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CCR7 mediates directed growth of melanomas towards lymphatics

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Abstract

Objective—To determine whether chemotactic-metastasis, the preferential growth of melanomas towards areas of high lymphatic density, is CCL21/CCR7 dependent in vivo. Lymphatic endothelial cells (LECs) produce the chemokine CCL21. Metastatic melanoma cells express CCR7, its receptor, and exhibit chemotactic-metastasis, whereby metastatic cells recognise and grow towards areas of higher lymphatic density.

Methods—We used two in vivo models of directional growth towards depots of LECs of melanoma cells over-expressing CCR7. Injected LEC were tracked by intravital fluorescence microscopy, and melanoma growth by bioluminescence.

Results—Over-expression of the chemokine receptor CCR7 enables non-metastatic tumour cells to recognise and grow towards LECs (3.9 fold compared with control), but not blood endothelial cells (0.9 fold) in vitro and in vivo, in the absence of increased lymphatic clearance. Chemotactic metastasis was inhibited by a CCL21 neutralising antibody (4-17% of control). Furthermore, CCR7 expression in mouse B16 melanomas resulted in in-transit metastasis (50-100% of mice) that was less often seen with control tumours (0-50%) in vivo.

Conclusion—These results suggest that recognition of LEC by tumours expressing receptors for lymphatic specific ligands contributes towards the identification and invasion of lymphatics by melanoma cells, and provides further evidence for a chemotactic metastasis model of tumour spread.

Keywords

lymphatic endothelial cells; melanoma; metastasis; CCR7; lymph flow

Introduction

Lymph node metastasis acts as an indicator for distant metastases in 75-80% of melanoma patients(7, 29). Metastasising melanoma cells are able to invade into and grow along lymphatic routes, and this can be seen clinically, where multiple lesions occur along the route of a lymphatic, termed in-transit metastasis. Patients presenting with in-transit metastasis have a poor prognosis(31). Although the mechanisms that underlie how tumour

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cells find and invade lymphatics are as yet uncertain, it is clear that this lymphatic route of entry is required for subsequent lymph node metastasis. The potential for lymph node metastasis in melanoma has been clearly linked to the presence of an increased lymphatic vessel density around the tumour, with those tumours that do not go on to metastasise presenting with a significantly lower lymphatic vessel density(5, 22). Several animal models have also been used to demonstrate that tumours over-expressing the lymphatic specific vascular growth factors, Vascular Endothelial Growth Factor C or D (VEGF-C or VEGF-D), demonstrate an increase in metastatic potential. They also stimulate a local lymphangiogenic response i.e. the growth of the nearby pre-existing lymphatics towards the newly formed tumour, thus increasing the local lymphatic vessel density(14, 26). Together, these results suggest that local lymphangiogenesis may be a mechanism underlying metastasis. However, a second mechanism has been proposed, in conjunction with this lymphangiogenic response, termed directed chemotactic metastasis(23). An increased lymphatic density surrounding a melanoma can also be explained if metastatic melanomas can grow preferentially towards areas of densely packed lymphatic vessels, either through chemokine mediated migration(23), physical flow routes(3), or a combination of both(24). There is an increasing body of evidence to support the concept that the metastasising tumour cells may have more than a passive role to play during dissemination and may be able to migrate towards the lymphatics in response to specific factors secreted by the endothelial cells(9). We have previously demonstrated that lymph node-metastatic but not non-metastatic melanoma will both migrate *in vitro* and grow *in vivo* specifically in response to lymphatic, but not blood, endothelial secreted factors and that this in vitro migration is mediated by the chemokine CCL21(23). However, we have not shown that in vivo that it is CCL21 or CCR7 dependent, nor whether it enables tumour cells to form in transit or tracking metastases. The role of chemokines in tumour migration is now receiving significant attention(1, 15, 21). The coordinated movement of immune cells around the body is under the control of chemokine signalling pathways(10). These chemokines form a superfamily of small chemotactic polypeptides that can be subdivided into groups depending upon the arrangement of two amino-terminal cysteine residues within the protein, giving rise to the CXC and CC families. These ligands bind to and activate G-protein coupled receptors to mediate a variety of effects including guiding cells along a concentration gradient of the chemokine and lymphatic transmigration(11). The receptor CCR7, and its ligand CCL21 have been implicated in lymphatic spread of tumours(17, 28), with CCR7 being expressed by melanoma cells(28), and CCL21, its ligand, by lymphatic endothelial cells(20), leading to the proposal that this axis may mediate a migratory response of tumours towards areas of high lymphatic density and increase metastatic potential to lymph nodes(22). Indeed, CCR7 expression has now been demonstrated in a variety of melanoma cell lines and in human samples of both primary and metastatic lesions(17, 19, 28). Over-expression of this CCR7 receptor in the mouse melanoma cell line, B16, has also been demonstrated to result in a 700-fold increase in metastatic lymph nodes in a mouse model of melanoma(30), and increase lymph node metastasis. Moreover, CCL21 has been implicated by the use of inhibitors of chemokines of that family (e.g. CCl21, CCL19, and possible other chemokines) as anti-metastatic agents in mouse tumour models(12), but it has not yet been shown that the chemotactic response of tumours to lymphatics is dependent specifically on CCL21 or CCR7 *in vivo,* nor whether this aids local in transit metastasis

CCR7 therefore appears to have a crucial role in melanoma metastasis through a lymphatic route, possibly by inducing migration of metastatic melanoma cells towards the local lymphatic vessels. This study aimed to investigate whether CCR7 chemokine receptor expressed by melanoma cells is able to mediate migratory potential in metastatic melanoma and thereby influence overall metastasis through this directed chemotactic metastasis.

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Methods

Cell Lines

Human amelanotic A375 melanoma cells (CRL-1619, ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) with 4mM L-glutamine, 1.5g/L sodium bicarbonate and 4.5g/L glucose, supplemented with 10% foetal bovine serum (FBS). Two subclones of the A375 cell line, the metastatic A375SM and non-metastatic A375P cells (A kind gift of IJ Fidler, MD Anderson Centre, Texas), and mouse B16-F10 cells transfected to over-express luciferase (Caliper Life Sciences) were all cultured in modified Eagle's medium (MEM) supplemented with 10% FBS, 0.5mM sodium pyruvate, non-essential amino acid solution, 1mM L-glutamine and 1x MEM vitamin solution. Pure populations of dermal neonatal human Lymphatic microvascular Endothelial Cells, LECs (cc-2812, HLMVECs, Cambrex, Wokingham, UK), and human Blood microvascular Endothelial Cells, BECs (cc-2813, HBMVEC, Cambrex, Wokingham, UK), were both grown in Endothelial Cell Basal Medium-2 (EMB-2) supplemented with 0.5ml hEGF, 0.2ml hydrocortisone, 0.5ml GA-1000 (Gentamicin, Amphotericin-B), 5% FBS, 0.5ml VEGF, 2.0ml hFGF-B, 0.5ml R3-IGF-1 and 0.5ml ascorbic acid (cc-3202, Cambrex, Wokingham, UK) per litre. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Transfection

Non-metastatic A375P cells and mouse B16-F10-Luc cells (Caliper life sciences) were transfected to over-express the human CCR7 receptor (UMR cDNA resource centre, Rolla, Missouri, USA) using Lipofectamine 2000 (11668-027, Invitrogen). Briefly, three hours prior to transfection, cells were fed with serum rich medium, to ensure all cells are in an active growth phase. 1μg of CCR7 cDNA, empty vector pcDNA3.1, or GFP control were added to 50μl Opti-MEM Reduced Serum Medium (31985-062, Invitrogen) and incubated at room temperature for 5 minutes. Concurrently, 4μl of Lipofectamine 2000 was also added to a separate aliquot of 50μl of Opti-MEM and then these were mixed together and incubated for a further 20 minutes. The A375P cells were then washed twice with serum free medium and then incubated in fresh serum free medium for 20 minutes prior to transfection. This was then replaced with the Opti-MEM and cDNA mixture and incubated for 1 minute before a further addition of 2ml of serum free medium. After 6 hours an additional 2ml of serum rich medium was added to the cells and incubated overnight before completely replacing the mixture with fresh serum rich medium. All the cDNAs carried a neomycin resistance gene, so cells expressing the newly transfected cDNA, A375P-CCR7 were selected and maintained by incubating with 500μg/ml Geneticin (10131-019, Gibco, Paisley, UK). CCR7 expression was assessed by RT-PCR using 2μg of total RNA extracted with Trizol reagent and primers for CCR7 and GAPDH as previously stated (9). Western blotting was performed on proteins extracted from cell lysate, run on a 10% acrylamide gel and transferred onto a PVDF membrane. The membrane was blotted using 1:5000 Rabbit [Y59] monoclonal anti-human CCR7 antibody (ab32527, Abcam) and 1:7000 Stabilised goat anti-rabbit IgG HRP conjugated (32460, Pierce). Stripping and re-probing was performed with 1:500 Actin (c-11) goat polyclonal IgG (sc-1615, Santa Cruz) and 1:10000 Donkey anti-goat IgG HRP (sc-2304, Santa Cruz). Primary antibodies were diluted in 5% BSA and secondary antibodies in 2.5% milk.

In Vitro Migration Assay

In vitro migration was assessed using a modified Boyden chamber in which cells were seeded into a cell culture insert with an 8μm pore polycarbonate membrane (Millipore) coated overnight with 5% collagen A at 4°C. That was then seated within an individual well of a 24-well plate, containing the chemoattractant. Cells were seeded into the top well at a density of 1×10^5 cells per well in serum free medium supplemented with 0.001% FBS,

whilst the lower well contained conditioned media. Conditioned media was collected by 24-hour incubation of 10ml of the appropriate serum free medium in an 80-90% confluent T75 flask of either LECs or BECs. This plate was then incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2 and 95% air to allow migration. These inserts were then fixed in 100% methanol for 10 minutes at 4°C, then for a further 2 minutes in 70% methanol and a final 2 minutes in 50% methanol. Any non-migrated cells were then removed from the inside surface of the membrane using a clean cotton swab and the remaining cells were then stained using haematoxylin. These membranes were then cut out using a scalpel blade before being mounted on glass slides with glass coverslips using Vectashield Liquid Mounting Media (Vector Laboratories, Peterborough, UK) and sealed with nail varnish. Migrated cells were then counted in 10 fields of view per insert using a Nikon E400 microscope and a 40x objective.

In Vivo Model

Directed chemotactic metastasis, was assessed *in vivo* as previously described (Shields *et al.*, 2008). Briefly, BalbC nu/nu nude mice (~25g, University of Bristol) in groups of 6 (unless stated) were injected with 1×10^6 A375 (metastatic), A375P (non-metastatic), or A375P-CCR7 cells suspended in 100μl PBS subcutaneously in the dorsal intrascapular region with a 25 gauge needle. The needle had been previously scored and dipped in Monastral Blue dye. The mice were then injected approximately 10mm caudal to this with 1×10^5 human lymphatic endothelial cells (LECs) or blood vascular endothelial cells (BECs) suspended in 100μl of PBS through a 25 gauge needle scored and dyed as above. When treated, mice were injected subcutaneously between the cell injection sites with an insulin syringe, thrice a week, with 5μg of goat anti human CCL21 neutralising antibody (nAb) (AF366, R&D) or goat IgG (AB-108-C, R&D). Once the tumours reached 8-10mm in diameter as measured through the skin, the mice were sacrificed by cervical dislocation, tumours excised, the skin laid out and photographed. Macroscopic measurements were then made from prosections of the excised tumour relative to the original injection sites by drawing a line on the image from the two tattoo points, then taking a perpendicular axis at the melanoma injection point. These measurements were done blind where possible and the percentage of tumour that grew towards or away from the endothelial cell injection site was calculated using NIH Image-J software. A value of 100% indicates that the tumour grew completely towards the endothelial cell injection site, whilst 50% indicates that half the tumour was on the LC side of the injection and 0% represents a tumour that grew away from the endothelial cell injection site (i.e. on the site that it was injected, without directional growth back towards the LEC).

Bioluminescence Imaging

One million B16-Luc melanoma cells were injected into the flank of CD1 mice. To image bioluminescence, mice were injected with 0.15mg/g luciferin intraperitoneally at 20mg/ml. Five minutes later they were anesthetised by halothane (5% in 95% O_2) and imaged using an IVIS Lumina (Caliper Life Sciences). Lesion maximum width and length were measured and the ratio used to calculate directional growth. Once tumours reached 16mm in size, the mice were injected with luciferin as above, imaged, then killed humanely and the flank dissected to investigate for in-transit metastases. Areas of black tracking along lymphatics were photographed using a Nikon Coolpix digital camera. Mice were again imaged for bioluminescence after dissection to visualise lymph nodes.

Fluorescence Lymphography

Mice were injected with 10^5 LEC (n=6), saline (n=8) or 2.5×10^8 pfu of Ad-VEGF-C prepared as previously described(2) (n=6) or saline (n=6). Cell injection sites were apparent by tattooing as described above. Three weeks later the mice were anaesthetised with

isofluorane, and injected with 0.16% FITC-dextran(150kDa) intradermally. Two mice were culled immediately and imaging carried out every 60 minutes for 6 hours using the IVIS Lumina. The mouse background fluorescence was measured and the fluorescence intensity of the region of interests (ROI) calculated. A monoexponential curve fit was applied to each set of measurements from each mouse, and rate constant calculated using Prism3.0. The half life is 0.69/k.

Immunohistochemistry

Paraffin embedded sections were first dewaxed in xylene for 15 minutes prior to re-hydration through a series of alcohols of 100%, 90% and 70% for 2 minutes each. Microwave antigen retrieval was carried out in 0.01M sodium citrate buffer, pH 6.0 for 8 minutes at 800W. Endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide for 5 minutes followed by two washes in phosphate buffered saline (PBS (mM): NaCl, 137; KCl, 2.68; Na2HPO4, 10; KH2PO4, 1.76). Non-specific binding was then blocked by incubation in 1.5% normal horse serum (1.5% v/v normal serum diluted in 1× PBS) for 30 minutes. Sections were then incubated overnight at 4°C in a humid chamber, with either a polyclonal goat anti-LYVE-1 antibody (AF2089, R&D Systems, UK) primary antibody; or, the same concentration of normal goat IgG (1-5000, Vector Laboratories, Peterborough, UK) as a negative control, diluted in non-immune block (Zymed Laboratories, San Francisco, USA). Slides were washed twice in PBS/Tween (PBS/Tween, 0.05% v/v) before repeating the non-immune block. Primary antibody was then detected by incubating with a biotinylated horse anti-goat IgG secondary antibody; 2μg/ml (BA-9500, Vector Laboratories, Peterborough, UK), diluted in blocking solution, in a humid chamber for 30 minutes. Any unbound antibody was removed by washing twice with 1× PBS/T for 5 minutes before incubating for a further 30 minutes with an avidin-biotin enzyme complex (Elite ABC Kit, Vector Laboratories, Peterborough, UK) at room temperature. Sections were visualised with diaminobenzidine (DAB, Vector Laboratories, Peterborough, UK) and washed in distilled water before counterstaining in haematoxylin and mounting with DPX.

Statistical analysis

Results represented mean ± standard error of the mean (SEM). Statistical significance was assessed by one-way ANOVA or t test or chi squared test, whilst correlation was assessed by Pearson's test for correlation.

Results

Metastatic tumour cells migrate towards endothelial secreted chemokines

We have previously shown that A375 metastatic melanoma cells but not non-metastatic melanoma cells (A375P) increase their migration *in vitro* when exposed to conditioned media from lymphatic endothelial cells, and that this migration is blocked by antibodies to CCL21. To determine whether this was mediated by chemokine receptors, and whether this was specific for lymphatic mediated metastatic melanoma cells we investigated whether migration towards different endothelial cell types could be induced. A375P cells, generated by selection of non-metastasising clones do not migrate towards lymphatic endothelial cells either *in vitro* or *in vivo*(23). To investigate the role of CCR7 in these cell lines, we transfected these cells with CCR7 or an empty vector (pcDNA3.1), and compared expression by RT-PCR (figure 1A) and Western blot (figure 1B). A375P-CCR7 cells demonstrated a significant migration towards LEC, but not BEC conditioned media (figure 1B), indicating CCR7 over-expression is sufficient to rescue the CCL21 mediated chemotaxis in vitro.

Tumours growth towards lymphatic endothelial cells is CCR7 dependent

To determine whether directed migration of tumour cells to LECs was maintained *in vivo*, 1×10^6 A375P-CCR7 cells were injected sub-dermally on the midline anterior dorsal surface of the skin of nude mice. 10mm caudal to this, the mice were then injected with either a pure population of 1×10^5 lymphatic endothelial cells, blood endothelial cells or saline alone. Both injection sites were marked by a Monastral Blue tattoo. All the tumours successfully took in the nude mice and there was no significant difference in the growth rates of the tumours, irrespective of whether injected with LEC, BEC or PBS alone. A375P tumours grew at their injection site, slightly on the other side of the tattoo from the LEC (as they were injected in an anterograde direction, away from the LEC site, figure 2A) or the BEC (figure 2B). In contrast A375P-CCR7 tumours demonstrated a significant increase in migration towards the LEC (figure 2C) but not the BEC injection site (figure 2D). Quantification of the area on the endothelial side of the tattoo showed that CCR7 significantly increased migration towards LEC (from 16.4±15.2% to 64.4±10.2% of the tumour on the LEC side, $p<0.01$, SNK) but not BEC (from 43.8±3.6 to 39.3±3.2% of the tumour on the BEC side $p>0.5$). To determine whether growth of metastatic melanomas towards LEC was CCL21 dependent, A375 metastatic melanoma cells were injected as above with LEC or BEC. BEC do not express CCL21 and the BEC cells did not attract tumour growth (not shown), but tumours grew towards the LEC cells, as previously described (figure 3A) Once tumours had formed in the LEC bearing mice, 5μg of a neutralising antibody was injected six times over the first two weeks after tumours became palpable between the A375 and LEC injection sites. Figure 3B shows that migration toward LEC could be blocked by injection of a CCL21 neutralising antibody (nAb). Equally growth of tumours generated from A375P cells expressing CCR7 towards LEC was inhibited by injection with the CCL21 nAb (figure 3C). Quantification of the amount of tumour on the LEC side of the injection site (figure 3D-F) showed that migration of the tumours towards the LECs was blocked by the CCL21 nAb (A375, 8.7±4.3%, A375P-CCR7, 1.3±0.5% figure 3G). Injection of antibody itself did not block migration as the migration observed in the presence of CCL21 nAb was significantly greater than when control goat IgG was injected ($p<0.05$, t test) for both A375 (50±9%), and A375P-CCR7 (31.8±8.3%). Moreover, injection of the CCL21 neutralising antibody did not prevent tumour growth as mice injected with A375 followed by thrice weekly injection of CCl21 nAb adjacent to the tumour still grew at the site they were injected into.

LEC injection results in increased lymphatic density

To determine whether the LEC injection was affecting lymphatic density in the mouse skin, LEC were incubated with an intravital green tracker dye. This dye is taken up intracellularly and last for up to 7 days in culture. These LEC were then injected subdermally into mice with a tattoo, and four days later the mice killed and tissue taken. Figure 4A shows a collection of human LECs, visualised by epifluorescence microscopy in a frozen section of mouse skin. Figure 4B shows a collection of lymphatics some 300μm from the original cell depot, which appear to be forming a cross section of a vessel. To determine whether the LEC injection resulted in increased lymphatic density, the skin of mice injected as above was taken after 21 days, and subjected to immunohistochemistry for LYVE-1 a lymphatic endothelial cell marker. Figure 4B shows that LYVE-1 staining detected many more lymphatics around the injection site ($7.6\pm1.2 cm^{-2}$) than at sites more than 1cm distant from the injection site (normal skin, $2.1\pm0.3 cm^{-2}$). Figure 4C shows that the lymphatic density in the region was almost four times higher than normal skin ($p<0.01$ paired t test). Migration of melanomas towards the site of high lymphatic density could occur either due to autologous chemotaxis down a flow pathway(24), or chemotactic metastasis down a chemokine gradient(23). The former requires the lymphatic endothelial cells injected into the skin to increase fluid drainage from the area, whereas the latter does not. To determine whether these lymphatics were able to increase lymph drainage, lymphography was carried out.

Figure 5A shows the disappearance of the fluorescently labelled tracer FITC dextran from the skin. This can be used to calculate the lymph flow(13). Figure 5B shows that the clearance rate between the LEC injected and the saline injected skin was not different indicating that the LECs injected do not increase functional lymph flow. To check that a lymphangiogenic stimuli did result in an increase in this assay we injected mice with AdVEGF-C, and this resulted in a more rapid clearance from the lymphatics (figure 5C). Calculation of the half life revealed that there was no significant difference in the half life ($p>0.1$) between LEC (114±35min) and saline injected cells (94±10 min, figure 5D), but a significant reduction in half life (increase in clearance) in the AdVEGF-C injected mice (51±6min) compared with their controls (80±7 min, $p<0.05$, t test).

CCR7 mediates in-transit metastatic melanoma

To determine whether CCR7 expression could induce the entry of melanoma cells into lymphatics a mouse model of melanoma was used. B16-F10-Luc cells were transfected either with control vector or CCR7 and injected subcutaneously into CD1 mice or nude mice. The tumours were then imaged for two weeks by intraperitoneal injection of luciferase, and when tumours reached 16mm the mice were killed and the tumours exposed. The B16 melanoma cell line is a mouse melanotic tumour, and metastatic deposits can be seen as black growths. Control tumours in both CD1 and nude mice grew as spheroid deposits (figure 6A) that did not metastasise (no evidence of lymph node metastasis either during imaging or post-mortem (figure 6B)). Interestingly, the CCR7 expressing tumours grew in an elongated shape in both immune competent (CD1)(figure 6C) and immune compromised (nude mice) and although they showed no evidence of lymph node metastases, in-transit metastases could be seen as deposits along the lymphatics draining the abdominal wall (figure 6D). In both strains of mice with CCR7 expressing tumours there was significantly higher proportion of mice carrying in-transit metastases (CD1, 100% compared with 50%, and nude mice 50% compared with 0%, $p<0.05$ χ^2 test, figure 6E).

Discussion

We show here that over-expression of a chemokine receptor can confer metastatic potential on melanoma cells by stimulating migration towards lymphatic endothelial cells. Transfection with CCR7, the receptor for the lymphatic-chemokine CCL21 confers the ability to recognise and grow towards areas of high lymphatic endothelial cell density. To our surprise, CCR7 expressing cells did not form lymph node metastases, but did form in-transit metastases. As part of normal physiological processes, CCL21 is produced primarily by Lymphatic Endothelial Cells (LEC) and regulates recruitment of CCR7 positive dendritic cells and lymphocytes back to lymph nodes along a chemical gradient(28). CCR7 positive cells have been shown to be strongly associated with lymphatic metastasis in breast cancer(4), squamous cell carcinoma(6), gastric cancer(32), small cell cancer of the lung(27)and melanoma(16, 23, 28) but the mechanism of its contribution to metastatic potential in humans is still unknown. Two non-mutually exclusive mechanisms have been proposed, chemotactic metastasis(23), as described here, and autologous chemotaxis, whereby cells expressing their own growth factors can move down a flow gradient that creates a concentration difference across the cell(24). The finding that LEC injection did not increase lymph flow points to a contribution of chemotactic metastasis in these experiments, but does not rule out a contribution from autologous chemotaxis.

In murine melanoma cells (B16) Wiley et al reported a significant increase in lymphatic metastasis by up-regulation of CCR7(30) in C57Bl/6 mice when injected into the foot pad, and we have recently confirmed this(12). In the model described here, lymph node metastases are not seen. This may be because the injection into the foot pad occurs in a low compliance area, and so interstitial fluid pressure is rapidly increased after injection of cells,

and hence lymphatic uptake is enhanced. In contrast the subcutaneous tissue on the flank is highly compliant, and so the cells are unlikely to be driven into the lymphatics so easily by pressure gradients. Interestingly, it has recently been shown that CCL21 expression by melanoma cells can result in a tumour mediated escape from immune surveillance, and tumour cells grow faster in the lymph node when there are CCL21 expressing tumours elsewhere in the mouse(25). Wiley et al did not demonstrate whether their B16-CCR7 tumours expressed CCL21, but we did not find upregulation of CCL21 expression in the A375-CCR7 tumours, or the B16-Luc-CCR7 tumours. The findings here that CCR7 over-expression leads to lymphatic specific endothelial recognition by tumour cells, suggests that CCR7 was responsible for entry into the lymphatic system at the level of the lymphatic capillary. The migration of melanoma and other cancer cells towards lymphatic secreted chemokines has been shown in culture, and this requires expression of the receptors, particularly CCR7 for CCL21. Moreover, generic chemokine block, inhibiting CCL21 and CCL19 (and possible other chemokines) can inhibit lymphatic metastasis(12). The regulation of CCR7 in cancer cells is poorly understood although recently prostaglandins have been implicated in regulating CCR7 expression in breast cancer cells through the EP2/4 receptors(18). However, cross talk between lymphatics and cancer cells can work both ways. Tumour cells have been shown to upregulate CCL21 expression by lymphatic endothelial cells by secreting VEGF-C, through VEGFR3 activation(9). This provides a positive feedback route for cancer cells to find their way towards depots of concentrated LEC, as seen in areas of high lymphatic density(22), or “hotspots”(5) which can predict metastatic outcome in melanoma(8).

The identification of in-transit metastases in mouse models of cancer in the CCR7 over-expressing tumours, indicate that it is the entry into the lymphatic system, rather than, or as well as, the lymph node that is regulated by CCR7 expression on the cancer cells. It remains to be seen whether CCR7 is involved in lymph node recruitment. The experiments carried out here involved killing the animals before removing the tumour, but it would be interesting to know whether CCR7 expressing tumours resulted in more lymph node involvement than tumours that had equal infiltration into the lymphatic vessels but did not express CCR7.

In summary, we have shown for the first time, that the in vivo chemotactic metastasis of tumours towards lymphatic endothelial cells is CCL21 dependent, and that entry into the lymphatic system is enhanced by tumours expressing CCR7, indicating that upregulation of CCR7 by tumour cells is a mechanism of tumour metastasis.

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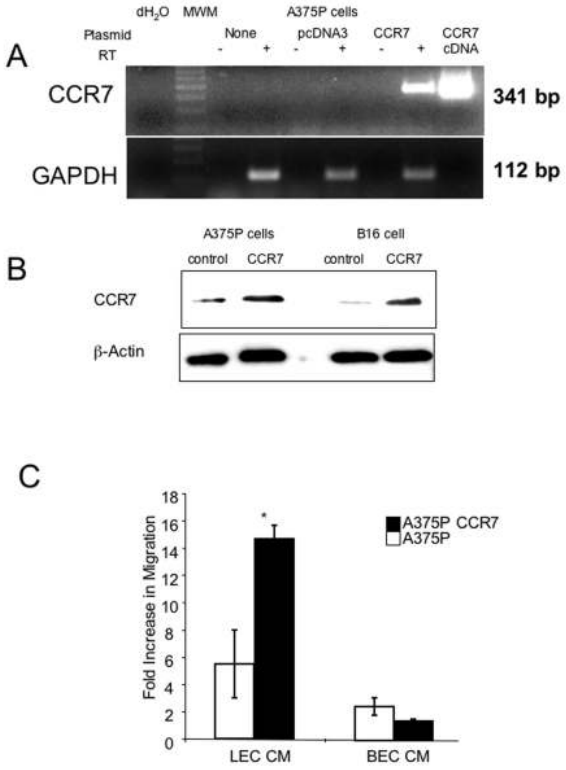

Figure 1.

CCR7 over-expression in A375P non-metastatic cells.

A) RT-PCR demonstrates CCR7 expression by A375P CCR7 cells *in vitro* and not A375P cells. B) Western blot demonstrates increased expression of CCR7 in A375P and B16 melanoma cells transfected with CCR7 expression plasmid. C *In vitro* A375P CCR7 cells actively migrate towards Lymphatic Endothelial Cell (LEC) and not Blood Endothelial Cell (BEC) conditioned media (CM) ($p<0.05$) whereas non-metastatic A375P cells show no significant increase in migration to either stimulus compared to control.

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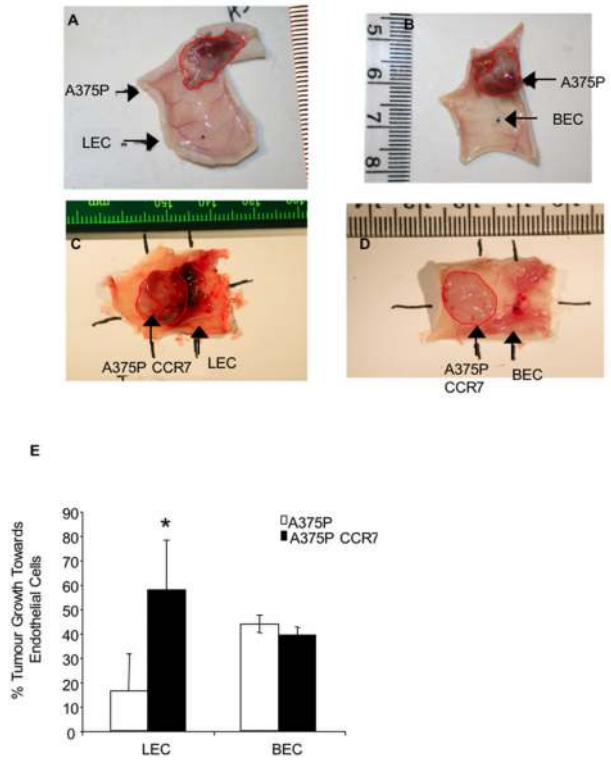

Figure 2.

CCR7 overexpression by non-metastatic A375P cells increases lymphatic metastatic potential in vivo.

A375P untransfected non-metastatic melanoma cells or A375P CCR7 transfected melanoma cells were injected subcutaneously into nude mice 8-10mm from a co-injection of LEC or BEC cells. A375P untransfected cells formed tumours that did not grow towards co-injected A) LEC or B) BEC, whereas A375P CCR7 transfected cell tumours specifically grow towards C) LEC but not D) BEC. Asterisk marks endothelial cell injection site. E) This increase in directed growth by A375P CCR7 over-expressing cells towards LEC not BEC was significant compared to untransfected non-metastatic A375P cells ($p<0.05$, ANOVA). 100% would be all of the tumour on the side towards the EC injection. *=$p<0.05$ compared with A375P

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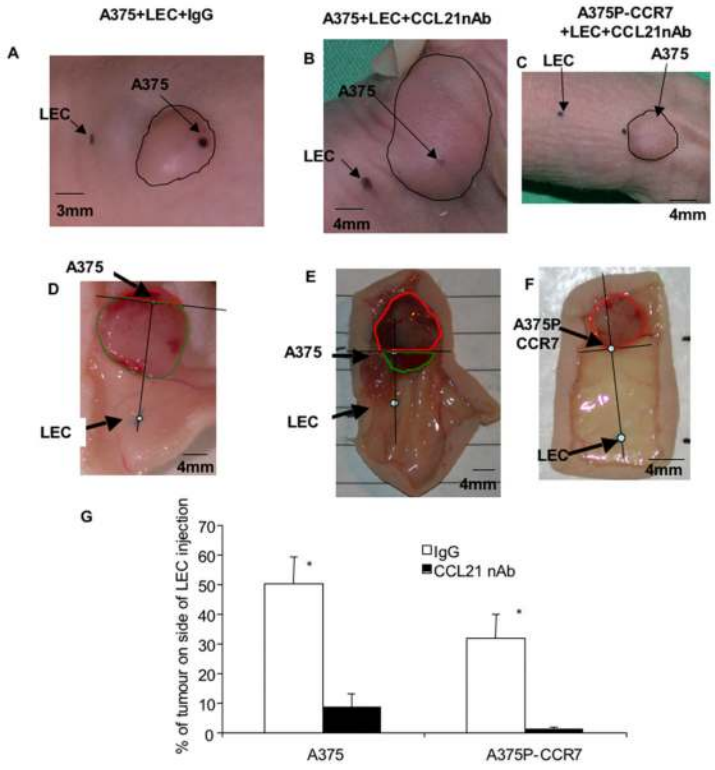

Figure 3.

Tumour cell growth towards lymphatics is CCL21 dependent.

Metastatic melanoma cells were injected subcutaneously into nude mice 8-10mm from a LEC depot. They were subsequently treated with 5μg of goat IgG or a CCL21 nAb between the two sites on six occasions over the two weeks following tumour development. A). A375 metastatic tumours grow towards LEC co-injected endothelial cells B) A375 tumours grow away from LEC when injected with CCL21 nAb. C). A375P non-metastatic tumours over-expressing CCR7 show no migration towards LEC when injected with an antibody to CCL21. D-F) Pronated pictures of A -C showing measurement on the side of the LEC injection depot. G. Quantitation of area shows that tumour growth towards LEC was significantly inhibited by CCL21 nAb. (*=p<0.05, t test compared with CCL21 nAb)

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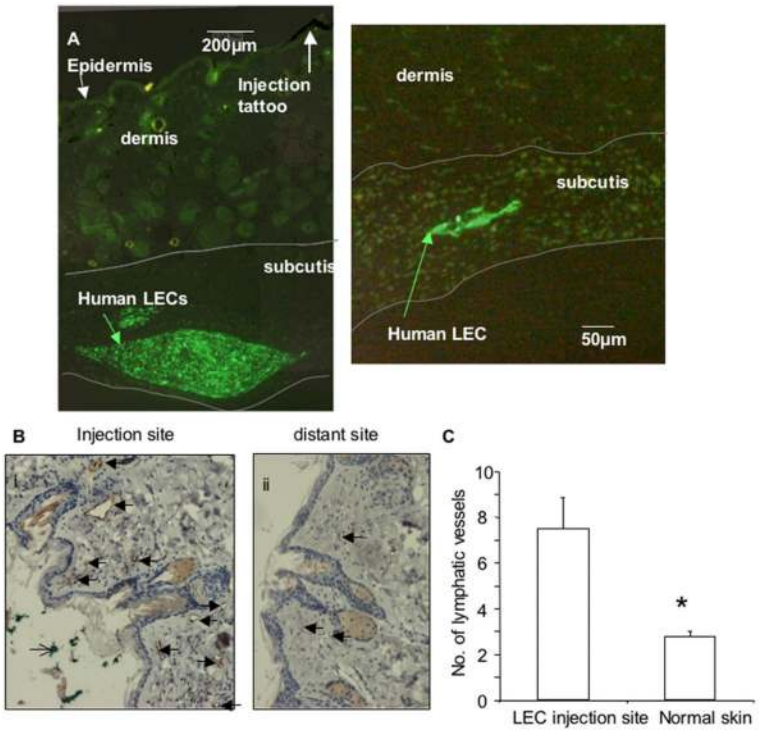

Figure 4.

The fate of injected human LECs in nude mouse skin.

A) LECs marked with green fluorescent cell tracker dye were injected as in the *in vivo* migration assay. 4 days post-injection, the cells mostly exist as a depot 1-2mm below the epidermis, in the subcutis. A collection of human LECs appear to be forming a separate structure with a lumen (right hand panel). B) 21 days after human LEC injection (without tracker dye) the skin surrounding the injection site has increased lymphatic vessel density. LYVE-1 staining (brown) specifically marks lymphatic vessels (solid arrows) in skin. (**i**) More lymphatic vessels, which appear to be larger and more dilated were seen at site of hLEC injection (Monastral blue tattoo visible in section, see skeleton arrow) compared to (**ii**) normal skin away from the endothelial cell injection site. C) Lymphatic vessel density was increased at the human LEC injection site compared to normal skin away from the endothelial injection site (*=t test $p<0.05$,n=4).

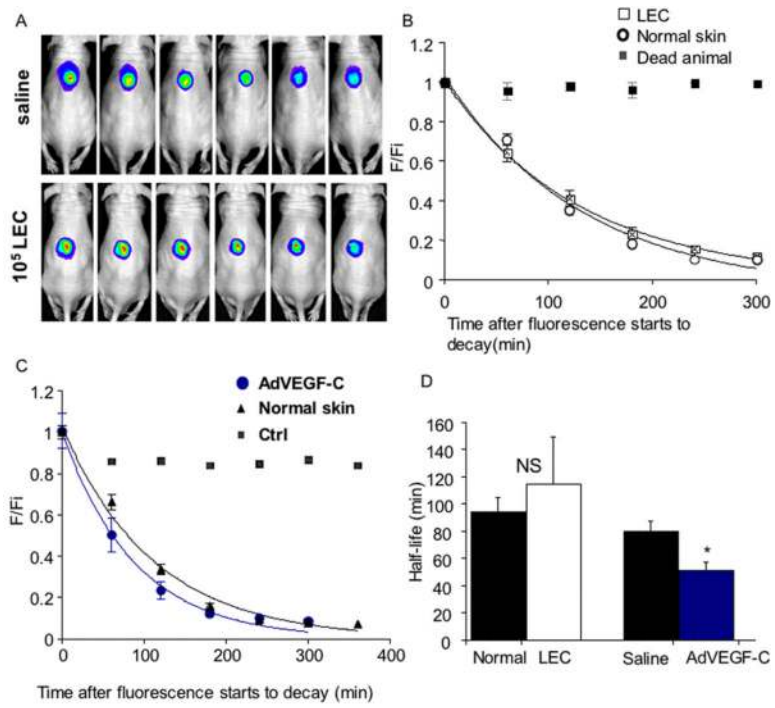

Figure 5.

Injection of LECs does not increase lymph flow.

A. LEC or saline was injected into nude mice and 3 weeks later lymphography was carried out to determine lymph flow from the injection site by measuring FITC-dextran clearance. Images of mice injected with FITC dextran. B) Clearance of FITC-dextran from LEC injected, PBS injected (normal skin) or from dead mice. C) Mice were injected with AdVEGF-C and 3 weeks later clearance measured as in B. D) Calculation of half life from the clearance curves. NS=not significant.*=$p<0.05$ compared with uninjected

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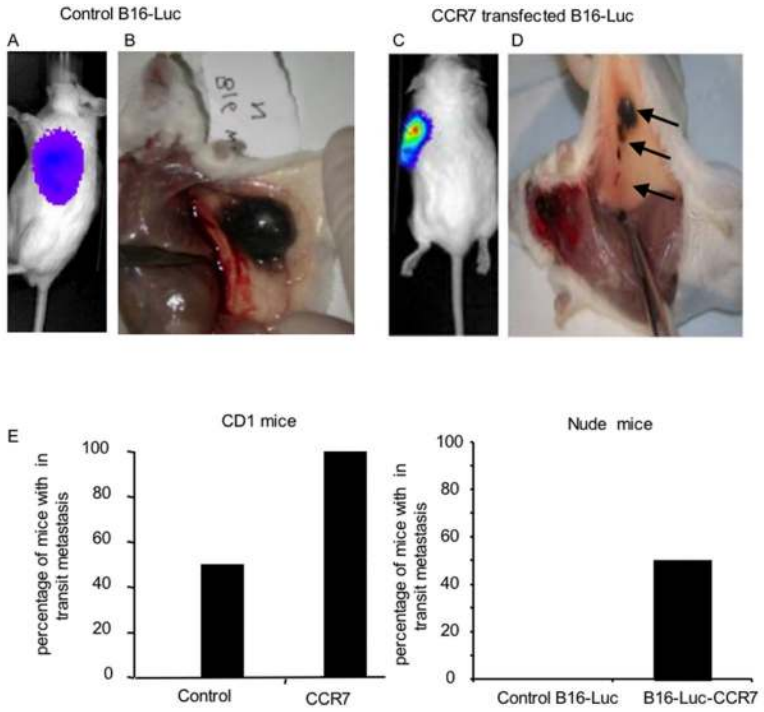

Figure 6.

CCR7 mediates tracking of melanomas to lymph vessels. B16-Luc melanoma cells were injected into CD1 mice and monitored for bioluminescence.

A) Bioluminescence image of tumour shows that untransfected cells grew with a circular profile. B) When tumours were examined postmortem solid tumour masses were seen. C) CCR7 transfected B16-F10-Luc grew asymmetrically. D) When exposed the B16-F10-Luc-CCR7 tumours were exposed postmortem, in-transit metastatic deposits could be seen along the lymphatic trunks. E) In-transit metastases were detected in more mice with CCR7 expressing melanomas than control ($p<0.05$ chi squared test). N=6 per group.

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