

# Melanoma cell lysate induces CCR7 expression and *in vivo* migration to draining lymph nodes of therapeutic human dendritic cells

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## Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that upon encounter with both pathogens and tumours efficiently trigger an adaptive immune response.<sup>1,2</sup> This is mainly due to their ability to capture and process antigens, as well as to respond upon

## Summary

We have previously reported a novel method for the production of tumour-antigen-presenting cells (referred to as TAPCells) that are currently being used in cancer therapy, using an allogeneic melanoma-derived cell lysate (referred to as TRIMEL) as an antigen provider and activation factor. It was recently demonstrated that TAPCell-based immunotherapy induces T-cell-mediated immune responses resulting in improved long-term survival of stage IV melanoma patients. Clinically, dendritic cell (DC) migration from injected sites to lymph nodes is an important requirement for an effective anti-tumour immunization. This mobilization of DCs is mainly driven by the C-C chemokine receptor type 7 (CCR7), which is up-regulated on mature DCs. Using flow cytometry and immunohistochemistry, we investigated if TRIMEL was capable of inducing the expression of the CCR7 on TAPCells and enhancing their migration *in vitro*, as well as their *in vivo* relocation to lymph nodes in an ectopic xenograft animal model. Our results confirmed that TRIMEL induces a phenotypic maturation and increases the expression of surface CCR7 on melanoma patient-derived DCs, and also on the monocytic/macrophage cell line THP-1. Moreover, *in vitro* assays showed that TRIMEL-stimulated DCs and THP-1 cells were capable of migrating specifically in the presence of the CCR7 ligand CCL19. Finally, we demonstrated that TAPCells could migrate *in vivo* from the injection site into the draining lymph nodes. This work contributes to an increased understanding of the biology of DCs produced *ex vivo* allowing the design of new strategies for effective DC-based vaccines for treating aggressive melanomas.

**Keywords:** CCR7; dendritic cells; immunotherapy; melanoma; migration; tumour lysates.

encounter with pathogen- and self-derived danger signals in peripheral tissues. DCs can migrate to draining secondary lymphoid organs where they can engage, in a proper cytokine context, antigen-specific naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, triggering their activation, proliferation and transference to peripheral tissues to perform effector functions.<sup>3,4</sup> In addition, DCs must express large amounts

Abbreviations: AM, activated monocytes; CCL19, C-C chemokine ligand 19; CCR7, C-C chemokine receptor type 7; DC, dendritic cell; MM, malignant melanoma; RT-qPCR, real-time quantitative polymerase chain reaction; TAPCells, tumour antigen-presenting cells; TRIMEL, melanoma-derived cell lysate

of co-stimulatory molecules, which provide crucial signals that ensure the effectiveness of the response mediated by T cells.<sup>5–9</sup>

During the maturation process, the phenotype of DCs undergoes several changes such as the up-regulation of co-stimulatory molecules, including CD40, CD80 and CD86, CD83 (a well known DC activation marker), the MHC class I and II molecules, and the C-C chemokine receptor type 7 (CCR7). Notably, CCR7 is recognized as a critical receptor for DCs migration into draining lymph nodes,<sup>10,11</sup> a process that is facilitated by CCL19 and CCL21, chemokines that are expressed by the stromal cells close to the T-cell area of lymph nodes.<sup>12–15</sup> The importance of CCR7 in DC migration and T-cell activation has been demonstrated in CCR7-deficient knockout (KO) mice, where lymph nodes are almost completely depleted of naive T cells and DCs and by contrast, the T-cell population is augmented in blood, spleen and bone marrow.<sup>16,17</sup> Moreover, these KO mice also have altered antibody levels and diminished delayed hypersensitivity responses.<sup>16,17</sup> In the same context, CCL19 KO mice showed that this chemokine is essential for DC homing to lymph nodes,<sup>13,18</sup> and in corresponding transgenic mice, the ectopic expression of CCL19 resulted in the retention of DCs in these tissues and the prevention of their migration to secondary lymphoid organs.<sup>19</sup>

It has also been shown that the development of a mature DC phenotype, capable of leading to T helper type 1, T helper type 2 or tolerogenic responses, is influenced by different stimuli, which elicit the maturation to qualitatively different states, suggesting that depending on the nature of the stimulus, DCs interpret signals from the microenvironment through pattern recognition receptors, for example Toll-like receptors.<sup>20</sup>

We have previously demonstrated that immunization with human tumour antigen-presenting cells (TAPCells), matured with the pro-inflammatory cytokine tumour necrosis factor- $\alpha$  and a melanoma cell lysate, referred to as TRIMEL, showed effectiveness in improving long-term survival in vaccinated patients with advanced malignant melanoma (MM).<sup>9,21</sup> Moreover, it was demonstrated that TRIMEL by itself can rapidly induce a mature and committed DC phenotype from activated monocytes (AMs), even in the absence of pro-inflammatory cytokines.<sup>22,23</sup> Furthermore, the presence of damage-associated molecular patterns, as derived from stressing the human metastatic melanoma cell lines constituting TRIMEL with heat-shock, is responsible for an efficient antigen cross-presentation by TAPCells.<sup>23</sup> However, the migration ability of TAPCells to draining lymph nodes, a relevant prerequisite for its clinical efficacy, remains to be studied.

To investigate whether patient-derived TAPCells are able to migrate to draining lymph nodes in an *in vivo* system, we established a xenograft ectopic animal model using immunodeficient or natural killer (NK) -depleted

immunocompetent mice. We also tested if TRIMEL was involved in the increased expression of surface CCR7 receptors during the differentiation and maturation of TAPCells from the monocytes of MM patients. Furthermore, it was important to test the lysate effect in a stable cell line model, such as the monocyte/macrophage THP-1, because monocytes derived from patients can show genotypic variations that could eventually affect the clinical outcome of treated patients.<sup>24</sup>

Using *in vitro* assays, we showed that TAPCells and TRIMEL-stimulated THP-1 cells were capable of specifically migrating in the presence of the canonical CCR7 ligand, CCL19. Finally, we demonstrated by flow cytometry and immunohistochemistry that TAPCells are able to migrate *in vivo* from the injection site into draining lymph nodes. This work contributes to a further understanding of the effect of tumour cell lysates on APCs generated *ex vivo* and helps in the design of new effective strategies for DC-based vaccine therapies for MMs.

## Materials and methods

### Patients

Peripheral blood mononuclear cells were obtained by leukapheresis from four advanced (stage IV) MM patients (codes MT-123, MT-197, MT-198 and MT-199), who were treated using a previously reported autologous TAP-Cell vaccination protocol.<sup>21,23</sup> Part of the peripheral blood mononuclear cells was then used for TAPCell generation for *in vitro* and *in vivo* assays. The present study was performed in agreement with the Helsinki Declaration and approved by the Bioethical Committee for Human Research of the Faculty of Medicine, University of Chile. All patients signed informed consent forms for the planned experiments.

### Mice strains

Six- to 8-week-old male C57BL/6J (C57) and NOD.Cg-*Prkdc<sup>scid</sup> IL2rg<sup>tmlWjl</sup>/Sz* (NOD-SCID) mice obtained from the high-security laboratory animal facility at the Institute of Biomedical Sciences of the Faculty of Medicine, University of Chile were used in this study. Mice were maintained in the animal facility under specific pathogen-free conditions and received sterilized food and water *ad libitum*. The Bioethical Committee for Animal Experimentation of the Faculty of Medicine, University of Chile, approved the animal protocol that was required for this study.

### Cell lines and melanoma cell lysate TRIMEL

The allogeneic cell lysate TRIMEL was prepared as previously described.<sup>21</sup> Briefly, three different melanoma cell

lines (Mel1, Mel2 and Mel3), established from three tumour-infiltrated lymph nodes from patients with metastatic melanoma at the Institute of Biomedical Sciences, University of Chile, were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen) until 95% confluence. Then, the cells were mixed in equal proportions ( $1 \times 10^7$  cells for each cell line) and heat-shocked at 42° for 1 hr and then incubated at 37° for 2 hr. Thereafter, the cell mixture was lysed through repeated freeze–thaw cycles in liquid nitrogen. Finally, the resultant cell lysate was sonicated and irradiated with a 60-Gy dose. All the melanoma cell lines were routinely tested for mycoplasma and were found to be free of contamination. THP-1 monocytic/macrophage cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

#### *TAPCells generation*

Peripheral blood mononuclear cells of melanoma patients were cultured in serum-free therapeutic AIM-V medium (GIBCO, Carlsbad, CA) at a concentration of  $13 \times 10^6$  cells/ml in six-well plates (Falcon Cat no. 3846; BD Biosciences, Hershey, PA) at 37° and 5% CO<sub>2</sub> for 2 hr. Thereafter, non-adherent cells were removed and the adherent cells (monocytes) were maintained and incubated for an additional 22 hr in the presence of 500 U/ml recombinant human interleukin-4 (rhIL-4) and 800 U/ml of granulocyte–macrophage colony-stimulating factor (GM-CSF; US Biological, Swampscott, MA). The obtained AMs, which showed an immature DC-like phenotype, were then stimulated for another 24 hr with either 100 µg/ml of TRIMEL and 20 U/ml of tumour necrosis factor- $\alpha$  (US Biological) or with only the medium.

#### *Flow cytometry*

TAPCells were characterized phenotypically by flow cytometry using the following conjugated antibodies: anti-HLA-DR-FITC, CD80-FITC, CD83-FITC, CD86-FITC, CD11c-PE-Cy7 and CCR7-FITC (eBioscience, San Diego, CA). Briefly, cells were gently removed from the culture plates using a cell scraper. Then, the cells were centrifuged at 193 *g* for 5 min at 4°, washed with PBS and incubated with antibodies for 30 min. After being washed twice with PBS, samples were acquired on a FAC-SCalibur (BD Biosciences) and analysed using FLOWJo software (Tree Star, Inc., OR). Cell viability was verified through trypan blue exclusion, and over 95% of treated cells in all cases excluded trypan blue. All the analyses were made in the CD11c<sup>+</sup> cell population of each condition and sample. To evaluate DC migration by FACS analysis, TAPCells and AMs were labelled with the fluorescent dye PKH67 (Sigma-Aldrich, St Louis, MO). Briefly,  $18 \times 10^6$  cells in 1.8 ml diluent C were mixed

with 27 µl PKH67 dissolved in 3-ml diluent C and stained for 5 min at room temperature. Labelling was stopped by incubation with 2.4 ml of 100% FBS. After that, cells were washed twice in RPMI supplemented with 10% FBS.

#### *Real-time quantitative polymerase chain reaction analysis*

For the isolation of total RNA, cells were first lysed using TRIzol reagent (Invitrogen) and purified following the manufacturer's protocol. RNA quality was determined in a microfluidic-based platform (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA). RNA was quantified using a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific, Wilmington, DE) and stored at –80° at a final concentration of 1 mg/ml. First-strand cDNA synthesis reverse transcription (RT) was performed using a synthesis kit (Roche Applied Science, Indianapolis, IN) and following the recommendations of the manufacturer. The mRNA expression levels of the chemokine receptor CCR7 were quantified using real-time quantitative PCR (RT-qPCR). As endogenous control, the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined and used for normalizing CCR7 expression. The first-strand cDNA product (50 ng) was used as template for RT-qPCR analysis using a mix for real-time PCR applications (iQ SYBER Green Supermix; Bio-Rad, Hercules, CA), specific primers, and a real-time PCR detection system (iCycler; Bio-Rad). Specific human primers were designed using the software PRIMER-BLAST (National Center for Biotechnology Information, Bethesda, MD); CCR7: forward 5'-CCTACAGCGCGG-CAAGTCC-3'; reverse 5'-CACGGTGGCGGTGAGCT-GAG-3'. GAPDH: forward 5'-GAAGGTGAAGTCCGGAGTC-3'; reverse 5'-GAAGATGGTGATGGGATTTTC-3'.

#### *In vitro transmigration assay*

Cell migration of TAPCells, AMs and THP-1 cell line in response to CCL19 (10 ng/ml) was evaluated using a 48-well Transwell double chamber (Neuroprobe, Gaithersburg, MD). Briefly, the lower chambers were loaded with media containing 30 µl per well of 10 ng/ml of CCL19 or AIM-V (negative control). Thereafter, corresponding inserts (upper chamber) with 8-µm pore size polycarbonate filters previously incubated for 24 hr in fibronectin 1 mg/ml (Invitrogen) were placed into the respective lower chamber. Finally, each well of the upper chamber was loaded with 50 µl of the cell suspension at a concentration  $1 \times 10^6$  cells/ml. After 4 hr of incubation at 37°, the upper inserts with the polycarbonate filter were removed. The polycarbonate filter was fixed in 100% methanol, stained with haematoxylin, washed twice with PBS, and placed onto a glass slide where the cells on the

upper surface of the filter were mechanically removed. This last step allowed us to maintain the cells that were able to pass through the filter's pore to the opposite side. Dry filters were further analysed using an inverted microscope (Leica DM IL, Wetzlar, Germany). Cells considered to have migrated through the pores were counted from five representative fields for each well in at least three independent experiments. Results were expressed as a migration index, which is the ratio between the number of migrated cells towards CCL19 and the number of migrated cells towards the medium alone for each cell condition studied.

#### *In vivo* ectopic xenograft model

After being labelled with PKH67 fluorescent cell linker (Sigma-Aldrich), equal numbers of TAPCells and AMs ( $2 \times 10^6$  cells/50  $\mu$ l of AIM-V) were administered by sub-mucosal lingual injection in previously anaesthetized NOD-SCID and C57BL/6 mice. In the case of the C57BL/6 mice, NK cells were depleted by pre-treating the animals, 48 and 24 hr before human cell administration, with two intraperitoneal injections (200  $\mu$ g) of an anti-NK1.1 antibody (kindly donated by Dr M<sup>a</sup> Inés Becker; Biosonda, Santiago, Chile). Twenty-four hours after injection of human DCs, the mice were killed and six cervical lymph nodes per animal were recovered for further analyses. Five of six lymph nodes were disaggregated with a 40- $\mu$ m cell-strainer (BD Biosciences) in PBS and the cells obtained were used for flow cytometry analysis to determine the percentage of PKH67-positive cells. One organ per animal was processed for immunohistochemistry as described below.

#### *Histological analysis*

Cervical lymph nodes from NOD-SCID and C57BL/6 mice injected with TAPCells or controls were recovered and fixed with 4% paraformaldehyde for 8 hr, dehydrated with graded alcohols (75–100%) and paraffin embedded. Next, 3- $\mu$ m sections were cut from these blocks using a microtome; these sections subsequently underwent treatment with proteinase K for antigen retrieval, followed by treatment with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and thereafter were washed twice with PBS. Finally, the sections were incubated with horse serum for 1 hr and then with anti-hCD11c (Abcam, London, UK) for an additional hour at 37° for lymph nodes obtained from NOD-SCID, and with anti-human mitochondria (US Biological) for lymph nodes obtained from C57BL/6. Next, sections were washed twice with PBS before incubation with the secondary antibody for 20 min at 37°. After further washing with PBS, the samples were incubated with ABC solution (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) for 20 min at 37°, washed twice with PBS and the

reaction was developed using DAB (Vector Laboratories). Human tonsils were used as positive controls and spleens ( $\alpha$ -CD11c) and lymph nodes ( $\alpha$ -mitochondria) from non-injected animals were used as negative controls.

#### *Statistical analysis*

Analysis of variance and the Kruskal–Wallis test for non-parametric variables were used to compare the significance of the differences between studied groups. Real-time qPCR data were expressed as  $C_t$  (cycle threshold),  $\Delta C_t$  ( $C_t$  mRNA blank –  $C_t$  GAPDH ribosomal RNA), and relative quantification (RQ). RQ was obtained by using the  $2^{-\Delta\Delta C_t}$  method, adjusting the blank mRNA expression to GAPDH mRNA expression and considering the adjusted expression in AMs as a reference (RQ = 1). Differences in mRNA expression of samples were determined by analysing the  $\Delta C_t$  data and the Mann–Whitney *U*-test. Differences were considered statistically significant at  $P < 0.05$ . The analysis of the association between variables was performed using GRAPHPAD PRISM 5 software (GraphPad Software, Inc., La Jolla, CA).

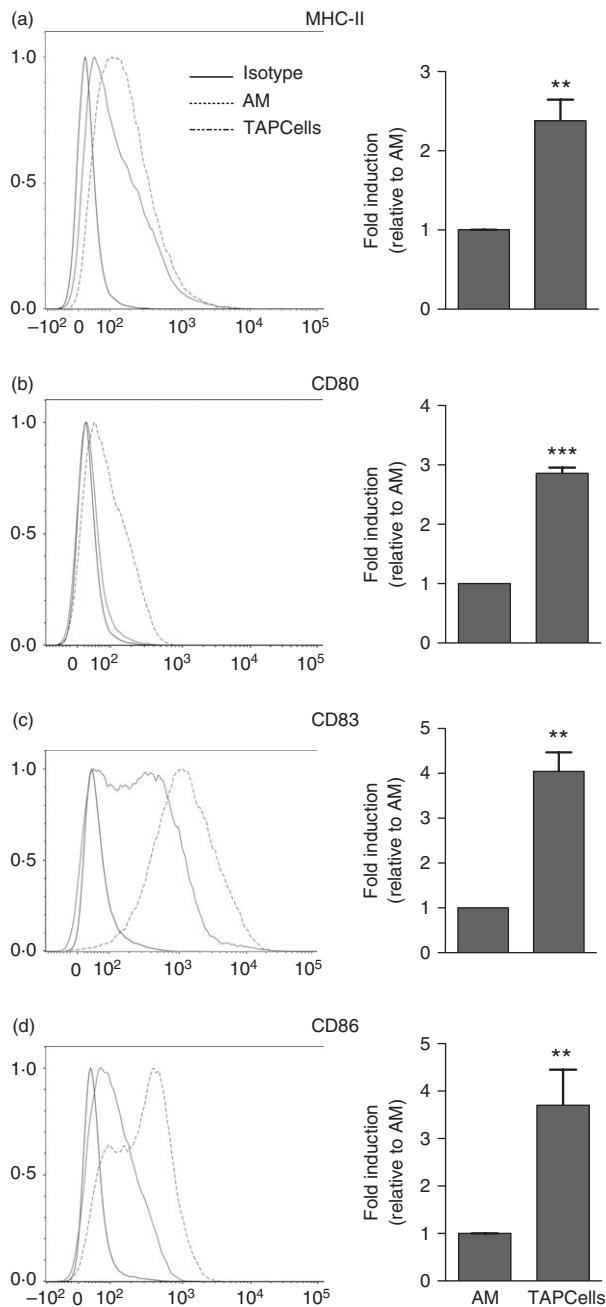
## Results

### TRIMEL induces the expression of CCR7 and *in vitro* transmigration of TAPCells and DC-like THP-1 cells

We have previously demonstrated that TRIMEL can rapidly ( $\approx$  24 hr) induce a mature and committed DC phenotype in TAPCells.<sup>23</sup> Here, we used flow cytometry to re-evaluate TRIMEL-induced expression of mature DC markers MHC-II, CD80, CD83 and CD86 on cytokine-activated monocytes (Fig. 1a–d). The addition of TRIMEL to TAPCells resulted in an induction of between twofold and fourfold of the above-mentioned maturation markers when compared with unstimulated cells (i.e. AMs) (Fig. 1), confirming our previous observations regarding TRIMEL function as a strong and reliable maturation stimulus for immature DCs.

Following this, we evaluated the effect of TRIMEL on the induction of the chemokine receptor CCR7, another well-established maturation marker of DCs, which is essential for the migration of DCs from the periphery to draining lymphatic tissues. Our results showed that a significant proportion of TRIMEL-activated TAPCells, compared with AMs, acquired surface expression of CCR7 molecules. Moreover, there was a 1.54-fold increase in the expression levels of CCR7 when the mean fluorescence intensity was evaluated in TAPCells and AMs derived from the monocytes of at least three different melanoma patients (Fig. 2a). In addition, we confirmed the induction of CCR7 with RT-qPCR, where the amount of CCR7 mRNA was also significantly higher in TAPCells (135-fold change) when compared with the AM control





**Figure 1.** TRIMEL induces a mature dendritic cell (DC) phenotype in tumour-antigen-presenting cells (TAPCells). Representative histograms and statistical quantifications comparing the expression of (a) MHC-II, (b) CD80, (c) CD83 and (d) CD86 between activated monocytes (AMs) and TAPCells. The quantification of the expression of the maturation marker considered the mean fluorescence intensity of TAPCells in relation to AMs. The expression of surface markers on AMs and TAPCells was assessed by flow cytometry (CD11c<sup>+</sup> cells were gated). Data in the bars represent at least four independent experiments with PBMC derived from different melanoma patients. Bars indicate SEM; \*\**P* < 0.01; and \*\*\**P* < 0.001.

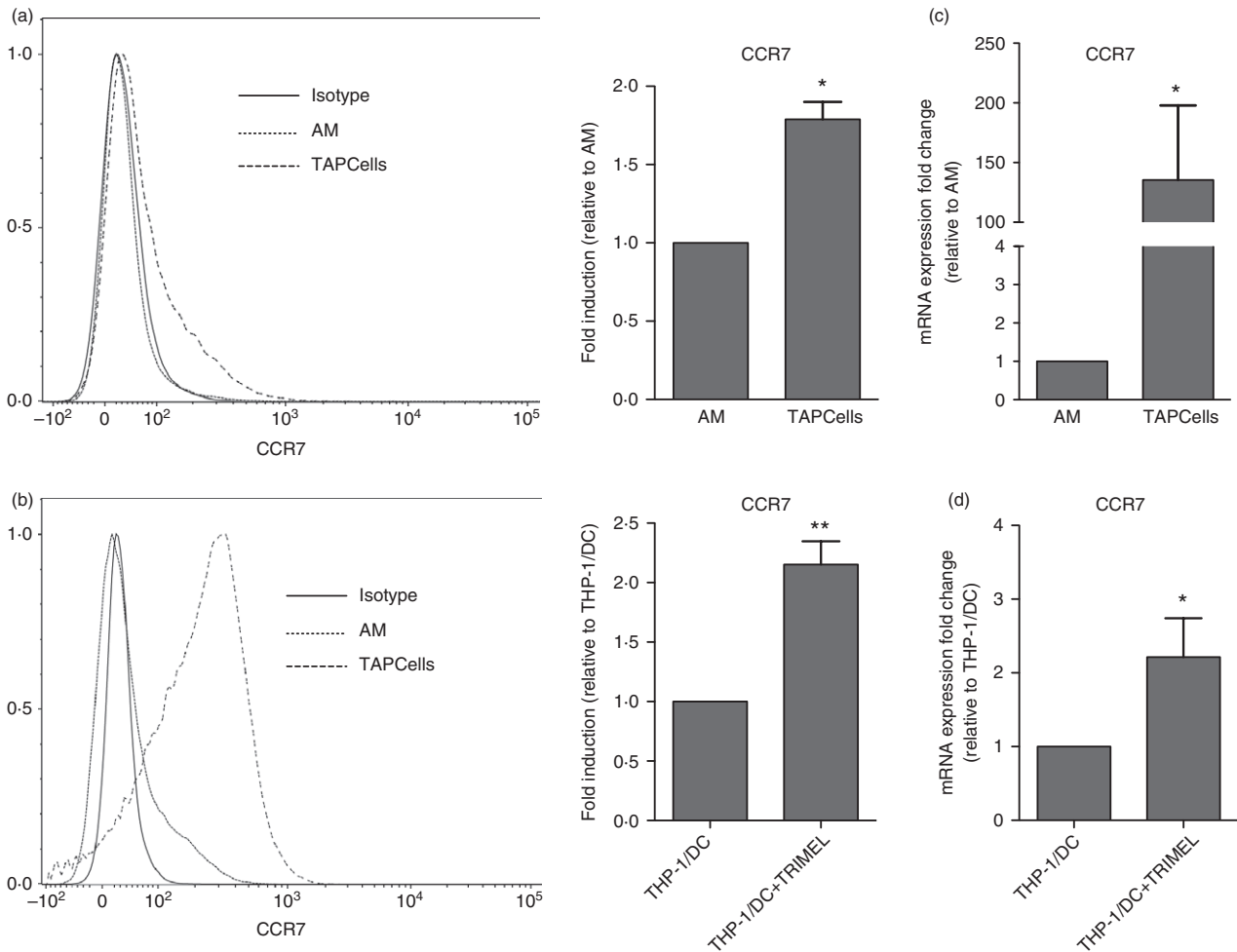
(Fig. 2c). This result was also consistent with previous reports from our group regarding the mature and committed phenotype of mature DCs exhibited by TAPCells.<sup>23,24</sup>

The differentiation of monocyte/macrophage THP-1 cells into immature and mature DCs using diverse cytokines and factors has been used as an experimental model for studying molecular events and pathways that govern the differentiation of human myeloid precursors into DCs.<sup>25</sup> However, the capacity of TRIMEL to induce a mature DC-like phenotype on THP-1 had not been demonstrated. In the present study, the TRIMEL lysate, with an absence of additional pro-inflammatory cytokines, induced a twofold increase in the expression of MHC-II and CD86 molecules in THP-1 cells and generated a similar DC-like phenotype compared with the unstimulated control cells (see Supporting information, Fig. S1). This result was similar to the effect observed in human monocytes derived from MM patients.<sup>23</sup> Additionally, we confirmed the induction of cell surface CCR7 proteins via flow cytometry (Fig. 2b) and RT-qPCR, where the amount of CCR7 mRNA was twice as high in THP-1 stimulated with TRIMEL as compared with unstimulated THP-1 control cells (Fig. 2d). These results demonstrate that TRIMEL could also significantly induce the differentiation of a well-established monocyte-derived cell line.

Further, we investigated the effect of TRIMEL stimulation in activated monocytes and in THP-1 cells in response to the chemokine CCL19, one of the canonical CCR7 ligands. To this end, we used an *in vitro* transmigration assay through a polycarbonate membrane in a double-well chamber. We observed that TRIMEL-stimulated TAPCells significantly increased their *in vitro* transmigration rate in response to CCL19 when compared with AM control cells (30.6 ± 4.0 versus 9.5 ± 3.0, respectively; Fig. 3a). Additionally, we observed that TRIMEL-stimulated THP-1 cells were also able to transmigrate significantly more in the presence of CCL-19 than cytokine-activated unstimulated THP-1 cells (DC-like THP-1; 48.3 ± 7.9 versus 12 ± 6.1, respectively; Fig. 3b). In summary, our data suggest that TRIMEL induces a mature DC-like phenotype characterized by an increased expression of the receptor CCR7, which in turn augmented TAPCells *in vitro* trans migratory potential in response to CCL19.

### TAPCells can migrate *in vivo* from the injection site to draining lymph nodes

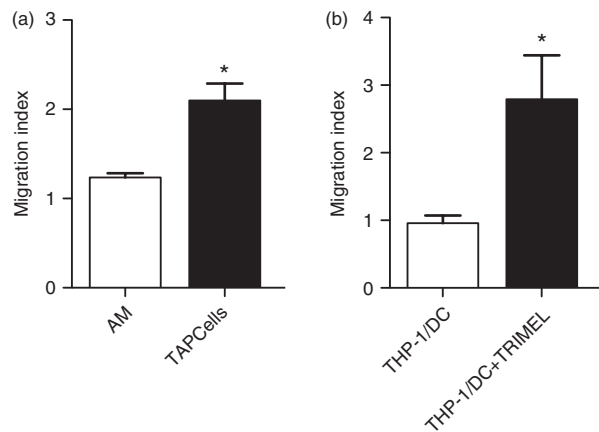
Because of the human origin of the TAPCells, when evaluating whether the *in vitro* migration ability displayed by TAPCells could also be recapitulated *in vivo*, we needed to use immunosuppressed mice capable of accepting xenogeneic tissues. For this reason, we injected TAPCells into an ectopic xenograft model in both immune-defi-



**Figure 2.** TRIMEL induces the expression of the chemokine receptor CCR7 on tumour-antigen-presenting cells (TAPCells) and THP-1 cells. Representative histograms of CCR7 surface expression on (a) activated monocytes (AMs) and TAPCells after 24 hr of stimulation with TRIMEL (100 µg/ml) or medium, and (b) on THP-1 cells previously stimulated with granulocyte-macrophage colony-stimulating factor and interleukin-4 for 5 days and additionally stimulated or non-stimulated with TRIMEL (100 µg/ml) for 24 hr [THP-1/dendritic cell (DC) + TRIMEL and THP-1/DC, respectively]. Quantification of the CCR7 mRNA expression (increasing in mean fluorescence intensity value) after TRIMEL stimulation in (c) TAPCells in relation to AMs and in (d) THP-1/DC like cells (THP-1/DC + TRIMEL cells) or controls (THP-1/DC cells). Cell surface protein expression on AMs and TAPCells was evaluated with flow cytometry of CD11c-positive cells. Data for flow cytometry and mRNA quantification were obtained from at least three independent experiments using peripheral blood mononuclear cells from different melanoma patients. Bars indicate SEM; \**P* < 0.05; and \*\**P* < 0.01.

cient NOD-SCID and immunocompetent NK cell-depleted C57BL/6 mice. Depletion of NK cells allowed for the survival of xenogeneic cells for a couple of days. Previously, PKH67-labelled TAPCells and AMs were prepared and injected into the lingual submucosa of mice for this purpose. Accumulation of xenogeneic cells into the draining cervical lymph nodes was compared 24 hr after injection by both flow cytometry and immunohistochemistry. Additionally, we evaluated the presence of PKH67<sup>+</sup> cells in the tongues of C57BL/6 mice. We observed that TAPCells were able to reach the cervical lymph nodes 24 hr after being injected in the lingual submucosa in both murine models (Fig. 4a,b). Positive TAPCells for PKH67 were found in a significantly higher

percentage compared with positive AMs in NOD-SCID mice with mean values of 2.1% versus 0.3%, respectively (Fig. 4a), and in C57BL/6 mice with mean values of 7.3% versus 1.2%, respectively (Fig. 4b). Although not significant, the percentage of TAPCells present in the tongues was lower when compared with AMs in NK-cell-depleted C57BL/6 mice showing mean values of 1.8% versus 2.7%, respectively (Fig. 4c). Importantly, the results were additionally confirmed by immunohistochemistry of the lymph nodes in NOD-SCID mice using anti-human mitochondria, which identify the human origin of the detected cells, and in C57BL/6 mice by anti-human CD11c antibodies, which can more specifically identify the dendritic cell phenotype of human cells (Fig. 5). In



**Figure 3.** TRIMEL induces the *in vitro* transmigration of tumour-antigen-presenting cells (TAPCells) and THP-1 cells. Evaluation of the CCL19-dependent, trans migratory activity of TRIMEL-activated cells. (a) Transmigration of monocyte-derived antigen-presenting cells obtained from different melanoma patients in the presence (TAPCells) or absence (activated monocytes; AMs) of TRIMEL. (b) The transmigration of THP-1/dendritic cell-like cells with or without TRIMEL stimulation. Transmigration capability of different cells was tested with a chemotaxis assay in a 48-well transwell chamber. Lower wells were loaded with CCL19 (10 ng/ $\mu$ l) or alternatively with only the medium, and the upper wells were loaded with 50 000 cells/well per condition and incubated for 4 hr at 37°. Data were obtained from at least three independent experiments. The migration index values correspond to the difference in transmigration between cells in the presence or absence of CCL19. Bars indicate SEM; \* $P < 0.05$ .

fact, lymph nodes derived from TAPCell-treated NOD-SCID mice showed a higher number of human CD11c-positive cells with a strong membrane distribution pattern compared with AM-treated animals (Fig. 5a, upper row). Similarly, lymph nodes from C57BL/6 mice treated with TAPCells showed a stronger cytosolic staining pattern than those from animals injected with AMs (Fig. 5a, lower row). Interestingly, a strong perivascular staining pattern was observed in lymph nodes from both mouse strains injected with TAPCells and, to a lesser extent, in animals injected with AMs (Fig. 5a, third and fourth columns). The quantification of positive cells for these human markers in the histological sections showed a significantly higher number of stained cells in the lymph nodes of mice treated with TAPCells than AMs in NOD-SCID and C57BL/6 mice (52 versus 22.6 cells, and 62.3 versus 30.3 cells, respectively; Fig. 5b), demonstrating that TRIMEL is also able to induce the migration of TAPCells towards the lymph nodes *in vivo*.

## Discussion

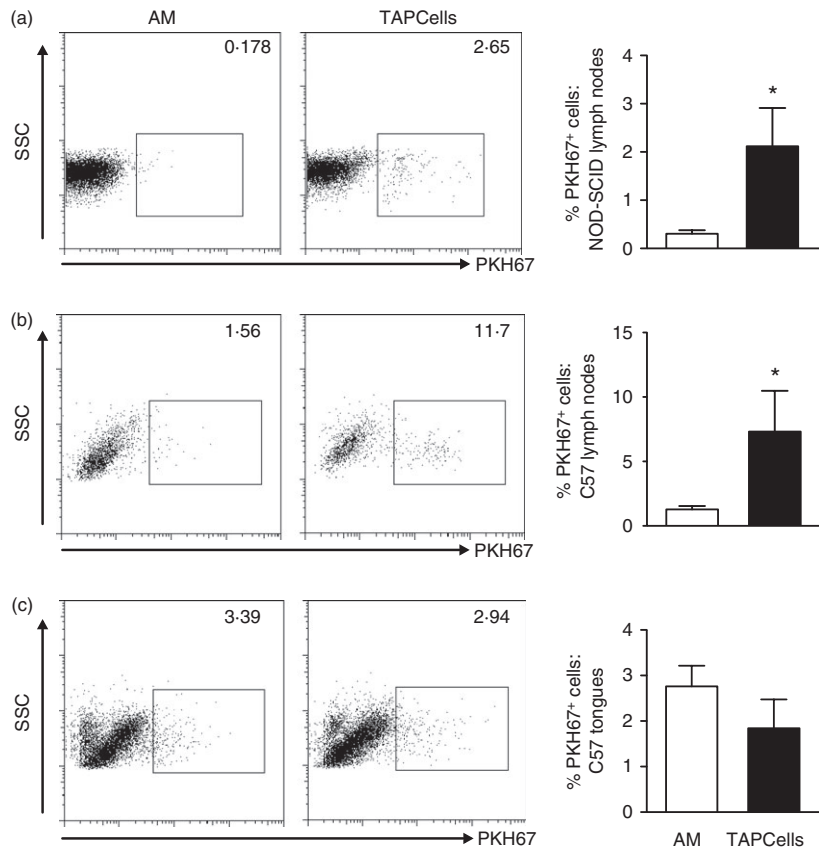
The homing of DCs is a fundamental feature for their ability to trigger a proper and effective immune response against both pathogens and tumours. Related with this,

DC-based immunotherapy is primarily dependent on these cells' unique antigen-presenting ability to prime naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and their capacity to migrate from the administration site to draining lymph nodes. Crucially, this migration capability of DCs is strongly regulated by chemokines (e.g. CCL19 and CCL21) and chemokine receptors such as CCR7, which are notably up-regulated on mature DCs upon stimulation by self or pathogen-associated danger signals. Naive T cells also express CCR7, allowing their convergence at the same lymph node area with DCs where they form an immunological synapse.<sup>14,26,27</sup>

In the present study, we demonstrate that TRIMEL-stimulated TAPCells showed a higher CCR7 expression than control AMs, correlated with an increased *in vitro* transmigration response to CCL19. Furthermore, our data are also complemented by the *in vitro* trans migratory ability of TRIMEL-stimulated TAPCells and DC-like THP-1 cells in response to the CCR7 ligand CCL19. Hence, the CCR7 surface expression is indicative of TAPCells' ability to migrate to the lymph nodes, which is an essential requirement for the activation of adaptive immunity.<sup>10</sup> Additionally, our present data also showed a significantly higher CCR7 expression on DC-like differentiated THP-1 cells compared with controls (Fig. 2), confirming several previous observations in which TRIMEL rapidly induced a mature and committed DC phenotype on activated monocytes (Fig. 1).<sup>23,24</sup>

We have previously described that the delayed hypersensitivity reaction, detected in 64% of TAPCell-vaccinated patients after challenge with TRIMEL, reflects the induction of a robust cellular response against the lysate and constitutes an excellent prognostic marker for clinical outcome.<sup>21,28</sup> In this context, TRIMEL-specific delayed hypersensitivity response has been associated with *in vivo* CD4<sup>+</sup>/CD8<sup>+</sup> memory T-cell accumulation at the reaction site, together with reduced rates of progression and prolonged patient survival, so linking *ex vivo* events, during TAPCell generation, with clinical anti-tumour responses.<sup>21</sup> These responses imply, although indirectly, that the ability of TAPCells to migrate *in vivo* from the injection site to regional lymph nodes plays a critical role for mounting the appropriate immune response. Specifically, TAPCells may migrate to the lymph nodes to reach the vicinity of their T-cell area, and to prime naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which in turn, may ultimately trigger an anti-tumour immune response in vaccinated patients.

Importantly, our data also demonstrate that human TAPCells – DC-like, therapeutically used immune cells in MM patients – can migrate *in vivo* from the injection site into draining lymph nodes in two robust xenograft mouse models, establishing for the first time this important capacity of TAPCells (Figs 4 and 5). Interestingly, the ability to migrate to the lymph nodes occurred within the first 24 hr after their injection, which means that they



**Figure 4.** Tumour-antigen-presenting cells (TAPCells) can migrate *in vivo* to draining lymph nodes in an ectopic xenograft mouse model. NOD-SCID and natural killer cell-depleted C57BL/6 mice, as described in the Material and methods, were injected submucosally with PKH67-stained activated monocytes (AMs) or TAPCells ( $2 \times 10^6$  cells/mouse) in the middle of the tongue. The animals were killed after 24 hr and cervical lymph nodes (NOD-SCID and C57BL/6 mice) and tongues (C57BL/6 mice) were recovered and processed. Representative dot plots show PKH67<sup>+</sup> human cell populations (left panels) and graphics show quantification of the gated positive cells for each strain and organ (right panel). (a) PKH67<sup>+</sup> cells in NOD-SCID lymph nodes (five per mouse). (b) PKH67<sup>+</sup> cells in C57BL/6 mice lymph nodes (five per mouse). (c) PKH67<sup>+</sup> cells in C57BL/6 tongues (one per mouse). Data obtained from three independent experiments. Bars indicate SEM; \* $P < 0.05$ .

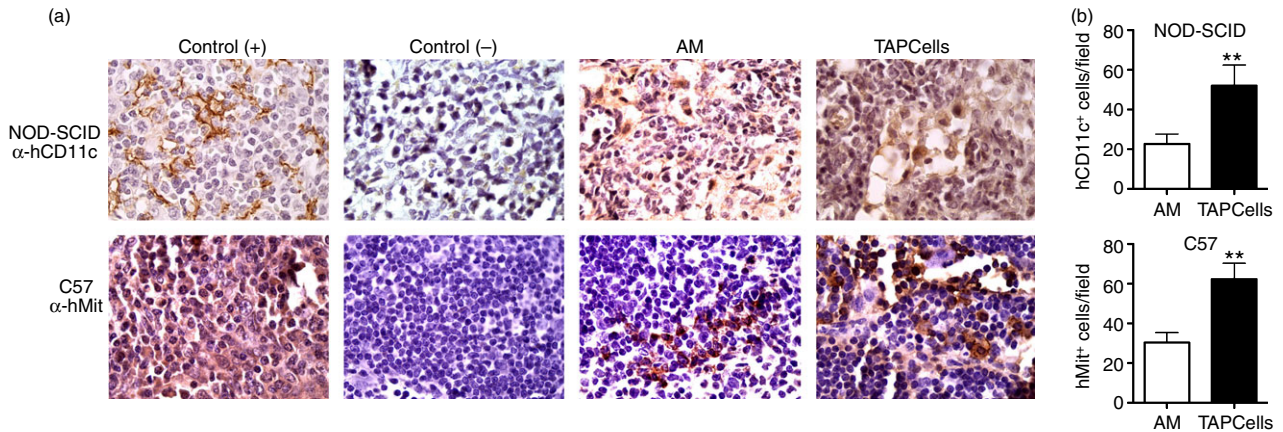
can mobilize rapidly from submucosa connective tissue, through lymphatic vessels, and reach local lymph nodes. The context of these observations appears even more remarkable considering that we did not use any adjuvant (e.g. aluminium hydroxide) during administration of the TAPCells to the animals. Migration of human cells in a murine model can, at least in part, be explained by the high homology between human and mouse CCL19 and CCL21 chemokines (83% and 86% amino acid sequence, respectively), which likely allows human TAPCells to recognize these mouse mediators. The latter has also been demonstrated in studies involving human cell metastatic capacity in mouse models.<sup>29–31</sup> In our experiments, although to a lesser extent compared with TAPCells, AMs were also able to migrate to lymph nodes, probably indicating an *in vivo* occurring maturation process triggered by danger signals generated at the injection site after inoculation-derived tissue injury. In this regard, we can speculate that the *in vivo* migratory efficiency of TAPCells could be even better in an autologous human context in

the presence of appropriate adjuvants, such as the case of MM patients, which could in turn explain at least in part their immunological effectiveness in approximately 60% of treated patients.

Optimal delivery of a wide-ranging pool of antigens coupled with the presence of factors promoting lymph node migration and antigen cross-presentation to cytotoxic T lymphocytes is critical for DC vaccine success.<sup>1,32</sup> Tumour lysates like TRIMEL, have been shown to induce all of these phenotypes on *ex-vivo*-produced DCs, prompting their use as a reliable vaccine against malignant cancers.<sup>21,33,34</sup>

Even though a relatively small percentage of human TAPCells was detected in mouse lymph nodes, we cannot discard the presence of other concomitant mechanisms involved in DC behaviour that amplify the *in vivo* response induced in MM patients, for example DC–NK and DC–DC cross-talk at the injection site microenvironment as well as in the draining lymph nodes. These mechanisms augment the possibilities of the transfer of





**Figure 5.** Immunohistochemical detection of human cells in lymph nodes from NOD-SCID and natural killer (NK) cell-depleted C57BL/6 mice injected with tumour-antigen-presenting cells (TAPCells). (a) Cervical lymph nodes derived from activated monocytes (AMs), TAPCells and PBS-injected NOD-SCID mice (top row) and NK cell-depleted C57BL/6 mice (bottom row) were stained with an anti-human CD11c monoclonal antibody (NOD-SCID mice) and an anti-mitochondria polyclonal antibody (C57BL/6 mice) and then analysed by immunohistochemistry. (a) Stained tissues. (b) Positive cell quantification in NOD-SCID injected mice (upper figure) and C57BL/6 injected mice (lower figure). Human tonsils were used as positive controls [Control (+)] and spleens ( $\alpha$ -hCD11c) and lymph nodes ( $\alpha$ -hMit) from PBS-injected animals were used as negative controls [Control (-)]. Eight representative photographic fields for each strain and condition were counted using IMAGEJ software (NIH, Bethesda, MD). Bars indicate SEM; \*\* $P < 0.01$ .

antigenic information from DCs to naive T cells during immunological synapses. In this regard, we have previously described the ability of TAPCells to transfer small molecules (i.e. peptides and second messengers) to other immune cell types through gap junction interactions.<sup>35</sup>

TAPCells represent a reliable and safe immune-therapy-based treatment against MM. Recently, we also described the therapeutic benefit of TAPCells in castration-resistant prostate cancer patients.<sup>36</sup> In fact, some patients vaccinated with TAPCells loaded with a mix of prostate cancer and TRIMEL lysates showed a significant decrease of PSA levels. Moreover, patients with a delayed hypersensitivity response showed a prolonged PSA doubling-time after treatment.<sup>36</sup>

In this study, we have demonstrated that the tumour cell lysate TRIMEL increases the expression of the chemokine receptor CCR7 on TAPCells, resulting in their increased *in vitro* response to CCL19. Furthermore, TAPCells can rapidly reach draining lymph nodes *in vivo*, a fundamental characteristic to be considered professional APCs. Certainly, the final proof-of-concept regarding the TAPCells' migratory ability by the analysis and *in vivo* visualization of TAPCell migration in vaccinated patients, remains to be addressed. This study contributes to an increased understanding of the biology of *ex vivo*-produced DCs allowing the design of new strategies for effective DC-based vaccines for treating cancer patients.

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## Disclosures

None of the authors has any potential financial conflict of interest related to this manuscript.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** TRIMEL induces the expression of MHC-II and CD86 in THP-1 cells.