Chemotrap-1: an engineered soluble receptor that blocks chemokine-induced migration of metastatic cancer cells in vivo

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Abstract.

Cancer and dendritic cells recognise and migrate towards chemokines secreted from lymphatics, and use this mechanism to invade the lymphatic system, and cancer cells, metastasise through it. The lymphatic secreted chemokine ligand CCL21 has been identified as a key regulatory molecule in the switch to a metastatic phenotype in melanoma and breast cancer cells. However, it is not known whether CCL21 inhibition is a potential therapeutic strategy for inhibition of metastasis. Here, we describe an engineered CCL21 soluble inhibitor, Chemotrap-1, which inhibits migration of metastatic melanoma cells \textit{in vivo}. Two-hybrid, pull down and co-immunoprecipitation assays allowed us to identify a naturally occurring human zinc finger protein with CCL21 chemokine-binding properties. Further analyses revealed a short peptide (\textasciitilde 70 amino-acids), with a predicted coiled-coil structure, which is sufficient for association with CCL21. This CCL21 chemokine-binding peptide was then fused to the Fc region of human IgG1 to generate Chemotrap-1, a human chemokine-binding Fc-fusion protein. Surface plasmon resonance (SPR) and chemotaxis assays showed that Chemotrap-1 binds CCL21 and inhibits CCL21-induced migration of melanoma cells \textit{in vitro} with sub nM affinity. In addition, Chemotrap-1 blocked migration of melanoma cells towards lymphatic endothelial cells \textit{in vitro} and \textit{in vivo}. Finally, Chemotrap-1 strongly reduced lymphatic invasion, tracking and metastasis of CCR7 expressing melanoma cells \textit{in vivo}. Together, these results show that CCL21 chemokine inhibition by Chemotrap-1 is a potential therapeutic strategy for metastasis, and provide further support for the hypothesis that lymphatic mediated metastasis is a chemokine-dependent process.
Introduction

Tumour metastasis occurs either through the lymphatic or the vascular systems. Lymphatic metastasis is the most common route for melanoma, breast, prostate, and other cancers. When they metastasise specifically through the lymphatic system, tumour cells locate and recognise lymphatic endothelial cells (LEC). The secreted lymphatic chemokine CCL21 is released from LEC(1), and is used by dendritic cells (DC) to guide their invasion into lymphatics by acting on its receptor CCR7, expressed on DCs(2). CCR7 expression is increased in patients with metastatic melanoma(3), prostate(4), head and neck(5) and squamous cell carcinoma(6) and breast cancer(7) compared with non-metastatic cancers. Thyroid, lung, oral squamous cell carcinoma, melanoma and breast cancer cells that upregulate receptors for lymphatic secreted chemokines such as CCL21 are more likely to metastasise(3, 8-10). It has therefore been hypothesised that CCR7 is used by metastatic melanoma cells to detect locally secreted lymphatic specific chemokines such as CCL21, and migrate towards it to find areas of increased lymphatic density(11). One potential therapeutic strategy to prevent metastasis would be to inhibit this chemokine mediated entry of cancer cells into lymphatics. Therapeutic strategies based on soluble human receptors prepared as Fc-fusion proteins, termed Traps, have been successfully developed for several cytokines, including TNFα (Etanercept)(12, 13), IL-1 (IL-1-Trap)(14) and VEGF (Aflibercept)(15). These are generated using the fixed chain (Fc) domain of the human IgG fused to a protein binding domain to provide a stable, dimeric protein with long plasma half-life. The protein binding domain has been either the ligand binding domain of the receptor (e.g Etanercept)(16), or a fusion protein of different ligand binding domains of multiple receptors (e.g. Aflibercept(15)) to
improve the affinity of the Trap. To determine whether CCL21 could be a potentially useful ant-metastatic target, we identified a naturally occurring human protein with CCL21-binding properties, and used its chemokine-binding domain to generate a human IgG1-Fc-fusion protein that binds CCL21 with high affinity and inhibits its activity. We then determined whether this protein, Chemotrap-1, could be inhibit melanoma growth towards areas of high lymphatic density.

**Materials and Methods**

**Cell culture and transfection** – melanoma cells, purchased from ATCC (CRL-1619), were maintained in 10% FBS-DMEM (A375) or 10% FBS-EMEM (mouse B16-Luc). Human dermal LEC (CC-2814, Cambrex) were maintained in EGM-2 MV media (CC-3202, Cambrex). For transfection, cells at 80-90% confluency were seeded in 6-well plates and transfected with 1 μg of DNA and 4 μl of Lipofectamine-2000 (Invitrogen) in Opti-mem according to manufacturer’s instructions. Antibiotic selection was started on day 2 following transfection.

**Plasmid constructions.** pGBT9-hCCL21 vector was generated by inserting a cDNA encoding the mature form of human CCL21 (GenBank Accession No:NP_002989; aa24-134) into the BamHI site of MATCHMAKER yeast two-hybrid (Y2H) system 2 vector pGBT9 (Clontech). pGBK7-hCCL21 vector was generated by subcloning the BamHI CCL21 fragment from pGBT9-hCCL21 into the BamHI site of pGBK7 (Clontech). pGADT7-hCCL21 and pGADT7-hCCL21ΔCOOH expression vectors were generated by subcloning the BamHI fragment (encoding CCL21 amino acids 24-134) or the BamHI-
PstI fragment (encoding CCL21 aa24-102) from pGBK7-CCL21 into pGADT7 expression vector (Clontech). pGADT7-mCCL21 and pGADT7-CCL27 expression vectors were generated by cloning the cDNAs encoding mature forms of mCCL21 (GenBank Acc: NP_035254; aa24-133) or hCCL27 (GenBank Acc: NP_006655; aa25-112) into pGADT7. Construction of pGBK7-THAP1 and pEGFP.C2-THAP1 expression vectors has been previously described (17). The GST-THAP1 expression vector was generated by cloning the full-length coding region of human THAP1 (amino acids 1-213) in the pGEX-2T prokaryotic expression vector (Amersham Pharmacia Biotech, Saclay, France). THAP1 deletion mutants THAP1-C1(aa90-213), THAP1-C2(aa120-213), THAP1-C3(aa143-213), THAP1-N1(aa1-90), THAP1-N2(aa1-166) and THAP1-N3(aa1-192) were amplified by PCR using pEGFP.C2-THAP1 as template. The PCR fragments were digested with EcoRI and BamHI, and cloned in-frame downstream of the Gal4-activation domain in the pGADT7 two-hybrid vector (Clontech).

**Y2H assays.** Two-hybrid screening of an HEV cDNA library(17) was performed using human CCL21 as bait. pGBT9-hCCL21 was co-transformed with the pGAD424-HEV cDNA library in yeast strain Y190 (Clontech). 1.5x10^7 transformants were screened and positive protein interactions were selected by His auxotrophy. Two-hybrid interactions were confirmed in strain AH109 using MATCHMAKER two-hybrid system-3 (Clontech). AH109 cells were cotransformed with pGBK7-THAP1 and pGADT7-hCCL21, -mCCL21, -hCCL27 or -hCCL21ΔCOOH expression vectors. Transformants were selected on media lacking histidine and adenine. Two-hybrid interaction between THAP1 mutants and chemokine CCL21 or wild type THAP1 was tested by co-
transformation of AH109 with pGADT7-THAP1-C1,-C2,-C3,-N1,-N2 or -N3 and pGBK7-CCL21 or pGBK7-THAP1.

**GST pull-down and co-immunoprecipitation assays.** GST and GST-THAP1 fusion proteins were produced and purified as previously described(17). In vitro-translated hCCL21, mCCL21, hCCL27 and hCCL21ΔCOOH, were generated with the TNT-coupled reticulocyte-lysate system (Promega, Madison, WI, USA) using pGADT7-hCCL21, -mCCL21, -hCCL27 or -hCCL21ΔCOOH as template. 25μl of 35S-labelled chemokines were incubated with immobilized GST-THAP1 or GST proteins overnight at 4°C, in 10mM NaPO4 pH8.0, 140mM NaCl, 3mM MgCl2, 1mM dithiothreitol (DTT), 0.05%NP40, 0.2mM phenylmethyl sulphonyl fluoride (PMSF), 1mM Na Vanadate, 50mM β-glycerophosphate, 25μg/ml chymotrypsin, 5μg/ml aprotinin, 10 μg/ml leupeptin. Beads were washed 5 times in 1ml binding buffer. Bound proteins were eluted with 2X Laemmli SDS-PAGE sample buffer, fractionated by 10% SDS–PAGE and visualized by fluorography using Amplify (Amersham Pharmacia Biotech). For co-immunoprecipitation experiments, U2OS cells were transfected with plasmids encoding CCL21-Flag and/or HA-THAP1 using a calcium-phosphate precipitation procedure. Two days after transfection, cellular extracts were prepared 15mM Tris-HCl, pH 7.5, 0.4M NaCl, 5mM MgCl2, 0.1%Tween 20 containing protease and phosphatase inhibitors (Roche). After three freeze/thawing cycles, cellular extracts were centrifuged at 12000g for 10 min, then precleared at 4°C for 1h in a rotating wheel with 20μl protein G-sepharose beads (Amersham). Beads were precipitated by centrifugation, and supernatants incubated overnight at 4°C with 5μg of Flag M2 antibody (Sigma). Immune
complexes were captured with 20µl of protein G-sepharose beads at 4°C for 1h, washed 4 times with 0.4M NaCl. Bound proteins were eluted and subjected to immunoblotting with an anti-HA antibody.

**In silico sequence analysis and molecular modeling** - Coiled-coil predictions were performed at PAIRCOIL ([http://paircoil.lcs.mit.edu/cgi-bin/paircoil](http://paircoil.lcs.mit.edu/cgi-bin/paircoil)) and MULTICOIL ([http://multicoil.lcs.mit.edu/cgi-bin/multicoil](http://multicoil.lcs.mit.edu/cgi-bin/multicoil)) web sites. The structure of the THAP1 dimeric coiled-coil was built by homology with the X-ray structure of the GCN4 parallel coiled-coil (PDB code:1KD8). The energy of the interaction interface was minimized by using the Affinity module within InsightII (Accelrys, San Diego, CA).

**Generation of Chemotrap-1 expression vector** – The sequence encoding residues 140 to 213 of human THAP1 was amplified by PCR with oligonucleotides THAP1-XhoI-5′(5′-ccgctcgaggtatacaatgcacc-3′) and THAP1-BamH1-3′(5′-gcgggatccgctggtacttcaacctttcaag-3′). The resulting XhoI-BamHI fragment was used to replace the XhoI-BamHI fragment encoding L-selectin in the pCDM8-L-selectin-IgG1 plasmid (18, 19). A linker encoding the Igκ chain signal peptide (SP,21aa) from plasmid pSecTag2 (Invitrogen) was ligated into the XhoI site of pCDM8-THAP1140-213-IgG1 to obtain the expression vector pCDM8-SP-THAP1140-213-IgG1. The whole construct was inserted into the pcDNA3.1 vector (Invitrogen) to generate pcDNA3.1-Chemotrap-1. pcDNA3.1-Chemotrap-189 expression vector was obtained by replacing the XhoI-THAP1140-213-BamHI fragment of pcDNA3.1-Chemotrap-1 by an XhoI-THAP1189-213-BamHI fragment generated by PCR.
**Chemotrap-1 production and purification** - pCDNA3.1-Chemotrap-1 or pCDNA3.1-Chemotrap-189 expression vectors were transfected into CHO cells using JetPEI (Ozyme). Cells were grown with Zeocin (Invivogen – 100mg/ml) to establish clonal lines. Supernatants were diluted in ImmunoPure-Gentle Ag/Ab Binding Buffer (Pierce) and mixed with protein A-Sepharose CL-4B (Amersham Biosciences) for 1h at room temperature, under gentle agitation. The packed resin was rinsed using 15 volumes of binding buffer, before elution with ImmunoPure-Gentle Elution Buffer (Pierce). The fractions containing Chemotrap were concentrated on an Amicon Ultra-4 (10,000 MWCO) and analyzed by immunoblotting with Goat α-Human IgG HRP 1/5000 and an enhanced chemiluminescence kit (Amersham Bioscience).

**Surface plasmon resonance (SPR) experiments**

Purified recombinant THAP1_{1-213}-Fc and Chemotrap-1 (THAP1_{140-213}-Fc) were covalently bound from their amino groups to the gold sensor chip surface (CM5 sensor chip). Purified recombinant human CCL21 (Chemicon) was used as analyte protein at a fixed concentration in the fluid phase (10mM HEPES pH 7.4, 150mM NaCl, 3mM EDTA, 0.005% P20 surfactant, as a running buffer), and at a constant flow rate of 20µl/min in a BIAcore 3000 system (BIAcore AB, Uppsala, Sweden). Association and dissociation curves were established for 12.5nM, 25nM, 50nM, 100nM, 200nM CCL21. Chemokine was injected during the association phase for 4 min (20µl/min). The dissociation phase was carried out over 5 min and flow cells were regenerated by injection of 0.05%SDS (30sec at a flow of 20µl/min) between each phase of association-
dissociation. Biaevaluation (Biacore) program was used for calculations of association/dissociation kinetic constants. $K_d = \frac{k_d}{k_a}$.

**ELISA.**

The CCL21 DuoSet ELISA was carried out as per manufacturers instructions. To determine the effect of chemotrap-1 on CCL21 release from LEC, cells were cultured for 24 hours in 0.5%EGM2, and 5ml media transferred to a T25 flask of B16 melanoma cells for a further 24 hours. Media was then collected, spun to remove cells, and then concentrated through an Amicon10000 spin column.

**In vitro Cell migration**

Migration assays were performed in a modified Boyden chamber consisting of a cell culture insert with an 8µm pore polycarbonate membrane (Millipore) seated in each well of a 24 well plate as previously described(11). For dose-response migration assays were performed as above in 150ng/ml CCL21 and increasing concentrations of chemotrap. The number of cells migrated through were normalised to the lowest concentration of chemotrap (10pM) and to 0.1% FCS alone (no CCL21), and EC50 determined by non-linear curve fitting with fixed top and bottom parameters (100% and 0% respectively) using a sigmoidal dose response curve.

**In vivo Metastatic chemotaxis model**

Experiments were carried out as previously described(11). Briefly, nude mice were injected with $1 \times 10^6$ tumour cells subcutaneously with a Monastral Blue coated needle. The mice were then injected with $1 \times 10^5$ human LEC approximately 10mm caudal to the melanoma injection site. Mice were left until tumors reached 8-10mm when measured.
through the skin. The animals were then killed, tumors excised, the skin pronated and photographed. Directional growth of the melanoma was measured as the plan area of the tumour below the perpendicular axis (i.e. closer to the endothelial cell injection site) and expressed as a percentage of the total area of the tumour.

**In vivo bioluminescent imaging of tumours.**

CD1 mice were injected subcutaneously with $1 \times 10^6$ B16-Luc cells. 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 imaged as above, then killed and the flank dissected to investigate in-transit metastases. Areas of black tracking along lymphatics were photographed using a Nikon Coolpix digital camera.

**Results**

**Identification of a CCL21-chemokine-binding protein.**

While searching for CCL21-interacting proteins in a two-hybrid screen of a high endothelial venule (HEV) cDNA library, we identified the cDNA encoding a novel nuclear factor, that we designated THAP1(17). Eight positive clones (of five million transformants) were identified with the CCL21 bait, and all corresponded to the C-terminal part of THAP1, aa 90-213 (Fig. 1A). To confirm the interaction in yeast, we performed *in vitro* GST pull-down assays. Full length THAP1-GST protein was incubated with radiolabelled *in vitro* translated CCL21. Both human and mouse CCL21
proteins bind to GST-THAP1 but not to GST (Fig. 1A). The interaction was also observed when the basic C-terminal extension of CCL21 was deleted, indicating that THAP1 interacts with the core chemokine domain of CCL21 (Fig. 1A). No binding of THAP1 was observed to CCL27, another CC chemokine. We next addressed whether THAP1 is able to interact with CCL21 in cells. We performed immunoprecipitation experiments in cells co-expressing epitope-tagged-CCL21 (Flag-CCL21) and -THAP1 (THAP1-HA). We observed specific immunoprecipitation of THAP1 with anti-Flag antibodies in cells co-expressing Flag-CCL21, whereas no precipitation of THAP1 with anti-Flag antibodies was observed in control cells (Fig. 1B). These findings demonstrated that THAP1 interacts with CCL21 both in vitro and in cells. To determine the kinetic parameters of the THAP1/CCL21 complex, we performed SPR. THAP1_{1-213}-Fc fusion protein was purified from cell supernatants and immobilized on a sensor chip. Addition of purified recombinant human CCL21 revealed association with immobilized THAP1_{1-213}-Fc with a Kd of 87 nM (Fig. 1C). Together, our observations indicated that the human THAP-zinc finger protein THAP1 is a chemokine-binding protein that binds CCL21 with nanomolar affinity.

**Mapping the CCL21 chemokine-binding domain.**

To identify sequences mediating CCL21-binding, a series of THAP1 deletion mutants were generated. These truncated versions were used as Gal4 DNA-binding domain fusion proteins (baits) in the Y2H system, together with preys corresponding to mature CCL21 (aa 24-134) in fusion with the Gal4 activating domain. Schematic representation of all THAP1 deletion mutants and summary of two-hybrid results are presented in Fig. 2A.
We found that neither the THAP1-N1 mutant, corresponding to the DNA-binding THAP-zinc finger(20) nor THAP1-N2 and –N3, which contain the THAP-zinc finger and the proline-rich linker region, interact with CCL21. In contrast, cells co-expressing CCL21 with N-terminal deletion constructs, THAP1-C1, -C2 or -C3, exhibited strong growth on selective medium, indicating that the C-terminal domain of THAP1 (aa143-213) is necessary and sufficient for CCL21 binding. To confirm the Y2H results we performed in vitro GST pull-down assays with GST-THAP1 deletion constructs that showed the C-terminal part of THAP1 (aa142-213) sufficient for interaction with radiolabeled in vitro translated CCL21 (Fig. 2B), but that it required amino acids 142-166, as deletion of aa142-166 reduced binding and deletion abolished it completely. These results show that the CCL21 chemokine-binding domain of THAP1 corresponds to the last 70 amino acids (aa 143-213).

The CCL21 chemokine-binding domain has characteristics of a coiled-coil.

Two-hybrid assays with THAP1 bait (Fig. 2A) revealed that the CCL21 chemokine-binding domain (aa 143-213) also mediates interaction of THAP1 with itself (homo-dimerization or oligomerization). The PAIRCOIL(21) and MULTICOIL(22) algorithms revealed that the CCL21 chemokine-binding domain of THAP1 has characteristics of a coiled-coil, one of the principal dimerization/oligomerization motifs found in proteins(23). The coiled-coil structure consists of two α-helices wrapped around each other with a slight superhelical twist. Its most characteristic feature is a heptad repeat pattern of primarily apolar residues that constitute the dimer/oligomer interface. Both PAIRCOIL and MULTICOIL programs predicted a parallel left-handed coiled-coil
domain with seven heptad repeats (49 residues), extending between amino acids 142 and 190 of human THAP1. The probability for formation of the coiled-coil structure of this region was 1 (maximum score) with both programs, indicating that this region of THAP1 has a very high probability of existing as a coiled-coil structure (Fig. 2C). Molecular modeling of the THAP1 coiled-coil domain was performed using the X-ray crystal structures of known coiled-coil domains as a template. The resulting three-dimensional model of the THAP1 coiled-coil is shown in Figure 2D. The parallel homodimer is stabilized by a perfect zipper, based on hydrophobic interactions between identical residue side-chains from each monomer. The CCL21–binding domain also includes the last 23 C-terminal amino-acids of THAP1 (aa 191-213), not required for homo- dimerization/oligomerization (Fig. 2A) but, with residues 142-166, essential for CCL21 binding.

**Chemotrap-1 – an engineered soluble inhibitor based on the CCL21 chemokine-binding domain.**

The CCL21 chemokine-binding domain of THAP1 could be useful to engineer a high affinity soluble inhibitor capable of blocking CCL21 chemokine activity *in vivo*. We thus generated a fusion protein between the Fc region of human IgG1 and the CCL21 chemokine-binding domain of human THAP1 (Fig. 3A). This fusion protein – Chemotrap-1 – was then expressed in chinese hamster ovary (CHO) cells and purified. As a negative control, we generated a fusion protein of human IgG1 with a 26 amino acid region (aa 189-213) of THAP1 that does not bind CCL21(Fig 2B). Chemotrap-1 bound to CCL21 with the same affinity as the full length THAP1-IgG1 fusion protein (~ 100 nM),
as determined by SPR (Fig. 3B). In contrast, Chemotrap-189 did not bind CCL21 (data not shown).

To determine whether Chemotrap-1 could inhibit CCL21 chemokine-mediated migration of cancer cells, we seeded A375P-CCR7 transfected melanoma cells on polycarbonate inserts, and measured migration across those inserts to CCL21, or CCL21 and Chemotrap-1. 1µg/ml Chemotrap-1 significantly inhibited migration of these melanoma cells towards 150ng/ml CCL21 (Fig 3C). To determine the potency of Chemotrap-1 in vitro we performed a dose escalating study using 150ng/ml CCL21 as a pro-migration agent. Figure 3D shows that chemotrap-1 dose dependently inhibited CCL21 mediated migration with an IC50 of 77pM, significantly more potent than to immobilised CCL21 on a chip in vitro. To determine the specificity of chemotrap-1 in vitro we investigated its effect on migration of A375P-CCR7 cells induced by three different chemokines – CCL19 (binds CCR7), CXCL12 (binds CXCR4) and CXCL10 (binds CXCR3). 150ng/ml of any of the four chemokines induced migration of melanoma cells, but only the CCR7 ligands, CCL21 and CCL19 were inhibited by Chemotrap-1 (figure 3E).

Chemotrap-1 blocks migration of metastatic melanoma cells towards lymphatics in vitro and in vivo.

We previously showd metastatic melanoma cells migrating towards LEC conditioned medium in a CCL21 dependent manner(11). To determine whether this could be inhibited by Chemotrap-1, A375 cells were transfected with expression vectors for Chemotrap-1 (pcDNA3.1-Chemotrap-1) or Chemotrap-189 (pcDNA3.1-Chemotrap-189). Expression
of the Fc-fusion protein was tested by immunoblotting, and the transfected A375 cells
were then seeded into the top of Boyden chambers. LEC conditioned media (CM) was
placed on the bottom part of the chamber, and A375 migration measured over 24 hours.
Figure 4A shows that expression of Chemotrap-1, but not Chemotrap-189, resulted in
complete inhibition of migration of A375 cells towards LEC-CM. We have also
previously shown that when implanted into nude mice 8-10mm from a depot of human
lymphatic endothelial cells, metastatic, but not non metastatic melanomas grow towards
the LEC injection depot. To determine whether Chemotrap-1 could inhibit directed
metastatic growth, 1x10^6 A375 cells transfected with pcDNA3.1-Chemotrap-1, pcDNA3.1-Chemotrap-189 or pcDNA3 (control vector) were implanted subcutaneously
into mice, 8-10mm from a simultaneous implantation of 1x10^5 human LEC. After 14
days, tumours were apparent in all mice. Whereas tumours in mice injected with the
pcDNA3 and pcDNA3.1-Chemotrap-189 transfected cells grew significantly back
towards the LEC depot, the pcDNA3.1-Chemotrap-1 transfected cells grew in the
location that they were injected (Fig. 4B). Tumours were excised, pronated and the area
of tumour on each side of the injection site was quantified. Figure 4C shows Chemotrap-
1 expression resulted in a statistically significant inhibition of tumour growth towards
LEC, to a level equivalent to non-directed growth (towards the site of a saline injection).

**Chemotrap-1 prevents in transit metastasis of melanoma cells in lymphatics in vivo.**

CCR7 over-expression increases lymph node metastasis of B16 mouse metastatic
melanoma cells, resulting from uptake into and tracking along the local lymphatics(24).
To determine whether Chemotrap-1 could inhibit this tracking, in a CCR7-dependent
manner, we imaged tumours growing in CD1 mice by bioluminescence. B16 melanomas formed tumours that were elongated (figure 5A). The maximum major to minor axis ratio was 4.1±1.1, (mean±SEM, n=6, figure 5Ai). In contrast, tumours expressing Chemotrap-1 had a mean ratio of 2.5±0.2 (figure 5Aiii), which was significantly lower than that observed with tumours expressing Chemotrap-189 (3.9±0.5, figure 5Aii). Moreover, upon excision, tracking of the black melanomas (figure 5A iv) could be seen along lymphatics in one third of the B16 melanomas and 60% of the cells expressing Chemotrap-189, whereas this tracking was rarely seen with cells expressing Chemotrap-1 (17%, figure 5Av). CCR7/Chemotrap-189 co-transfected cells all showed evidence of tracking (figure 5B i, ii, iv). In contrast, CCR7-Chemotrap-1 transfected cells showed a significant inhibition of tracking (figure 5A iii, v) to 33%, (p<0.01 chi squared test, figure 5C), indicating that co-expression of Chemotrap-1 completely inhibited the effect of CCR7 overexpression. To determine whether recombinant Chemotrap could prevent metastasis in vivo we used a CCR7 dependent model of lymph node metastasis in syngeneic mice. B16-Luc cells were stably transfected with CCR7, and 50µl of 1x10⁵ cells injected into the foot pad of 10 C57/Bl6 mice. Three animals were treated twice weekly with 50µg Chemotrap-1 by intraperitoneal injection. After 22 days the mice were injected with luciferin and tumours imaged. Mice were killed, and lymph nodes exposed and imaged. Figure 5D shows luminescence of the primary tumour and of a lymph node metastasis in the popliteal lymph node. Metastases were seen in all 7 control mice, but in only 1 of 3 treated mice (p<0.02 Chi squared test), suggesting that systemic administration could reduce lymph node metastases in this mouse model.
To confirm that Chemotrap-1 was being secreted from the transfected cells used a commercial DuoSet CCL21 ELISA. Figure 5F shows that the melanoma cells do not express CCL21 at levels detectable on the CCL21 Duoset Kit, (confirmed by RT-PCR, data not shown). LEC expressed CCL21 protein (1.79pg/ml per flask per hour) and mRNA as determined by RT-PCR (data not shown). When LEC CM was mixed with media from Chemotrap-1 expressing cells (B16-CT1), there was a reduction in available CCL21 indicating binding of CCL21 to Chemotrap-1 to below the detection limit of the assay. In contrast when LEC-CM was added to Chemotrap-189 there was no reduction in CCL21.

**Discussion.**

Metastatic mechanisms are potential therapeutic targets, as cancers can be detected and excised before metastasis is apparent, yet spread months or even years after excision. For melanoma, prognosis is poor once metastasis has occurred and there are no widely accepted therapies. We show here that one mechanism of metastasis, chemokine mediated LEC targeting, is potentially therapeutically amenable, as inhibition of the CCL21/CCR7 axis can prevent melanoma cells recognising and migrating towards areas of high lymphatic density, and invading those lymphatics.

Chemokine receptors, including the CCL21 receptor CCR7, and the CXCL12 receptor CXCR4 have been associated with metastatic melanoma in humans, and increase metastatic growth in mouse and human tumours in experimental animals(3, 25). Two non-mutually exclusive mechanisms have been proposed for CCR7-CCL21 mediated metastasis, metastatic chemotaxis towards areas of high lymphatic density(11), and autologous chemotaxis(26). Current experiments did not determine the relative
contributions of these two mechanisms towards invasion of melanoma cells into the lymphatic system. Although we showed that secreted Chemotrap-1 bound to CCL21 secreted from LEC, and that recombinant Chemotrap-1 could inhibit metastasis we do not exclude the potential for synthesis of Chemotrap-1 acting intracellularly or autocrinely on the tumour cells. We show here that CCR7-dependent lymphatic recognition by metastatic melanoma is inhibited by the CCL21 binding agent Chemotrap-1. Chemotrap-1 is a human IgG1-Fc-fusion protein containing the CCL21 chemokine-binding domain of human THAP1, a physiological regulator of endothelial cell proliferation(17)(27). CCL21 contains a functional nuclear localization sequence in its carboxyl-terminal extension and may localize to the nucleus under certain conditions (JPG, unpublished), but the physiological relevance of the THAP1/CCL21 interaction has not yet been demonstrated. Chemotrap-1 is the first example of a human recombinant chemokine-binding Fc-fusion protein. The THAP1 chemokine-binding domain, predicted to adopt a coiled-coil helical structure, presents no sequence or structural homologies with CCR7 and other chemokine receptors. Determination of the three-dimensional structure of the complex will be therefore required to understand the molecular basis for specific recognition of the chemokine core domain of CCL21 by the THAP1 chemokine-binding peptide. However, inhibitors of chemokine receptors have been shown to be effective in animal models of melanoma – specifically the CXCR4 receptor inhibitor AMD3100(25). However, there are no specific small molecule inhibitors of CCR7, and CCR7-IgG1 soluble receptors cannot be prepared because of the 7-transmembrane nature of chemokine receptors.
Chemotrap-1 blocked CCR7-mediated tracking of metastatic melanoma cells into and along lymphatics indicating that Chemotrap-1 can inhibit CCL21 activity \textit{in vivo}. Another chemokine-binding protein, the M3 protein encoded by murine gammaherpesvirus-68, has previously been shown to bind CCL21 with high affinity and to inhibit CCL21-mediated recruitment of lymphocytes \textit{in vivo}(28). A major advantage of Chemotrap-1 is that it is a fully human recombinant protein. The M3 protein also binds and blocks the activity of other chemokines \textit{in vivo}(28) and it will be important in future studies to widen the chemokines against which Chemotrap-1 has been tested to understand the full binding potential of this protein.

Most human melanomas metastasise through the local lymphatics and, through the lymph node, gain access to the circulation and reach distant organs such as the lung, brain, liver, etc. We therefore used a model in experimental animals whereby lymphatic metastasis is generated from subcutaneous injection of primary tumour cells, in contrast with experimental models using intravenous injection of tumour cells. This is not the usual route for melanoma metastasis. In patients, melanomas can form secondary lesions either locally within the skin (local metastasis), along the lymphatics (in transit metastasis), or distantly (lymph node, primarily, followed by brain, lung, liver, etc). We show here that chemokine-binding agents can prevent melanoma migration towards areas of high lymphatic density, invasion into lymphatics, and in transit metastasis.

The findings here indicate that inhibition of chemokines acting on tumour cells can prevent tumour migration towards and into draining lymphatics. This suggests that chemokine antagonists may be potential therapeutic agents. Further evidence for this would be required before moving to clinical studies, particularly in terms of generating
pharmacologically relevant inhibitors, and protein therapeutics, but the results here show for the first time that an endogenous human protein sequence can be used to block lymphatic metastases.

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Figure legends

Figure 1. Interaction between CCL21 and THAP1.

A-Two-hybrid and GST pull-down interaction assays demonstrate interaction between human CCL21 (hCCL21) and human THAP1. This interaction was also observed with mouse CCL21 (mCCL21), indicating evolutionary conservation. Schematic representation of results obtained in two-hybrid and GST pull-down assays using human THAP1 together with human CCL21, mouse CCL21, human CCL21 deleted from its C-terminal extension (hCCL21DCterm) and human CCL27, another CC-chemokine used as a control. B-Co-immunoprecipitation of THAP1-HA with Flag-CCL21 in cellular extracts (0.4M NaCl) followed by immunoblot with anti-HA antibody. Stars represent Ig subunits, arrows the HA tagged THAP1. C-Surface plasmon resonance analysis of THAP1/CCL21 association. Full length THAP1 protein (aa 1-213) was immobilized on the sensor chip as an IgG1-Fc fusion protein and incubated with increasing concentrations of soluble CCL21 protein. The Kd for the THAP1/CCL21 association had a calculated value of 87 nM.

Figure 2. The CCL21 chemokine-binding domain of THAP1 has characteristics of a coiled-coil domain. A-Summary of two hybrid results obtained with THAP1 deletion mutants and CCL21 or wild type THAP1 (homodimerisation). B-GST pull-down interaction assays demonstrate interaction between human CCL21 and human THAP1 residues 126-213 and 142-213, less so for 166-213 and not for 186-213. C-THAP1 contains a predicted coiled-coil domain between residues 142 and 190. The 213 amino acid sequence of THAP1 was analyzed using the PAIRCOIL program. The probability of
each residue within the THAP1 sequence contributing to a coiled-coil structure, is plotted (maximal probability score=1). **D**-Molecular modeling of the THAP1 coiled-coil domain based on the crystallographic structure of the GCN4 homodimeric parallel coiled-coil. Molecular modelling was performed as described in the Materials and Methods.

**Figure 3. Chemotrap-1 binds CCL21 and blocks CCL21-induced melanoma cell migration in vitro.** A-The chemokine-binding peptide of THAP1 was fused to the Fc region of human IgG1 to generate Chemotrap-1, a fully human chemokine-binding Fc fusion protein. **B**-Surface plasmon resonance analysis of Chemotrap-1/CCL21 association. Chemotrap-1 was immobilized on the sensor chip as an IgG1-Fc fusion protein and incubated with increasing concentrations of soluble CCL21 protein. The Kd for the Chemotrap-1/CCL21 association had a calculated value of 102 nM. **C**-Chemotrap-1 blocks CCL21-induced melanoma cell chemotaxis in vitro. Cell migration of media containing 150ng/ml CCL21 or 150ng/ml chemotrap transfected cells Mean±SEM. ***=p<0.01 compared with media and chemotrap-189. ANOVA, SNK. **D**. Chemotrap 1 has a high affinity for inhibition of CCL21 mediated migration. A75P-CCR7 cells were subjected to migration assay towards 150ng/ml CCL21 or increasing concentrations of chemotrap1. The IC50 was 77pM. **E**. Chemotrap-1 inhibition of migration is restricted to CCR7 ligands. Migration of A375P-CCR7 melanoma cells across polycarbonate filters towards 150ng/ml of CCL21, CCL19, CXCL12 or CXCL10 was measured in the presence or absence of 1µg/ml Chemotrap. +=p<0.05 compared with control (value of 1), *=p<0.05, ***=p<0.001 compared with cytokine alone, one way ANOVA, Dunnet’s test.
Figure 4. Chemotrap-1 inhibits directed growth of melanoma towards lymphatic endothelial cells. A. Migration of melanoma cells or melanoma cells expressing empty vector (pcDNA3.1), negative control Fc (chemtrap189) or Chemotrap-1 was measured in vitro to LEC CM. B. Melanoma cells as above were injected into nude mice 8-10mm from a depot of LEC, and direction of tumour growth measured. The red highlighted area is growing towards, and the blue away from the LEC. C. The % growth towards the LEC is reduced in A375 cells expressing chemotrap-1 to the same level as in the absence of lymphatic endothelial cells (PBS). Mean±SEM. ***=p<0.001, **=p<0.01, *=p<0.05 compared with pcDNA3 control (ANOVA).

Figure 5. Chemotrap1 inhibits tracking of melanoma cells into and along lymphatics. A. B16-Luc melanoma cells (i), transfected with chemotrap-189 (ii) or chemotrap-1 (iii) were injected into CD1 mice and circularity of tumour growth measured by bioluminescence. Upon excision tracking (arrow) of the tumour was seen in control (iv) but not in chemotrap-1 transfected (v) tumours. B. Effect of over-expression of CCR7 (i) or CCR7 and chemotrap-189 (ii) increased non-uniform growth but CCR7-chemotrap-1 expression again results in more circular growth (iii). Tracking was increased in CCR7 transfected tumours (iv) but reduced in CCR7 and chemotrap-1 expressing tumours (v). C. Percentage of tumours tracking. **=p<0.01, *=p<0.05 chi squared test compared with control. D. C57Bl6 mice were injected with B16-Luc-CCR7 melanoma cells in the foot pad. 22 days later the tumours were imaged by bioluminescence (i) and then the mouse killed and the lymph node exposed and imaged (ii). E. Chemotrap-1 treatment significantly reduced the incidence of lymph node
metastasis (p<0.02 Chi squared test). F CCL21 Duo set ELISA kit was used to determine the concentration of CCL21 in the LEC-CM, LEC-CM incubated with B16 cells expressing Chemotrap-1, or Chemotrap-189. *=p<0.05 Dunnet’s test.
References

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Figure 3: Chemotrap-1 binds CCL21 with high affinity and blocks CCL21-induced melanoma cell migration in vitro.
Figure 5. Chemotrap1 inhibits tracking of melanoma cells into and along lymphatics.
Chemotrap-1: an engineered soluble receptor that blocks chemokine-induced migration of metastatic cancer cells in vivo

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