positive for IL4Ra and pStat6 (pY641). Therefore, for these experiments, we used the 4T1 cell line with the IL4Ra-shRNA construct in combination with PyVT-R221a cells to examine IL4Ra expression and activation in an orthotopic model of mammary cancer.
Using commercially available lentiviral particles carrying shRNA, we generated two clones representing different knockdown sequences for the 4T1 line. A separate mix of IL4Rα-targeted shRNA sequences was used to make two different clones of R221A cells. Corresponding sh-control lines made by expression of nontargeting shRNA sequences were also generated. Western blot analysis confirmed a reduction (62%–90%) in IL4Rα protein expression (Fig. 2A), and in Stat6 activation in R221a and 4T1 IL4Rα knockdown clones compared with sh-controls.

Combined sh-control or IL4Rα knockdown clones for each cell line (R221a or 4T1) were injected into the tail vein or spleen of age-matched female mice to examine colonization of lung and liver, respectively. At endpoint, the number of surface tumors on the lungs of tail vein injected mice was significantly reduced in tumors originating from IL4Rα knockdown clones for both cell lines (Supplementary Fig. S1A). In addition, gross liver weights of mice receiving splenic injections of R221a IL4Rα knockdown clones were significantly reduced compared with sh-controls, but no significant difference in liver weight was seen with the 4T1 line (Supplementary Fig. S1B). Following gross examination, lungs and livers were serially sectioned for further analysis. IHC analysis confirmed that functional knockdown of IL4Rα was retained in vivo, as IL4Rα expression (Fig. 2C) and nuclear pStat6 levels (Fig. 2D) were significantly reduced in IL4Rα knockdown lung tumors compared with sh-control for both cell lines. Serial lung (Supplementary Fig. S1C) and liver (Supplementary Fig. S1D) sections were also stained with H&E, and used to calculate tumor burden. In both the R221a and 4T1 cell lines, reduced IL4Rα expression resulted in a significant decrease in metastatic lung and liver tumor burden (Fig. 3A and B).

We previously determined that neither R221a nor 4T1 cells produce detectable levels of IL4 by Luminex analysis (data not shown), suggesting that the cellular source of IL4 in vivo is likely the host. To determine the contribution of host IL4 to mammary cancer metastasis to the lung, as well as the significance of the IL4/IL4R interaction in vivo, wild-type (WT) or IL4−/− (IL4 KO) BALB/c mice were injected via tail vein with either combined sh-control or IL4Rα knockdown 4T1 clones. Supporting the role of IL4 in promoting metastatic tumor growth, the number of lung surface tumors (Fig. 3C) and the lung tumor burden (Fig. 3D) at endpoint were significantly decreased in IL4 KO mice compared with WT mice receiving sh-control cells. These results suggest that the IL4/IL4Rα interaction is a strong promoter of lung metastatic tumor growth in vivo.

**IL4Rα-associated survival and proliferation contribute to metastatic colonization and outgrowth**

The metastatic seeding and outgrowth ability of R221a and 4T1 clones were estimated by quantifying tumor

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**Figure 1.** IL4R expression and activation in human breast cancers and murine models. A, left, representative images of a human tissue microarray of breast carcinoma samples (n = 50) stained by IHC for IL4Rα or pSTAT6. Right, summary of categorical (positive/negative) tissue microarray staining results. B, representative images of IL4Rα and pStat6 immunostaining in murine mammary tumors (n = 3 each). Scale bar, 100 μm. Inset, negative staining controls and one ×40 image (scale bar, 10 μm) of pStat6 in an R221a tumor.
number and area, respectively, from the H&E-stained lung and liver sections (Supplementary Fig. S1B and S1C). The ability of R221a IL4Rα knockdown clones to seed tumors was significantly reduced in both the lung (Fig. 4A) and liver (Fig. 4B) compared with sh-control clones. Although the same was true for 4T1 IL4Rα knockdown clones compared with sh-controls in the lung (Fig. 4A), the decrease in seeding ability in the liver was not significant (Fig. 4B). However, in both cell lines, reduced IL4Rα expression resulted in a significant decrease in lung (Fig. 4C) and liver tumor outgrowth (Fig. 4D).

In addition to quantifying tumor number and size, lung and liver metastases were stained for cleaved caspase-3 (apoptosis marker) and Ki67 (proliferation marker) to further examine the role of IL4Rα in promoting the survival and outgrowth of metastatic tumors (Supplementary Fig. S2). Differences in cleaved caspase-3 in the lungs of mice receiving either R221a or 4T1 IL4Rα knockdown cells by tail vein injection were not statistically significant (Fig. 4E). In R221a tumors, this was likely due to the lack of assessable staining within very small knockdown tumors. Quantification of cleaved caspase-3 staining in liver metastases revealed a significant increase in apoptosis in IL4Rα knockdown tumors of R221a, but not 4T1 recipient mice compared with sh-control (Fig. 4F). Ki67 staining results were more consistent in that mice receiving either R221a or 4T1 IL4Rα

Figure 2. Functional knockdown of IL4Rα expression in vitro and in vivo. A and B, Western blot analysis illustrating the percent knockdown of IL4Rα protein expression (A) and Stat6 activation in response to 20 ng/mL IL4 in individual R221a and 4T1 control (ctl) and IL4Rα knockdown (KD) clones (B). C and D, representative images and quantification of IHC staining for IL4Rα (×40, scale bar, 10 μm; C) or pStat6 (×20, scale bar, 100 μm; D) in R221a and 4T1 sh-control and IL4Rα knockdown lung metastases (n = 4–6). Inset, negative staining controls.
knockdown cells exhibited a significant reduction in proliferation in both lung (Fig. 4G) and liver metastases (Fig. 4H). These data suggest that IL4Rα promotes the growth of murine mammary cancer cells at metastatic tumor sites through enhanced survival and colonization ability and increased proliferation for outgrowth.

**Changes in immune infiltration contribute to IL4Rα-enhanced metastasis**

It is possible that increases in infiltrating immune cells could aid in the clearance of IL4Rα knockout tumor cells with reduced survival ability, thus decreasing metastatic tumor size. We therefore quantified infiltrating immune cells in R221a and 4T1 sh-control and IL4Rα knockout lung and liver metastases by IHC staining of neutrophils (Ly6B.2 positive), macrophages (F4/80 positive), and T lymphocytes (CD3 positive; R221a: Supplementary Fig. S3; 4T1: Supplementary Fig. S4). There was an upward trend or significant increase in the number of each of these cell types in R221a and 4T1 lung and liver IL4Rα knockout metastases compared with sh-control (R221a: Fig. 5A–C; 4T1: Supplementary Fig. S5).

Higher levels of immune infiltrates within knockdown tumors could result from an increased expression of proinflammatory cytokines. To examine this, a murine cytokine array was used to test conditioned media from combined R221a or 4T1 sh-control or IL4Rα knockout clones for the expression of 62 secreted factors. Five factors associated with recruitment of immune cells (MIG, IL1a, Lix, Eotaxin, SCF, and MIP-3) were modestly upregulated by IL4Rα knockdown clones in comparison with sh-control clones for both cell lines (Fig. 5D). Macrophage inflammatory protein-2 (MIP-2) was the only cytokine that increased more than 2-fold in knockdown clones for both cell lines. Leptin receptor has no known direct role in immune cell recruitment; however, this protein was also upregulated more than 2-fold by both R221a and 4T1 knockdown clones. Thus, although it is possible that increased MIP-2 production from IL4Rα knockdown clones may lead to enhanced leukocyte recruitment, we were unable to identify robust patterns of cytokine production that differed between sh-control and IL4Rα knockdown cells.

**IL4-activated Akt, Erk, and mTOR mediate colonization ability of mammary cancer cells**

Our in vivo data establishes that the IL4R mediates enhanced colonization of mammary cancer cells at metastatic sites, and that host IL4 promotes the growth of lung tumor metastases. Therefore, we used exogenous IL4 to elucidate signaling pathways that control IL4Rα-induced colonization ability in vitro. Demonstrative of functional IL4/IL4Rα signaling, treatment of R221a and 4T1 mammary cancer cells with IL4 resulted in the activation of Stat6 (Fig. 6A). Erk1/2 and Akt (ser473) were also phosphorylated.
in response to IL4 in both cell lines, whereas Akt (thr308) was only robustly phosphorylated in R221a cells (Fig. 6A). Consistent with IRS1 inactivation and degradation (15, 16), IRS1 (ser612) was also phosphorylated in response to IL4 in both cell lines in a temporal manner, and total IRS1 levels initially increased then rapidly decreased in the 4T1 cell line.

Figure 4. IL4Rx-associated survival and proliferation contribute to metastatic tumor growth. A and B, enumeration of R221a or 4T1 IL4Rx knockdown (KD) and sh-control lung (A) and liver (B) metastatic tumors per mouse (lung, n = 4–6; liver, n = 5–8) from H&E-stained sections (three per mouse). C and D, quantification of individual lung (C) or liver (D) tumor areas from the same H&E sections. E and F, quantification of total cleaved caspase-3–positive area per total IL4Rx knockdown or sh-control lung (E) or liver (F) tumor area for each mouse (lung, n = 4–6; liver, n = 5–8). G and H, quantification of the total number of Ki67–positive cells per IL4Rx knockdown or sh-control tumor area for each mouse lung (G) or liver (H; lung, n = 3–6; liver, n = 5–8).
We next confirmed the specificity of the IL4/IL4Rα interaction in promoting colonization ability in vitro. R221a or 4T1 combined sh-control or IL4Rα knockdown cells were plated at clonal density in the presence or absence of IL4 with an IL4-blocking decoy receptor (IL4Rα-FC), or an IgG control. The ability of IL4Rα-FC to neutralize IL4-induced Stat6 activation at the dose used in clonogenic assays was confirmed by Western blot analysis (Fig. 6A). As expected, the significant increase in colonization ability in response to IL4 was blocked by the addition of IL4Rα-FC in control clones, but neither IL4 nor IL4Rα-FC had a significant effect on the colonization ability of IL4Rα knockdown clones for either cell line.

Figure 5. IL4Rα expression is associated with changes in immune infiltration and cytokine production. Quantification of neutrophils (A), macrophages (B), and lymphocytes (C) from immunostained lung and liver sections of mice receiving R221a or IL4Rα knockdown (KD) or sh-control cells (n = 4–7). D, quantitation of the fold-change in protein levels from R221a and 4T1 IL4Rα knockdown and sh-control conditioned media, assessed using a commercial cytokine array and densitometry. Proteins increased in IL4Rα knockdown clones for both cell lines that have potential to regulate immune recruitment are bracketed.
We then used small-molecule inhibitors to test the contribution of IL4-activated Erk and Akt to IL4-induced colonization ability. U1026 was used to inhibit the activity of Erk1/2 (17), and LY294002, a known PI3K inhibitor, was used to inhibit Akt phosphorylation at both sites (thr308 and ser473; ref. 18). First, the effective dose of inhibitor that
decreased colony formation by 50% (ED$_{50}$) was determined for each cell line (data not shown). Before their use in clonogenic assays, selected drug doses were also confirmed to inhibit IL4-induced activation of pErk1/2 or pAkt (thr308/ser473), by Western blot analysis (Fig. 6B). Suggesting that IL4-activated Erk1/2 promotes initial colonization, U1026 treatment in both cell lines blocked IL4-induced colony formation (Fig. 7A). Treatment with LY294002 also blocked IL4-induced colony formation in the 4T1 cell line, but not the R221a line (Fig. 7B).

A potential complication with LY294002 is that it also inhibits mTOR at concentrations slightly higher than the IC$_{50}$ of PI3K (19). We therefore also tested the effect of rapamycin. In both cell lines, combined IL4 and rapamycin treatment significantly reduced colony formation compared with IL4 treatment alone (Fig. 7C). We then discovered by Western blot analysis that rapamycin treatment resulted in strong inhibition of IL4-activated mTOR ser2481, but not mTOR ser2448, indicating that activated mTORc2 rather than mTORc1 may be important in regulating IL4-induced colony formation (Fig. 6B).

To evaluate whether the signaling pathways identified in vitro were relevant in vivo, we immunostained for pAkt (ser473), pErk1/2, and pmTOR (ser2481) in R221a and 4T1 sh-control and IL4Rx knockdown lung metastases (Supplementary Fig. S7). The anti-pmTOR (ser2481) antibody selectively labeled mitotic cells with high intensity and did not provide useful information. However, we were able to confirm a significant reduction in both pErk1/2 and pAkt (ser473) in IL4Rx knockdown R221a and 4T1 lung metastases compared with sh-controls (Fig. 7D and E).

**Discussion**

The biologic effects of the IL4/IL4Rx interaction have recently been extended from immune cells to epithelial cancer cells expressing the type II IL4R (IL4Rt and IL13Ralpha1 chains). Previous studies have established the importance of this interaction in promoting protumorigenic phenotypes, including proliferation and survival ability in several types of epithelial cancers (20). However, the majority of these studies utilized in vitro assays and subcutaneous xenograft models in nude mice. Here, we have established the relevance of IL4Rx expression in human breast cancer and in two syngeneic murine models of breast cancer (Fig. 1). Using murine models, we have defined a novel role of epithelial IL4Rx in promoting metastatic mammary tumor growth.

Using R221a and 4T1 IL4Rx knockout clones and WT mice in experimental metastasis assays, we demonstrated that reduced IL4Rt expression results in significantly attenuated metastatic tumor burden in both the lung and the liver (Fig. 3). This reduced burden was also seen in the lungs of IL4^-/- mice injected with control cells, indicating that reducing either the ligand or receptor can attenuate metastatic growth (Fig. 3C and D). Notably, IL4^-/- mice injected with IL4Rx knockout cells showed a greater reduction in tumor burden than either IL4^-/- or IL4Rx knockout alone. This could be partially explained by incomplete knockdown of IL4Rx, but may also reflect a role for the other type II IL4R ligand, IL13 *in vivo*. Decreased metastatic ability of IL4Rx knockout clones was attributed to (i) reduced tumor cell survival as determined by seeding ability (number of tumors) and an increase in apoptosis by cleaved caspase-3 positivity, and (ii) a reduction in proliferation determined by tumor outgrowth ability (tumor size) and a decrease in Ki67 positivity (Fig. 4). In some cases, differences in these parameters were not significant at endpoint, which may be attributable to changes that occurred earlier in tumor development. In addition, there are large differences in the growth rates of the cell lines. The aggressive and highly metastatic 4T1 line is typically used to establish models representative of clinical stage four breast cancer, whereas R221a cells rarely metastasize spontaneously, and may represent less aggressive breast cancers. This growth disparity is most evident in the H&E-stained images of the 4T1 livers (Supplementary Fig. S1D), where large tumors were difficult to delineate from each other.

In addition to proliferation and survival, we examined whether immune infiltrates contributed to the reduction in knockdown tumor size. We saw increases in almost all leukocyte populations examined in both R221a and 4T1 knockdown metastases (R221a: Fig. 4; 4T1 Supplementary Fig. S5). Thus our results overall suggest that more efficient clearance of knockdown tumor cells could contribute to the reduction in tumor size visualized at endpoint compared with sh-control tumors.

It is feasible that enhanced production of proinflammatory cytokines from IL4Rx knockout cells could recruit immune cells to these tumors. However, out of 62 secreted proinflammatory factors examined by cytokine array, only MIP-2 was increased more than 2-fold by IL4Rx knockout cells in comparison with sh-controls for both cell lines (Fig. 5D). MIP-2 secreted from epithelial cells enhances neutrophil and lymphocyte recruitment (21). Leptin/leptin receptor signaling reportedly induces MIP-2 production in preneoplastic colon cells (22). Therefore, the increased expression of leptin (>1.5-fold) and its receptor (>2-fold) within IL4Rx knockout tumors could also facilitate neutrophil and lymphocyte recruitment. Still, strong patterns of cytokine production from IL4Rx knockdown cells were lacking, indicating that other mechanisms may contribute to IL4Rx-induced survival and proliferation ability at metastatic sites.

The Jak/Stat6 pathway is activated by the IL4/IL4Rx interaction in lymphoid cells (5), and in several epithelial cancer cell types, including breast cancer (Fig. 1; ref. 7). We were able to confirm activation of IL4R *in vitro* via phosphorylation of downstream Stat6 in response to IL4 (Fig. 6A). Stat6 has been shown to regulate protumorigenic processes, including migration and invasion (23) and enhanced survival and proliferation *in vitro* (4, 7, 21), although one study showed that Stat6 activation decreased between ductal carcinomas *in situ* and invasive breast ductal carcinomas (24). That study did not examine IL4R expression, or other IL4/IL4Rx-activated pathways, including PI3K/Akt and MAPK/Erk, that may be important for the promotion of metastatic phenotypes *in vivo*.

Using the Mek inhibitor, U0126, we determined that IL4-activated Erk1/2 promotes the colonization ability of
Figure 7. The IL4/IL4Rα interaction promotes colonization ability via Erk, Akt, and mTOR activation. R221a or 4T1-combined sh-control clones were seeded ± IL4 (R221a, 2 ng/mL; 4T1, 10 ng/mL) and ± drug. A, U1026 (R221a, 10 μmol/L; 4T1, 15 μmol/L) inhibits IL4-induced colony formation. B, LY294002 inhibits colony formation in 4T1 cells (8 μmol/L), but not R221a cells (5 μmol/L). C, rapamycin (R221a, 5 nmol/L; 4T1, 3 nmol/L) inhibits IL4-induced colony formation. D and E, quantification of pErk1/2 (D) and pAkt (ser473) (E) immunostaining in R221a and 4T1 IL4Rα knockdown (KD) and sh-control lung metastases. The percentage of positive area per mouse (n = 4–6) is shown.
mammary cancer cells in vitro (Fig. 7A), the PI3K/Akt pathway inhibitor, LY294002, blocked IL4-activated Akt in both cell lines, but only abrogated colony formation in the R221a line (Fig. 7B). The R221a cells express the middle T oncoprotein, a strong driver of constitutive PI3K/Akt and Src kinase activity, which would be unaffected by LY294002 treatment (25). These results confirm previous reports that activation of Akt in response to IL4 is cell type/context dependent (5, 12).

We next examined whether IL4-activated mTOR could promote IL4-induced colony formation for two reasons: (i) Akt was phosphorylated at ser473 in both cell lines in response to IL4 (Fig. 6A) and (ii) LY294002 is known to fit in the active catalytic domain of mTOR and inhibit its activity at slightly higher concentrations than the IC50 for PI3K inhibition (19). Rapamycin, a potent mTORC1 inhibitor, has also been shown to inhibit ser2481 phosphorylation and mTORC2 (26). Use of this compound in vitro demonstrated that IL4-activated mTORC2 (ser2481), but not mTORC1 (ser2448), is a potent promoter of colonization ability in both the R221a and 4T1 cell lines (Fig. 6B and Fig. 7C). Our findings are consistent with a previous report demonstrating that mTORC2 but not mTORC1 mediates the survival of breast cancer cells (27).

IRS proteins mediate the activation of several signaling pathways in response to IL4 (27–29). IRS1 expression in breast cancers is often decreased in high-grade invasive tumors compared with well-differentiated tumors (24, 30–32). Also, IRS1 in murine lung metastases is inactivated by serine phosphorylation, and loss of IRS1 expression in murine mammary cancer cells led to increased lung metastases (32). Serine phosphorylation of IRS1 for inactivation and/or proteosomal degradation is known to be mediated by several kinases, including mTOR, Akt, and Mapk (33), three effector proteins activated in response to IL4 (Fig. 6). In R221a and 4T1 cells, ser612 is phosphorylated in a time-dependent manner after IL4 (Fig. 6A). These results indicate that IL4 signaling may induce the inactivation and proteosomal degradation of IRS1, thus removing a potential metastasis suppressor.

In conclusion, we have demonstrated for the first time that the IL4/IL4Rα interaction promotes mammary metastatic tumor growth, and that loss of IL4Rα in mammary cancer cells results in reduced survival and proliferation and decreased colonization and outgrowth at metastatic sites. It is known that the Mapk/Akt, and PI3K/Akt/mTOR signaling axes are potent drivers of breast cancer growth (26, 27, 34, 35). We have shown that Erk, Akt, and mTOR are downstream effectors of the IL4Rα-associated prometastatic colonization phenotype. In addition, suppression of IRS1 signaling may be another mechanism by which IL4/IL4Rα promotes mammary cancer metastatic tumor growth. Overall, systemic inhibition of IL4Rα may have promise as an anticancer therapy, provided that any adverse effects on immune cells that signal through the type I IL4R could be minimized. Significantly, modified IL4 ligands termed "superkines," are capable of specifically targeting the type II IL4R, and are being tested for improving cytokine therapy selectivity (36). We raise the possibility of using such agents to target the type II IL4R on breast cancer cells as a novel means to thwart metastatic tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.T. Venmar, B. Fingleton

Development of methodology: K.T. Venmar, D.G. Hwang, E.A. Dozier

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.T. Venmar, D.G. Hwang

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Writing, review, and/or revision of the manuscript: K.T. Venmar, B. Fingleton

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.T. Venmar, K.J. Carter

Study supervision: B. Fingleton

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