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ORIGINAL ARTICLE

CXCR1 as a novel target for directing reactive T cells toward melanoma: implications for adoptive cell transfer immunotherapy

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Abstract Adoptive cell transfer therapy with reactive T cells is one of the most promising immunotherapeutic modalities for metastatic melanoma patients. Homing of the transferred T cells to all tumor sites in sufficient numbers is of great importance. Here, we seek to exploit endogenous chemotactic signals in order to manipulate and enhance the directional trafficking of transferred T cells toward melanoma. Chemokine profiling of 15 melanoma cultures shows that CXCL1 and CXCL8 are abundantly expressed and secreted from melanoma cultures. However, the complimentary analysis on 40 melanoma patient-derived tumor-infiltrating lymphocytes (TIL) proves that the corresponding chemokine receptors are either not expressed (CXCR2) or expressed at low levels (CXCR1). Using the in vitro transwell system, we demonstrate that TIL cells preferentially migrate toward melanoma and that endogenously expressing CXCR1 TIL cells are significantly enriched among the migrating lymphocytes. The role of the chemokines CXCL1 and CXCL8 is demonstrated by partial abrogation of this enrichment with anti-CXCL1 and anti-CXCL8 neutralizing antibodies. The role of the chemokine receptor CXCR1 is validated by the enhanced migration of CXCR1-engineered TIL cells toward melanoma or recombinant CXCL8. Cytotoxicity and IFNγ secretion activity are unaltered by CXCR1 expression profile. Taken together, these results mark CXCR1 as a candidate for genetic manipulations to enhance trafficking of adoptively transferred T cells. This approach is complimentary and potentially synergistic with other genetic strategies designed to enhance anti-tumor potency.

Keywords Melanoma · T cell · CXCR1 · Chemotaxis · Immunotherapy

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Introduction

The homing of T cells toward tumors depends on an intricate network of guiding cues that is only beginning to be understood and involves chemokines secreted from the tumor milieu [1–4]. Chemokines are small (8–10 kDa) cytokines, acting through seven transmembrane domain G-protein-coupled receptors to elicit a signaling cascade

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culminating in directed locomotion. They are classified into four groups (C, CC, CXC, and CX3C), according to the number and spacing of cysteines in a conserved N-terminal motif. With more than 50 known chemokines and 20 receptors so far, the chemokine system is characterized by redundancy, with some receptors binding several chemokines (ex. CCR1–CCR5), others only one (ex. CXCR4–CXCR6) and some function as "deceptors" that bind chemokines but do not transmit signals. Though originally identified in the control of leukocyte chemotaxis, especially during infection and inflammation, it is now known that virtually all cells, including tumors, express chemokines, and chemokine receptors. The pleiotropy in the chemotactic system is reflected by the diverse physiological and pathological processes it coordinates, including patterning of neuronal cells in the developing nervous system, homeostatic transport of hematopoietic stem cells, lymphocytes and dendritic cells, inflammatory diseases, tumor growth, metastasis, angiogenesis, and recruitment of macrophages by tumors [1, 3–5]. The published data regarding chemokines that direct anti-tumor T cells to melanoma are scarce and sometimes contradictory [2]. It was found, for example, that CCL2 and CCR4 play a role in T cell chemoattraction by melanoma in vitro [6], and that tumor infiltration of T cells is strongly associated with high CXCL9 and CXCL10 expression in melanoma in in situ hybridization studies [7]. CXCL12 was shown to enhance T cell migration toward melanoma in vitro [8], but to cause chemorepulsion in other systems [9]. In a pioneering paper published recently by Harlin et al., 44 biopsies of malignant melanoma patients were analyzed by gene array and the expression level of chemokines in highly and poorly T cell infiltrated biopsies was compared. Combining validation and functional assays, Harlin et al. [2] pointed on the combination of melanoma-secreted CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 as the chemotactic cues for infiltrating T cells. However, the complete T cell/melanoma chemotactic network is still to be explored, as, for example, the pattern of chemokine receptors on clinically derived, ex vivo cultured T cells as well as the chemotactic signals secreted from melanoma versus those secreted from normal melanocytes were never studied. Moreover, our understanding of how to exploit chemotactic signals in order to manipulate reactive T cells to better reach melanoma sites is far from being complete.

In the promising adoptive cell immunotherapy (ACT), metastatic melanoma patients are infused with autologous T cells, which originated in surgically removed metastases and were massively expanded in culture [10–15]. It has been shown that ACT and other immunotherapeutic regimes, such as vaccination, enhance the level of circulating anti-melanoma effective T cells [16–21]. Many efforts are currently invested in engineering of highly potent T cells with a broad spectrum of cancer cell recognition by using various genetic technologies, with some clinical success [22–24]. However, the effective trafficking of reactive T cells into tumor sites remained uncertain [21, 25, 26] and is considered to be a major barrier toward the achievement of durable and effective anti-melanoma immune responses [2, 27, 28].

In this work, we aimed to define the most common determinants that could be exploited for efficient enhancement of trafficking of adoptively transferred T cells toward melanoma. This approach is complimentary to the major line of research that focuses on engineering of T cell receptors or chimeric antigen receptors. By profiling clinically derived melanoma cells and tumor-infiltrating T cells (TIL) for the expression of chemokines and chemokine receptors, we identified two chemokines that are ubiquitously expressed by melanoma cells. We studied the potential of targeting this chemotactic signature by blocking antibodies and overexpression of the appropriate chemokine receptor, CXCR1, on TIL cells. Engineering of T cells to overexpress CXCR1 improved their specific migratory capabilities and implies that manipulation of CXCR1 expression may enhance the efficacy of ACT therapy for the vast majority of melanoma patients.

Materials and methods

Cells and media

Low passage primary metastatic melanoma cultures were developed from surgically resected tumors as previously described [29] and included 001mel, 02mel, 003mel, 007mel, 008mel, 08mel, 009mel, 09mel, 14mel, 15mel, 37mel, 39mel, and 42mel. Melanoma cell lines were 526mel (obtained from Dr. SA Rosenberg, Surgery Branch, NCI, Bethesda, MD, USA) and C8161mel (obtained from Dr. Hendrix, Children's Memorial Research Center, Chicago, IL, USA). Melanoma cells were maintained in complete RPMI medium, as previously described [29]. Normal epidermal melanocytes were purchased from PromoCell GmbH (Heidelberg, Germany) and maintained as recommended by the supplier in melanocyte growth medium (PromoCell GmbH). Non-melanoma human tumor cells included 721.221 (Epstein–Barr virus-transformed B-cell lymphoma), NK92 (natural killer cells), and K562 (chronic myeloid leukemia) that were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); PC3 (prostate cancer), MCF7 (breast cancer), and RCC (kidney cancer) that were gifts from Dr. R. Berger (Sheba medical center, Israel). 721.221 and K562CML were maintained in complete RPMI, PC3, and RCC in complete Dulbecco's modified Eagle's medium (Gibco)

and NK92 as suggested by the ATCC. Primary tumor-infiltrating lymphocytes (TIL) were generated from melanoma metastases and maintained in TIL growing medium supplemented with 3,000 IU/ml rhIL-2 (Chiron B.V.) as previously described [30].

Bioethics

Generation of primary melanoma and TIL cultures was performed as part of clinical adoptive transfer protocols, which were approved by the Israel Ministry of Health (Approval no. 3518/2004, ClinicalTrails.gov Identifier NCT00287131), after obtaining an informed consent from the patients.

Antibodies

The following antihuman chemokine receptors fluorophore-conjugated antibodies (and their isotype controls) were used in flow cytometry: anti-CCR4-FITC, -CCR5-PE, -CXCR1-PE, and anti-CXCR3-APC were purchased from R&D Systems (Minneapolis, MN, USA); anti-CCR7-APC and anti-perforin-FITC from eBiosciences (San Diego, CA, USA); anti-CXCR2-PE and -CXCR1-APC from BioLegend (San Diego, CA, USA); anti-MCSP-PE and anti-NGFR-APC from Miltenyi Biotec (Germany) and anti-CD8-FITC from DakoCytomation (Cambridgeshire, England). The blocking antihuman antibodies anti-CXCL1 and -CXCL8 were purchased from R&D Systems (Minneapolis, MN, US). The following antihuman antibodies were used in immunohistochemistry: anti-CXCL1 from Proteintech Group Inc. (Chicago, IL, USA); anti-CXCL8 from Abnova (Taipei City, Taiwan); and anti-CXCR1 and anti-CXCR2 from R&D Systems (Minneapolis, MN, USA).

Lymphocyte migration assay

Lymphocyte chemotaxis was tested by modified Boyden chamber procedure using 5-μm pore size polycarbonate membranes (Costar, Corning Inc., NY, USA) and 24-well culturing plates (Greiner bio-one). TIL were pre-incubated in RPMI/1 % human serum containing 10 μM Calcein AM (Molecular Probes, Inc.) at 37 °C for 1 h. Dye-loaded cells were pelleted, washed, resuspended in TIL media containing 10 % human serum and then added (1×10^5 cells in 100 μl) to each upper well of the transwell apparatus. Melanoma cells (or other cells when indicated) that were seeded in duplicates or triplicates at 3×10^5 cells/well in 24-well culturing plates 2 days before the experiment served as the targets for TIL migration and constituted the lower well (LW) of the Boyden chamber apparatus. The conditioned media of these cells served also as target for TIL migration when indicated. After assembling, the apparatus was incubated at 37 °C for 4 h (2 h if indicated). Then, the upper wells were removed and lower well cells were pelleted by 400 g at 4 °C for 5 min, washed in HBSS, and lysed in HBSS with 0.5 % SDS. Calcein fluorescence was measured using the FLX800 Fluorescence Microplate Reader (BioTek, VT, USA) with excitation at 485 nm and emission read at 538 nm. The fluorescence intensity was converted to TIL number with a corresponding standard curve. The relationship between cell number and fluorescence intensity was linear over the range of the experimental values obtained. Results are presented as Migration Index, a value which represents the fold migration toward specific target (cells or conditioned media) over non-specific migration toward fresh melanoma growing media.

Flow cytometry

We used standard flow cytometry procedures as was described previously [29, 31]. For the analysis of migrating and non-migrating cells, 1×10^6 unlabeled TIL were loaded on each upper transwell chamber and were allowed to migrate toward melanoma-conditioned media for 4 h, then counted and subjected to FACS. In the perforin experiments, the same procedure was performed, except that following migration, each TIL subpopulation was supplemented with 14mel cells (at E:T 10:1), to activate TIL, and with 2 μM monensin (eBiosciences), to block perforin secretion, for 5 h at 37 °C. Cells were then stained with anti-CXCR1, anti-perforin, or both and analyzed by FACS. For peforin, CXCR1 and CXCR2 intracellular (total) stainings cells were fixed (4 % PFA for 15 min on ice), permeabilized in saponin buffer (0.2 % BSA, 0.1 % saponin, 0.02 % sodium azide; 10 min on ice), and then stained in saponin buffer. In PHA and IL6 experiments, 20 μg/ml PHA or 200 ng/ml IL6 was added to 14TIL for various incubation times as indicated. Following incubation, the cells were washed twice in PBS and FACS stained for CXCR1. In the blocking antibodies experiments, 40 μg/ml anti-CXCL1 and 22 μg/ml CXCL8 were added to the lower wells prior to TIL loading on the transwell upper wells.

In vitro cytotoxic assay

Killing assays were performed as previously described [29, 32]. Briefly, CFSE (Sigma-Aldrich, Israel)-labeled target melanoma cells were co-cultured with TIL cells at effector-to-target ratio of 10:1 for 4 h. Cells were then centrifuged at 500 g for 5 min, resuspended in PBS containing 40 μg/ml PI (Sigma-Aldrich, Israel), and analyzed by FACS. The percentage of dead melanoma cells was calculated as $\%PI^+$ cells out of gated $CFSE^+$ cells. Specific killing activity is expressed as $\%PI^+CFSE^+$ values after subtraction of

background dead melanoma in a sample containing merely melanoma cells. Background level did not exceed 20 % in all experiments.

IFNγ release assay

Melanoma cells and TIL cells were cultured for 4 h. The concentration of IFNγ in the supernatants was quantified using standardized commercial sandwich ELISA (R&D Systems), as was previously described [29, 32].

RNA isolation, RT-PCR, and quantitative (real-time) PCR

Total cellular RNA was extracted using Tri-Reagent (Sigma-Aldrich, Israel) and converted to cDNA by the High capacity cDNA Reverse Kit (Applied Biosystems, CA, USA) or the GeneAmp RNA PCR Kit (Applied Biosystems). PCR on 20 selected human chemokines was performed using the primers specified in Supplementary Table 1. For quantitative (real-time) PCR, we used SYBR Green and the ABI Prism 7500 sequence detection system (Applied Biosystems). Relative expression levels were calculated using experimentally determined primer efficiency and the ΔCT method [33]. Briefly, sample amplicon levels during the linear phase of amplification were normalized against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) control. The resulted values (ΔCT) were further calibrated to their ΔCT counterparts in the normal melanocytes, which served as a reference tissue. Final values (fold over melanocytes) are $2^{-\Delta\Delta CT}$. Assays were performed in triplicate, and the means $\pm$ SD were determined. The primers used for real-time PCR are listed in Supplementary Table 2.

Chemokine concentration levels

Chemokine concentration levels were quantified in the conditioned media of normal melanocytes and of 8 low passage primary cultures of metastatic melanoma. The chemokines CCL2, CCL4, CXCL1, CXCL8, CXCL9, and CXCL10 were detected by semiquantitative ELISA-based protein array (Multi-analyte ELISArray kit, SA Biosciences, MD, USA). Following subtraction of background (growth media), values were processed and presented as percents of the positive control (1,000 pg/ml). Noteworthy, all results were within the sensitivity limits of the kit. Quantification of another set of chemokines, CCL3, CCL5, CCL7, CCL11, and CCL22, was performed with quantitative Multi-analyte profiling based on Luminex technology (Upstate, Dundee, Scotland). Results were normalized according to the positive controls and were presented as percents of 1,000 pg/ml, which enabled unification of the results of the first and second chemokine sets.

Immunohistochemistry

Histopathological slides were warmed up to 60 °C for 1 h and further processed with a fully automated protocol. Detection was performed with View detection kit (Ventana) and counterstained with hematoxylin. After the run on the automated stainer, we dehydrated the slides in 70 % ethanol, 95 % ethanol, and 100 % ethanol for 10 s each. Before coverslipping, sections were cleared in xylene for 10 s and mounted with Entellan. Stained sections were reviewed by an expert pathologist, and suitable digital images were captured with Olympus BX51 microscope.

RNA electroporation

CXCR1 was cloned into pGEM-4EZ-64A expression vector using the primers 5′-gcTCTAGAatgtcaaatattacagatccacagatg-3′ (containing XbaI site) and 5′-taaaGCGGCCGCtcagaggttggaagagacattga-3′ (containing NotI site). For cloning of CXCR1/CCR5 chimera, CXCR1 lacking the cytoplasmic tail (aa 310–351) was obtained by PCR on CXCR1/pGEM plasmid using the primers 5′-gcTCTAGAatgtcaaatattacagatccacagatg-3′ (containing XbaI site) and 5′-gaggtagtttctgaacttctcgccgatgaaggcgtagat-3′ and the cytoplasmic tail of CCR5 was obtained by PCR on NK92 cDNA using the primers 5′-atctacgccttcatcggcgagaagttcagaaactacctc-3′ and 5′-taaaGCGGCCGCtgacaagcccacagatatttcc-3′ (containing NotI site). PCR on the two fragments using the fwd primer for CXCR1 and the rev primer for CCR5 yielded the chimeric insert, which was cloned by XbaI and NotI into pGEM. A pGEM clone containing truncated NGFR was previously described [34]. Plasmids were linearized by SpeI, RNA was transcribed in vitro using the AmpliCap-Max™ T7 High Yield Message Maker Kit (EPICENTRE Biotechnologies, WI, USA), purified using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), and eluted in RNase-free water at 1–0.5 mg/ml. Electroporation was based on previously published protocols [35, 36]. Briefly, TIL were resuspended in OptiMEM (Invitrogene) at 1×10^6 per 50 µl OptiMEM, mixed with 2 µg RNA and transferred to electroporation cuvettes (Biosmith, San Diego, CA, USA). Cells and cuvettes were prechilled by putting them on ice for <5 min before electroporation. Following a 400 mV pulse for 500 ms in the ECM830 Square Wave Electroporation System (BTX, Harvard Apparatus Inc., MA, USA), electroporated cells were transferred to fresh TIL growing media, supplemented with 6,000 IU/ml rhIL-2 (Proleukin, Chiron B.V.), and were incubated at 37 °C. NGFR and CXCR1

expression levels were determined by FACS 18-h post-electroporation.

Results

Profiling of chemokine receptors in clinical melanoma-derived infiltrating T cells

T cells are thought to reach melanoma lesions by chemotaxis [2, 6, 7]. In order to reveal which receptors participate in this process, we profiled the expression of chemokine receptors on bulk cultures of ex vivo expanded tumor-infiltrating lymphocytes (TIL) that were derived from melanoma metastases and were about to be adoptively transferred back into melanoma patients as part of the ACT immunotherapy regimen [30, 37] (for patients' clinical characteristics, see Supplementary Table 3). Five melanoma-related chemokine receptors [2, 6, 38, 39] were selected for profiling by flow cytometry: CCR4, CCR5, CCR7, CXCR1, and CXCR3. Although the expression level of these receptors varied markedly between patients (Fig. 1a), CCR5 and CXCR3 were expressed at high levels (68.82 and 95.48 % mean positive cells, respectively), CCR4 at intermediate level (20.5 %), and CCR7 and CXCR1 at low levels (1.4 and 3.64 % mean positive cells, respectively). Similar analysis on CD8+ T cells derived from peripheral blood of healthy donors has been previously published and yielded similar results, i.e., high expression of CXCR3, moderate CCR5 levels [2, 39, 40], low CCR7 expression confined to naïve cells [2, 40], and low CCR4 and CXCR1, confined to small subsets of antigen-experienced CD8+ T cells [40]. Importantly, the expression of chemokine receptors has never analyzed before on clinical samples of melanoma-derived cultured T cells, and is presented here for the first time.

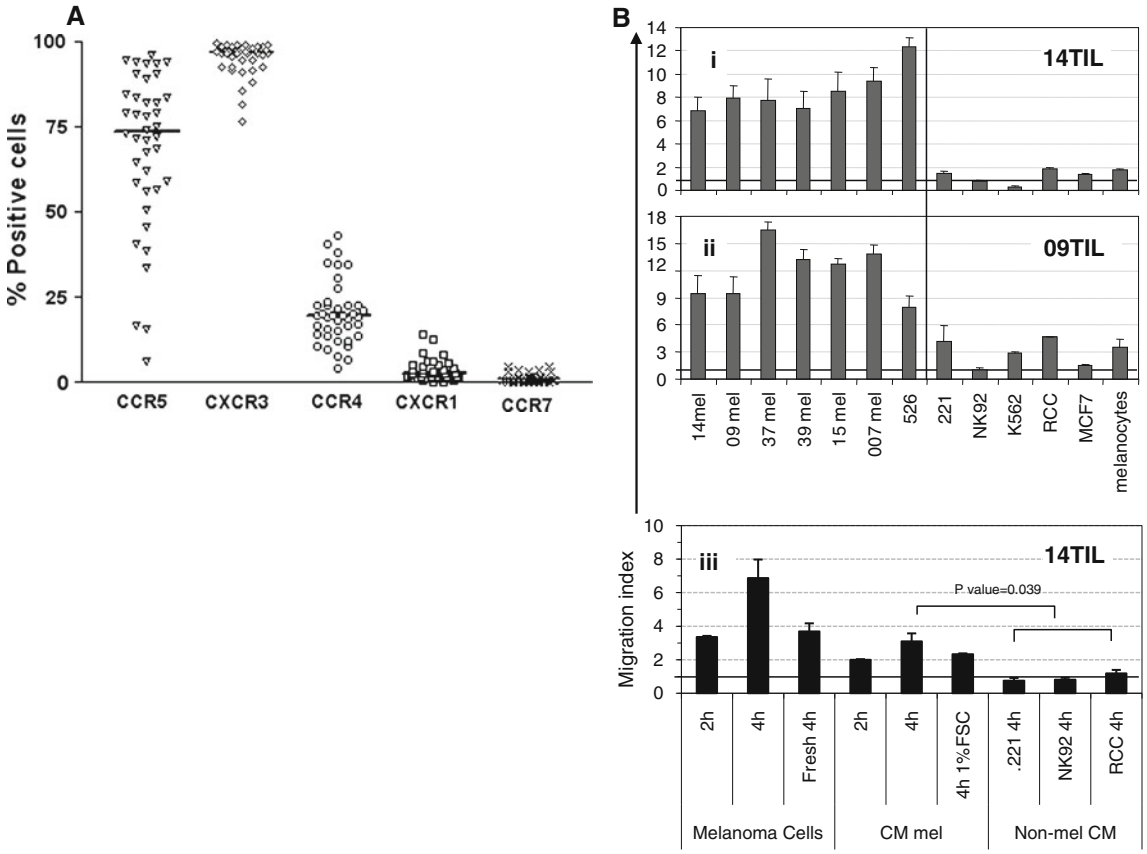

Fig. 1 Expression pattern of major chemokine receptors on clinically derived TIL cultures and preferential in vitro migration of TIL toward melanoma. The expression of 6 chemokine receptors that were previously described on lymphocytes with reference to melanoma was analyzed by FACS on 40 patient-derived clinical TIL samples that were infused to the patients. The *horizontal line* denotes the mean (**a**). The in vitro chemotaxis of TIL toward melanoma was analyzed in the transwell system (**b**): Calcein-labeled 14TIL (*i*) or 09TIL (*ii*) were allowed to migrate toward melanoma and non-melanoma cells; (*iii*) migration of 14TIL toward conditioned media (containing 10 or 1 % FCS) or cells supplemented with fresh media. 4-h migration was determined by chemotactic transwell system. Migration index was calculated as the fold of specific migration toward cells or conditioned media over the migration toward fresh media. The figure shows the average of three independent experiments

Melanoma-derived TIL cells preferentially migrate toward melanoma in vitro

In order to recapitulate T cell directional migration toward melanoma in vitro, and to check whether it is preferentially mediated by melanoma-derived chemotactic cues, we used Boyden chambers (transwells). Calcein-AM-labeled TIL were loaded onto the upper transwell chamber and allowed to migrate for 4 h toward either 7 different melanoma cultures, 5 non-melanoma tumor cultures, or normal melanocytes (Fig. 1b). Following incubation period, the amount of migrated cells was quantified by standardized fluorometry. Remarkably, all TIL cultures tested, which were derived from different patients (TIL from patient 09, patient 14 and two other TIL that are not shown), exhibited a robust migration preference toward all melanoma cultures (Fig. 1b, i–iii). The migration indices toward different melanoma samples were similar, including autologous and non-autologous as well as primary cultures and cell lines, suggesting that melanoma-derived TIL were attracted by common melanoma-derived soluble chemotactic cues (Fig. 1b, i–iii). Migration indices were 7–16.5 toward melanoma cells, as compared to 0.5–4 toward melanocytes and non-melanoma tumor cells (P value $= 3.2 \times 10^{-8}$) (Fig. 1b, i–ii). Background migration toward fresh media was 6.5 %, while specific migration toward melanoma cells was on average 31 % over background for 14TIL (average migration index = 8.5) and 40 % for 09TIL (average migration index = 11.87).

The presence of common melanoma-derived chemokines was further implied by the preferential migration of TIL cultures toward conditioned media of melanoma cells, as compared to conditioned media of other non-melanoma cells (Fig. 1b, iii). Notably, migration of 14TIL cells toward conditioned media from non-melanoma cells did not exceed background levels, while it reached migration indices of 2–3.5 in the case of conditioned media derived from melanoma cells (Fig. 1b, iii) (P value = 0.039). Similar results were obtained with 09TIL cells (data not shown). Noteworthy, the migration toward melanoma cells was higher than that toward the condition medium. Further, replacing the medium of melanoma cells with fresh medium, just before applying TIL cells onto the upper chamber, significantly reduced the TIL migration index but it was still comparable to conditioned media (Fig. 1b, iii). Migration rates toward both melanoma cells and conditioned media were time-dependent and increased with the time allowed for migration (Fig. 1b, iii). It should be emphasized that there was no serum gradient, as the media in both upper and lower wells included 10 % FCS. Nevertheless, in order to exclude the possibility that the serum present in the lower target well masks chemotactic signals, the assay was repeated with melanoma-conditioned medium that contained 1 % serum. In this case, T cells on the upper were also seeded in 1 % serum. Apparently, T cells migrated at a similar rates toward conditioned media containing 10 and 1 % FCS (Fig. 1b, iii), ensuring that neither serum gradients nor serum-driven migration were involved in the chemotaxis system. Together, these results suggest that the chemotactic receptors on TIL cells direct a specific and robust migration toward melanoma-specific chemoattractants, which are rapidly produced and secreted, as was previously shown; for example, for CXCL8 [41].

Screening of chemokines' expression in melanoma cells

To get more profound understanding of TIL chemotactic guidance, RT-PCR was used to screen 20 selected chemokines in 13 different low passage primary cultures of metastatic melanomas and 2 melanoma cell lines, normal melanocytes and a set of non-melanoma tumor cells including lymphoma, acute and chronic leukemia, prostate cancer, breast cancer, and kidney cancer cells. Interestingly, a common pattern of chemokines emerged, which was unique to melanoma and was shared by all melanoma cells tested, regardless of donor or site of metastasis (Fig. 2). Specifically, 7 chemokines (CCL2/MCP-1, CCL4/MIP-1β, CCL19/MIP-3β/ELC, CXCL1/GROα, CXCL8/IL-8, CXCL9/Mig, and CXCL12β/SDF-1) were expressed by at least 80 % of melanoma cultures, while the others were either not expressed or were shared by 50 % or less melanomas (Fig. 2). Primary melanocytes showed a similar pattern, expressing CCL2, CCL4, CCL7, CCL19, CCL27, CXCL1, CXCL9, and CXCL12β (not shown).

Real-time PCR analysis performed on the same set of samples was further used to quantify the results. GAPDH expression level served as an internal control and normal melanocytes as a reference value for all samples. We found that CXCL1 and CXCL8 were clearly overexpressed by nearly all melanoma cultures, while they were not detected or even down-regulated in non-melanoma samples (Fig. 3a, b). The expression of CXCL9 was below detection threshold in melanocytes (not shown) and was therefore normalized to GAPDH only. CXCL9 was weakly expressed in most melanoma cultures as well as in some non-melanoma cell samples (Fig. 3c). CCL4 was overexpressed in melanoma; however, an additional, non-relevant, product produced in melanocytes prevented reliable quantification of the results (not shown). There were no apparent differences in CCL19 expression between melanoma cells and melanocytes (not shown). Finally, the expression of CCL2 and CXCL12β was down-regulated or shutdown in the great majority of melanoma samples (Fig. 3d, e). To conclude, CXCL1, CXCL8, CXCL9, and CCL4 were up-regulated in melanoma relative to normal

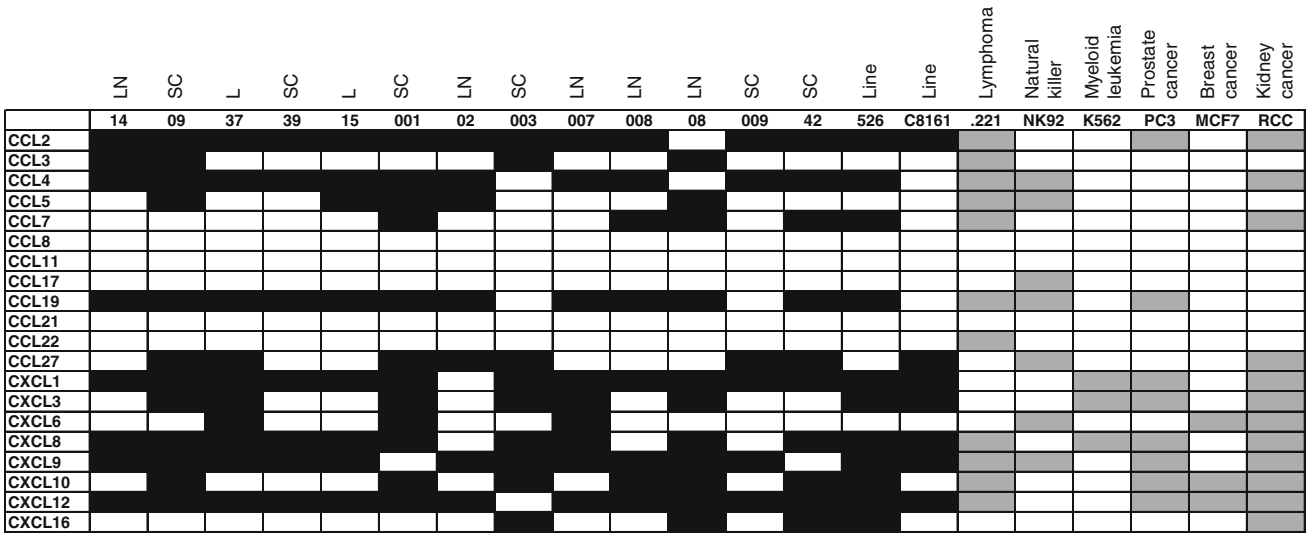

Fig. 2 Screening of chemokine expression profile in melanoma, melanocytes, and non-melanoma cancer cells. The expression of 20 selected chemokines was examined in 13 primary cultured metastatic melanoma cells, 2 melanoma cell lines, normal melanocytes, and various non-melanoma cancer cell lines by RT-PCR. *Empty squares* indicate no expression and *black* or *gray squares* indicate expression in melanoma and non-melanoma cells, respectively. *LN* lymph node, *SC* subcutaneous, *L* lung, respectively (sites of the metastases)

melanocytes and constituted a chemokines set unique to melanoma samples.

CXCL1 and CXCL8 are the major chemokines secreted from melanoma cells

Normal melanocytes and 8 primary melanoma cultures were subjected to chemokine analysis at the protein level. Protein analysis included the chemokines of interest: CCL4, CXCL1, CXCL8, and CXCL9; other chemokines that were previously reported to play a role in melanoma chemoattraction [2]: CCL2, CCL3, CCL5, and CXCL10; and negative controls: CCL7, CCL11, and CCL22. In accordance with the RNA expression, findings (Fig. 3a–e), CXCL1 and CXCL8, were found in remarkable levels in the conditioned media of the vast majority of melanoma cultures (Fig. 3f), while melanocytes secreted no CXCL1 and very low concentration of CXCL8. CXCL9 was not secreted from melanoma, and CCL4 was detected at very low levels in only some of the melanoma samples. CCL2 was secreted at low to moderate levels from most melanoma cultures, but it was secreted from melanocytes at even higher levels than from most melanoma samples. CXCL10 was secreted in intermediate levels, but only by few of the melanoma samples. CCL3, CCL5, and the negative controls CCL7, CCL11, and CCL22 were either not secreted or secreted at low levels by only some of the melanoma cells (Fig. 3f). Immunostaining of paraffin-embedded sections from metastatic melanoma patients also demonstrated that CXCL1 and CXCL8 are abundantly expressed by melanoma cells (Sup. Fig. 1A). Combining the RNA expression and protein expression and secretion data, CXCL1 and CXCL8 are the major common chemokines secreted from melanoma cells and their high expression level is shared across melanoma cultures, regardless of donor patient or metastasis site. These results present a most interesting situation when compared to the receptors data depicted in Fig. 1a, showing that TIL express low CXCR1 and no CXCR2. Moreover, melanoma-infiltrating CD8-positive T cells were mostly negative for membranous CXCR1 and CXCR2 in immunostaining of histopathological sections of melanoma patients (Sup. Fig. 1B, C), and the low extracellular expression of the receptors was maintained over culturing time (Sup. Table 4). These receptors are the corresponding CXCL1 and CXCL8 receptors [25, 42, 43], suggesting that they do not play a dominant physiological role in the recruitment of TIL cells to tumor sites. However, they point on the potential relevance of manipulating T cells to express these receptors in order to enhance their migration toward melanoma.

Lymphocytes that migrate toward melanoma are enriched in functional CXCR1-positive cells

In order to test whether this concept has a physiological basis, we assayed the functional involvement of CXCR1 and CXCR2 in chemotaxis of TIL cells toward melanoma in vitro, using the transwell system depicted in Fig. 1b. The expression of the two chemokine receptors was monitored by flow cytometry in melanoma cells (Fig. 4a) and in three TIL subpopulations (Fig. 4b): TIL that migrated toward

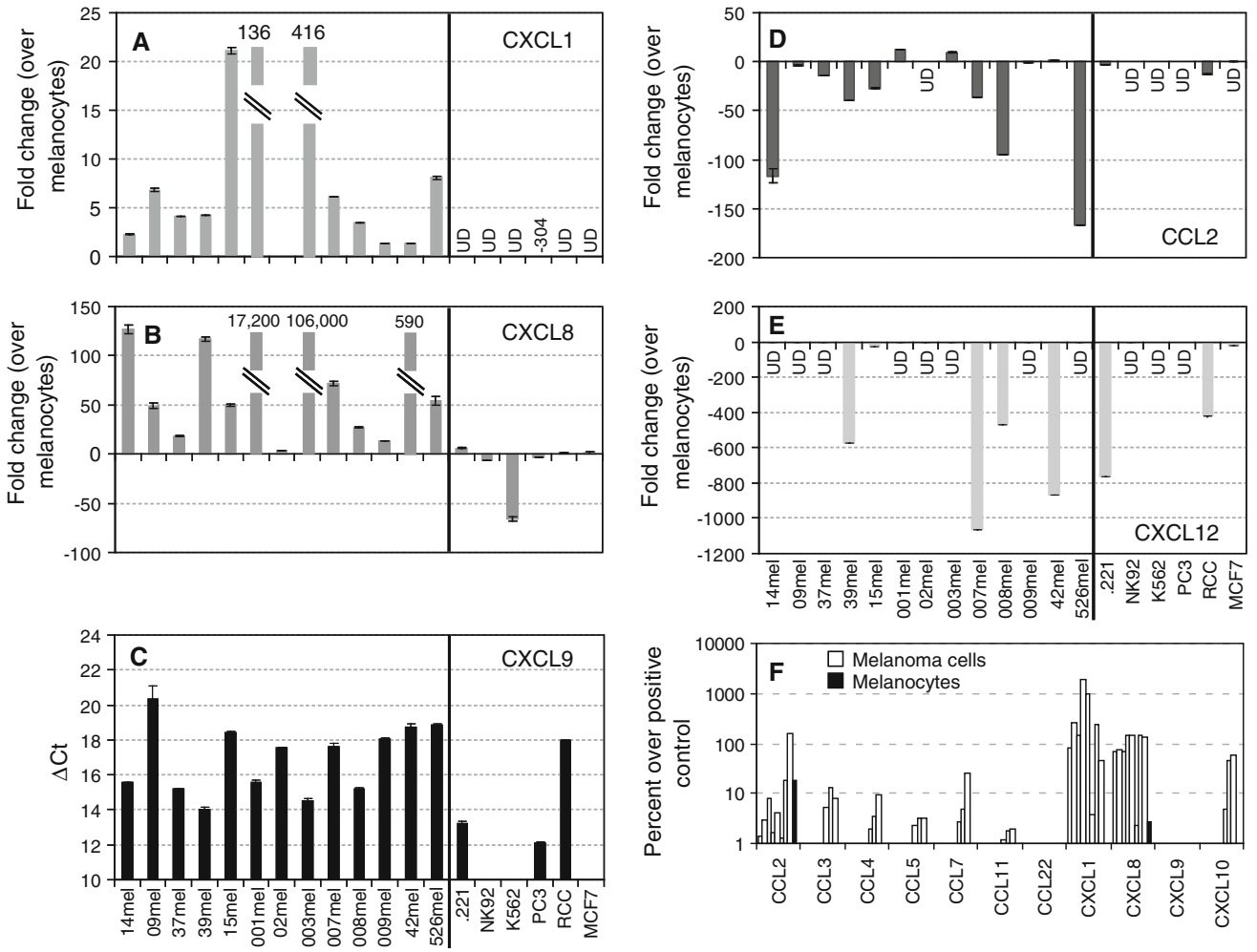

Fig. 3 Quantitative chemokine production in melanoma and non-melanoma cells. Five chemokines that were expressed by at least 80 % of melanoma samples in RT-PCR were analyzed by real-time PCR (**a**–**e**). GAPDH served as an internal control. Expression in normal melanocytes served as reference tissue for CXCL1, CXCL8, CCL2, and CXCL12. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ equation. CXCL9 was not expressed in normal melanocytes, and its expression is therefore presented as ΔCt; (**f**) secretion of selected chemokines at the protein level was tested by semiquantitative ELISA-based protein array in the conditioned media of 8 melanoma cultures (*white*) and normal melanocytes (*black*)

melanoma-conditioned medium and resided in the lower transwell well (LW) following 4-h migration, TIL that did not migrate and were left at the upper transwell system (UW) and in the total TIL population (TIL that were not exposed to transwells; unselected, US). Extracellular FACS staining revealed that 003mel (Fig. 4a) and 14mel (not shown) melanoma cells express low levels of surface CXCR1 and no CXCR2. This suggests that CXCL1 and CXCL8 are not acting in an autocrine manner on lower well melanoma cells in the transwell system. Intracellular (total) staining proved that both receptors are expressed by melanoma cells in a moderate (CXCR2) to high (CXCR1) levels. Examining 14TIL cells, we found a remarkable differential expression of the chemokine receptors between TIL subpopulations (Fig. 4b). While in TIL–US and TIL–UW about 10 % of the cells were positive for CXCR1 extracellular staining, a remarkable enrichment in cell surface CXCR1 was exhibited by TIL–LW cells, reaching fivefold–ninefold increase in positive CXCR1 cells in comparison with TIL–US and TIL–UW cells (Fig. 4b). CXCR1 was shown before to reside mainly in intracellular pools in T cells [44, 45]. Indeed, total (intracellular) staining showed that CXCR1 is expressed at a similar extent (~80 %) in all TIL subpopulations. CXCR2 staining yielded a similar but moderate trend of results, with almost no extracellular staining in TIL–US and TIL–UW cells and 22 % positive cells for membrane CXCR2 in TIL–LW cells. Intracellular CXCR2 staining proved very low CXCR2 expression in all TIL subpopulations (Fig. 4b). Similar results were obtained also with 09TIL

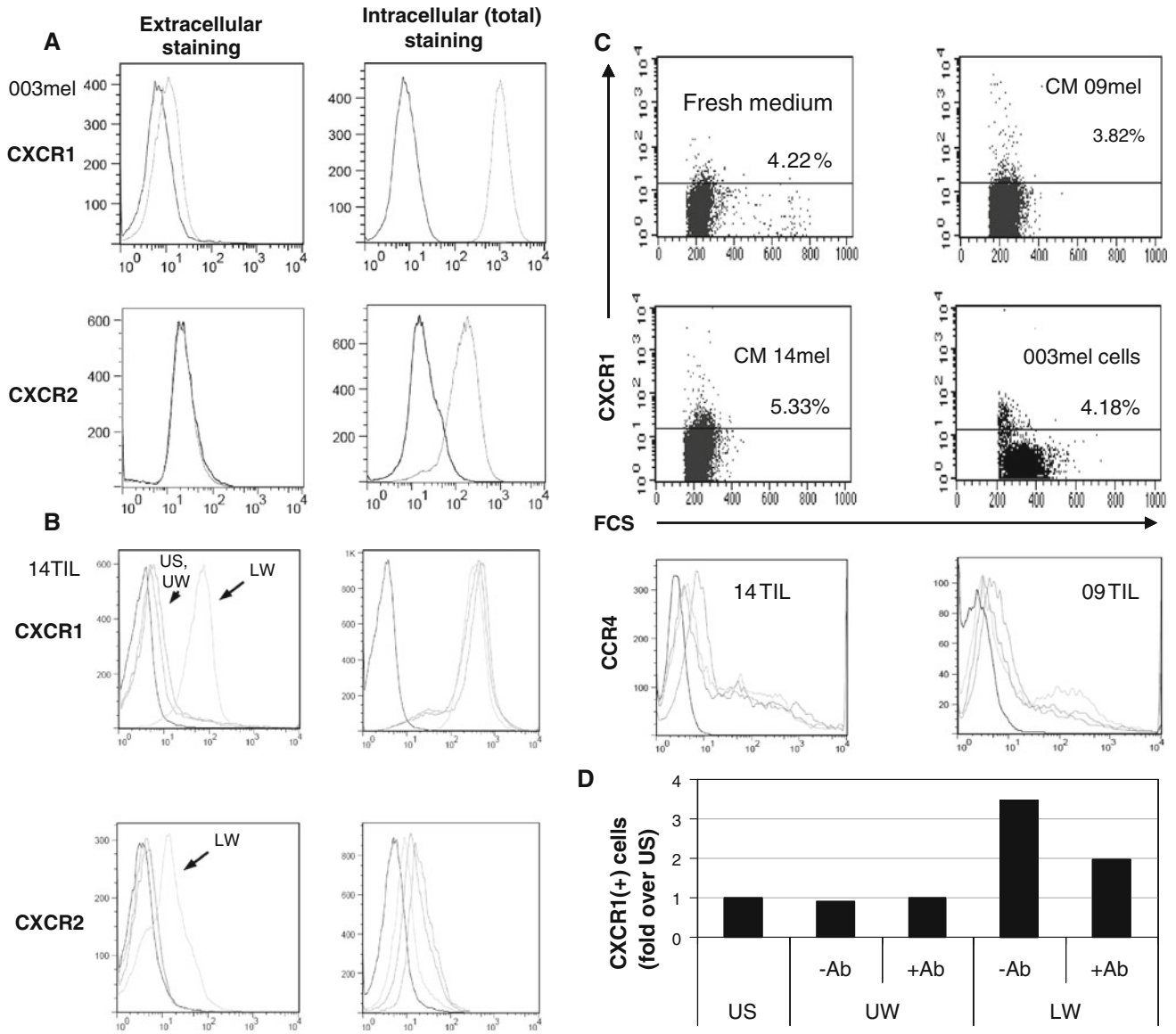

Fig. 4 CXCR1 involvement in TIL migration toward melanoma. The expression of CXCR1 and CXCR2 was measured by extracellular and total (intracellular) FACS staining in 003mel (**a**) and in 14TIL cells subpopulations obtained following 4-h migration toward melanoma-conditioned medium in the transwell system (**b**). In each of the histograms in A and B, the isotype control staining appears in *black*; **c** CXCR1 staining of 14TIL cells after 4 h co-incubation with conditioned media of 09mel or 14mel or with 003mel cells (*upper 2 panels*). In the experimental setup of migration toward melanoma cells, melanoma and TIL cells residing together in LW were clearly discriminated by the differential expression of MCSP and CD8, respectively (Sup. Fig. 2); CCR4 staining of 14TIL and 09TIL subpopulations following migration toward melanoma-conditioned medium. Isotype control staining appears in *black* (*lower panel*). The figure shows a representative experiment out of 3 performed. *LW* lower well, *UW* upper well, *US* unselected, respectively. **d** Reduction in the enrichment of CXCR1-positive cells in the lower well following addition of anti-CXCL1 and -CXCL8 blocking antibodies. *Y*-axis denotes the fold increase over control (US TIL) in the percentage of CXCR1-positive cells of each subpopulations, as measured by flow cytometry

(not shown) and when TIL migration was tested toward melanoma cells instead of conditioned media (not shown).

Recently, it was shown that melanoma-secreted IL6 induces the expression of CXCR1 on regulatory CD4-positive cells [46]. In our experimental system, TIL cultures were comprised almost entirely of CD8+ cells and did not contain regulatory T cells (data not shown). However, incubation of 14TIL–US with melanoma-conditioned media or with melanoma cells for 4 h did not alter their CXCR1 cell surface level (Fig. 4c, upper panels and Sup. Fig. 2). Moreover, the enrichment of CXCR1 and CXCR2 among migrating TIL cells was unique, as CCR4, which is

moderately expressed in TIL (Fig. 1a), was not enriched in 09TIL–LW and 14TIL–LW cells (Fig. 4c, lower panel). The confined expression of CXCR1 and CXCR2 in TIL–LW cells suggests that they respond to the flux of CXCL8 and CXCL1 secreted from melanoma and are able to direct the migration of $CXCR1^{high}$ and $CXCR2^{high}$ TIL toward melanoma in the lower well.

For further functional studies, we focused on CXCR1 due to its significantly stronger expression in TIL–LW as compared to CXCR2 (Fig. 4b). CXCR1 is the receptor for CXCL8, though several articles pointed on its ability to bind also CXCL1 at low affinity [42, 43]. To test the effect of CXCR1 on 14TIL cells, blocking anti-CXCL1 and anti-CXCL8 antibodies were added to the conditioned media of 14mel cells. As shown (Fig. 4b), the great majority of TIL do not express CXCR1 and CXCR2 and thus were not expected to be affected by neutralizing CXCL1 and CXCL8. Indeed, the addition of blocking antibodies did not significantly affect the total TIL migration (not shown). However, it significantly reduced the amount of CXCR1-positive cells among 14TIL-LW by about twofold (Fig. 4d), while not affecting the amount of CXCR1-positive cells in the UW (Fig. 4d).

Enhanced migration of T cells overexpressing CXCR1 toward melanoma cells

The exclusive enrichment of functional CXCR1-expressing cells among migrating TIL cells suggests that CXCR1, and perhaps also CXCR2, mediates the migration of a subset of TIL toward melanoma. Unfortunately, the enrichment in CXCR1 among 14TIL-LW and 09TIL-LW was transient and dropped to low levels following a few days of cultivation (not shown), preventing subsequent exploration of this unique subpopulation or implementation of this strategy for the establishment of CXCR1-positive T cells for adoptive cell transfer therapy. Attempts to expand other TIL specimens, which showed 20 % CXCR1 expression before expansion (Fig. 1a), similarly resulted in loss of CXCR1 expression after a few culturing days (data not shown). In order to validate whether CXCR1 and CXCR2 expression enhances the migratory capabilities of T cells, we ectopically overexpressed CXCR1 and CXCR2 by RNA electroporation [35, 36]. Electroporation with truncated NGFR, incapable of signaling, or with GFP, served as negative controls [34]. The great majority of electroporated 09TIL–US and 14TIL–US were viable, and NGFR and GFP were highly overexpressed in the electroporated TIL09 cells. Disappointingly, CXCR1 was mildly overexpressed and CXCR2 was very poorly overexpressed on the cell surface of the electroporated TIL09 cells (Fig. 5-a).CXCR1, NGFR, and GFP expression levels peaked at 18-h post-electroporation and decreased afterward. CXCR1 expression reached basal levels at 72-h post-electroporation while NGFR and GFP were still detectable 96-h post-electroporation (not shown). Using this method, we could not reach high expression levels of CXCR1 or CXCR2, similar to the endogenous CXCR1 expression level in TIL–LW cells (Fig. 4b as compared with Fig. 5a). Importantly, CXCR1-electroporated TIL still exhibited a reproducible and statistically significant 23 % increase in the migration index over NGFR-electroporated TIL cells, toward melanoma-conditioned media (Fig. 5b). Similarly, CXCR1-electroporated TIL exhibited a superior migration toward recombinant CXCL8 by 31 % over NGFR-electroporated TIL (Fig. 5b). These results indicate that TIL migration toward melanoma can be improved by the expression of CXCR1. The relatively low enhancement in the migration activity is probably due to the mild ectopic CXCR1 expression.

In order to check whether the low surface expression of CXCR1 and CXCR2 results from a defect in their transport machinery to the plasma membrane, non-electroporated T cells were incubated with PHA and IL6, which were previously shown to up-regulate cell surface CXCR1 in cultured T cells [38, 44, 45]. Incubation with PHA yielded a time-dependent increase in surface CXCR1, while not affecting its total (intracellular) expression, suggesting that the transport machinery of CXCR1 was intact (Fig. 5c). The up-regulation of CXCR2 to the cell surface was lower and slower than that of CXCR1 (Fig. 5c). IL6 did not up-regulate surface CXCR1 and CXCR2 (Fig. 5c).

CCR5 is highly expressed by TIL cultures (Fig. 1a), and its cytoplasmic tail was shown to drive membrane targeting [47]. We cloned a chimeric CXCR1 construct, in which the cytoplasmic tail of CXCR1 (aa 310–351) was replaced by that of CCR5 (aa 302–353). The CXCR1/CCR5 chimera was transfected into 09TIL by RNA electroporation. Flow cytometry demonstrated a mild overexpression of the chimera, which was similar to results obtained with transfection of the wild-type CXCR1 (Fig. 5d). In contrast, extracellular FACS staining revealed that surface CXCR1 expression did not increase following transfection with the chimera, as opposed to the reproducible mild increase following transfection with the wild-type CXCR1 (Fig. 5d). This indicates that the cytoplasmic tail plays a crucial role in targeting CXCR1 to the surface membrane.

Migrating and non-migrating TIL cells exhibit similar effector functions

It was previously shown that high CXCR1 expression is confined to a small subset of T cells with a potent effector phenotype, including elevated perforin, granzyme B, and IFNγ expression levels, which exerts an increased cytotoxic activity [16, 40]. Accordingly, migrating (LW) and

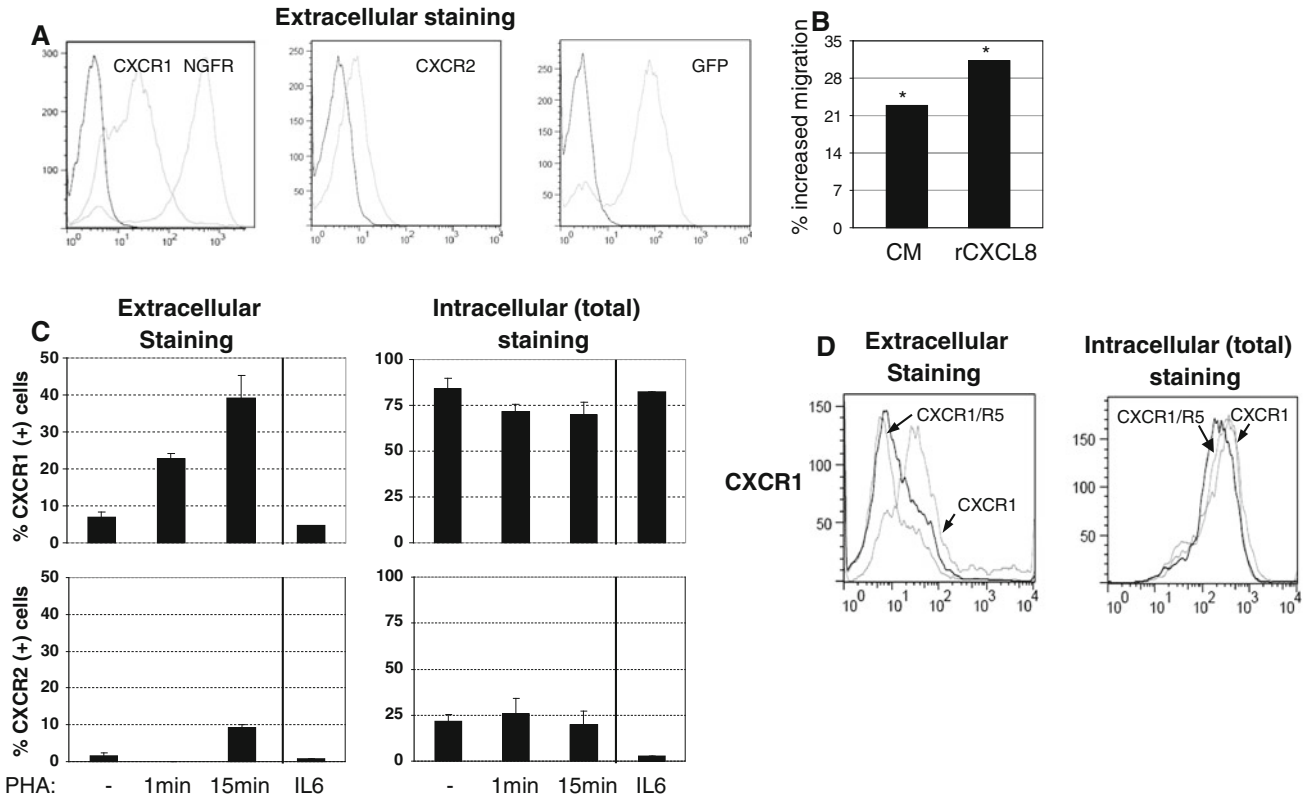

Fig. 5 Overexpression of CXCR1 enhances TIL migration toward melanoma. **a** 09TIL cells were electroporated with CXCR1, truncated NGFR, CXCR2, or GFP RNA, and stained by FACS for extracellular expression of the corresponding proteins. Isotype controls appear in *black*; **b** the effect of overexpressed CXCR1 was measured in the transwell system in electroporated 09TIL, which was allowed to migrate toward melanoma-conditioned medium. Migration index was calculated as the fold of specific migration toward conditioned medium or recombinant CXCL8 over the migration toward fresh complete medium. The figure (*right*) shows the additional migration indices obtained when the indices for NGFR-electroporated cells were subtracted from those obtained for CXCR1 cells. The figure shows a representative experiment out of 3 performed. *Asterisk* denotes *P* value <0.05. *LW* lower well, *UW* upper well, *US* unselected, respectively. **c** 14TIL cells were incubated with 20 μg/ml PHA for 1 or 15 min, with 200 ng/ml IL6 for 3 h, or with PBS, as control. Figure shows extracellular and total (intracellular) FACS staining of CXCR1 and CXCR2. Results are the average of 3 experiments. **d** 09TIL were either non-electroporated (*black histogram*), or electroporated with wild-type CXCR1 (as in **a**) or with chimeric CXCR1/CCR5. 24 h following electroporation, TIL were subjected to FACS staining using anti-CXCR1 antibodies. Endogenous CXCR1 staining serves as background (*black*)

non-migrating (UW and US) 14TIL were tested for their anti-melanoma reactivity. We performed an in vitro cytotoxic assay, in which the different 14TIL subpopulations were co-cultured together with CFSE-labeled HLA-A2-matched target 526mel cells at an effector-to-target ratio of 10:1, and the percentages of killed melanoma cells were calculated by flow cytometry. This assay demonstrated that all 14TIL subpopulations exhibited similar killing potentials (Fig. 6a), indicating that migration per se does not impair TIL cells function, nor select for TIL cells with a reduced effector potential. We next analyzed Perforin expression. As it is not expressed by 14TIL, Perforin was tested in 09TIL subpopulations by intracellular FACS staining. As expected, LW-09TIL cells were enriched in CXCR1 (Fig. 6b). However, the Perforin content was actually higher in total UW- than in the total LW-09TIL (57 vs. 36 % perforin-positive cells in UW and LW, respectively). The percentage of $CXCR1^{+}Perforin^{+}$cells was similar in UW and LW TIL (about 6 % double-positive cells) (Fig. 6b). Finally, IFNγ release by US, UW, and LW 14TIL was measured by ELISA following co-incubation with target melanoma cells and found to be similar between the different TIL subpopulations (Fig. 6c). To conclude, although CXCR1-positive TIL cells do not exhibit an enhanced effector phenotype as previously reported for cytotoxic T cells against CMV [16], they retain their functional activity against melanoma cells. Therefore, it could be safely speculated that CXCR1-mediated TIL migration toward melanoma does not reflect an immune-manipulation mechanism of the melanoma cells, which attract inert or suppressive lymphocytes. To conclude, we show that CXCR1 on T cells is functional, correlates with enhanced migration and does not interfere with cytotoxic potential, and that ectopic CXCR1

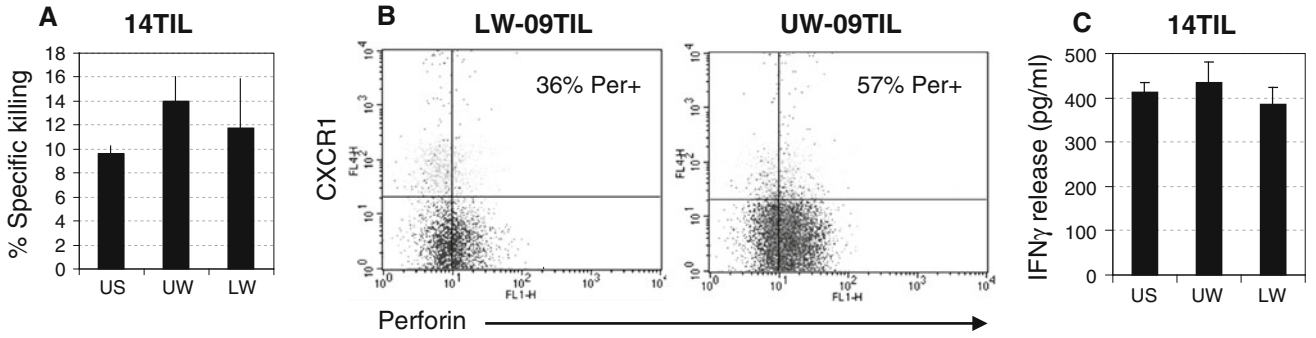

Fig. 6 Retention of effector functions by migrating TIL. **a** Killing of CFSE-labeled 526mel by 14TIL subpopulations as measured by FACS; **b** triple staining of 09TIL cells co-incubated with melanoma cells in the presence of monensin with anti-perforin, anti-CD8, and anti-CXCR1 mAbs. The figure shows staining results of gated CD8-positive lymphocytes; **c** IFNγ release by 14TIL subpopulations following co-incubation with 526mel cells. Figure shows a representative experiment out of 3 performed. *LW* lower well, *UW* upper well, *US* unselected, respectively

overexpression renders T cells with improved chemotaxis toward melanoma. We therefore suggest that manipulating T cells to express CXCR1 will enhance their directional migration toward lesion sites and may be beneficial for metastatic melanoma patients receiving adoptive T cell transfer.

Discussion

Adoptive cell transfer (ACT) of reactive anti-tumor T cells is a promising immunotherapy for metastatic melanoma patients [10–12, 48, 49]. One of the major obstacles of this regimen, however, is the insufficient homing of T lymphocytes to tumor sites [2, 21, 25–28]. In this work, we sought to identify guiding signals which are secreted from melanoma and to exploit them for re-directing adoptively transferred T cells. We started by profiling chemokine receptors/ligands in the melanoma/T cells scenario. The screening of chemokine receptors on ex vivo cultured T cells that comprised the clinical infusions that were actually administered to melanoma patients was done here for the first time and proved very low expression of CXCR1 (Fig. 1a). This was somewhat contradictory to the finding that CXCL1 and CXCL8 are the major melanoma-secreted chemokines, and that they are expressed and secreted at exclusively high levels from nearly all melanoma samples tested, regardless of donor or metastases site (Figs. 2, 3). Noteworthy, other chemokines were also secreted at various levels from different melanoma cultures (Fig. 3f), consistent with a recently published chemokine profiling in melanoma cultures [2] and with the idea that in different melanoma patients, T cell chemotaxis is mediated by different combinations of chemokines and their receptors [6]. Indeed, the chemokine system is characterized by redundancy and overlapping signaling, and the cellular expression profile of chemokine receptors is influenced by microenvironmental factors (i.e., chemokine concentration, inflammatory cytokines, and hypoxia) and thus may vary among individuals [3, 4].

CXCL1 and CXCL8 are potent cytokines, acting to enhance melanoma cell proliferation and angiogenesis [50, 51] and to attract immune cells, such as neutrophils, monocytes, NK cells, and eosinophils [52–56]. The finding that T cells express low levels of CXCR1 (Figs. 1a, 4b) [40] and no CXCR2 on their cell surface (Fig. 4b) [39, 40, 57] and thus lack the potential to respond to the CXCL1/8 flux, posed an intriguing situation. We proceeded by both analyzing the endogenous and relatively rare high CXCR1 and CXCR2 expressing T cells and by overexpressing these chemokine receptors in the general T cells population (Figs. 4, 5). Endogenous CXCR1- and CXCR2-positive cells were characterized in the chemotactic context using the in vitro transwell system (Figs. 1b, 4). We found that melanoma-infiltrating TIL cells preferentially migrate toward melanoma cells and melanoma-derived conditioned media, as compared with melanocytes or non-melanoma tumor cells (Fig. 1b). Further, although CXCR1 expression in the general TIL population was low (Fig. 1a), it was enriched by fivefold–ninefold among TIL cells that migrated toward melanoma-derived conditioned media (Fig. 4b). CXCR2 was also enriched in migrating TIL, but at lesser extent. These enrichments were specific, as the expression of CCR4 was similar among migrated and non-migrated TIL (Fig. 4c). Moreover, they were mediated in a CXCL1- and CXCL8-dependent manner, as the addition of anti-CXCL1 and anti-CXCL8 blocking antibodies reduced the amount of CXCR1-positive cells among migrating TIL cells (Fig. 4d). Characterization of the anti-melanoma reactivity of migrated versus non-migrated TIL cells (Fig. 6) showed that migrated, CXCR1-positive cells are not impaired in their cytotoxic potential.

Unfortunately, the expression of CXCR1 on migrating TIL was transient in all CXCR1-positive TIL and did not

persist in subsequent culturing, due to yet to be defined reasons. This precluded further development of a selection strategy for T cells with enhanced migration capacity, which is based on fast migration toward melanoma cells in vitro, but set the grounds for the manipulation of T cells to overexpress CXCR1. A proof of concept was demonstrated by RNA electroporation. Overexpression of CXCR1 as compared with control receptor incapable of signaling resulted in a moderate though reproducible and statistically significant 23 % enhancement in the migration of TIL cells toward melanoma-conditioned media (Fig. 5a). The moderate increase in migration is probably due to the modest overexpression of CXCR1, which was significantly lower than the endogenous CXCR1 expression in CXCR1^{high} T cells (Fig. 4b). The low endogenous and overexpressed CXCR1 at the cell surface probably did not result from impaired intracellular trafficking, as incubation with PHA shifted CXCR1 from intracellular pools (Fig. 4b) to the cell membrane (Fig. 5c). Attempts to force CXCR1 expression at the cell surface by replacing its cytoplasmic tail with that of CCR5 failed and resulted in a reduction in surface expression as compared with naïve CXCR1 overexpression (Fig. 5d). This suggests that the cytoplasmic tail of CXCR1 is involved in its surface membrane targeting, which may operate via a different pathway than CCR5. Collectively (Figs. 4, 5), our results show that CXCR1 is functional and mediates a part of T cell chemotaxis toward melanoma. They suggest that genetic engineering of human T cells, such as by viral transduction of CXCR1, may substantially enhance the migration of T cells toward melanoma. The advantage of focusing on CXCR1 stems from the strong expression of the CXCR1 ligands, CXCL1, and CXCL8, from all melanoma cultures. CXCR1 engineering of T cells would enable enhancement of specific migration of the adoptively transferred cells toward melanoma cells in virtually all patients. Recently, Peng and his colleagues have shown that transduction of murine T cells with CXCR2 enhanced their directional migration and augmented antitumor responses. In their pioneering work, pmel-1 transgenic T cells, which recognize the melanoma antigen gp100, were virally transduced with the murine CXCR2 protein. Transduced lymphocytes were then adoptively transferred to mice bearing gp100-expressing and CXCL1-secreting tumors (B16 melanoma and MC38 colon adenocarcinoma) and were shown to preferentially accumulate in tumor sites and to enhance tumor regression and survival in these mice models [57].

Genetic engineering of human T cells to enhance antitumor cell-based therapy is still pioneering. Few clinical trials have already been performed with genetically modified transferred T lymphocytes, which focused mainly on endowing T cells with an improved specificity and/or avidity to cancer cells by engineering of the T cell receptor [22–25, 58]. Although clinical results yielded relatively low response rates, they proved the feasibility and the exciting opportunities of this novel immunotherapeutic direction. Importantly, the future implementations of our results are expected to be synergistic with the current efforts and focus on TCR engineering. Potentially, future engineering of T cells for adoptive transfer could involve multi-cistronic constructs that will include enhanced specific migration toward melanoma cells along with improved recognition and killing functions.

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Conflict of interest The authors declare that they have no conflict of interest.

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