Manipulating the expression of chemokine receptors enhances delivery and activity of cytokine-induced killer cells

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Background: Cytokine-induced killer (CIK) cells are ex vivo-expanded immune cells that express NK-cell and T-cell markers and that are routinely used in the treatment of many cancers. One key advantage of CIK cells is their ability to efficiently traffic to many solid tumours. Although likely to be mediated by chemokine receptor (CKR) expression, a thorough examination of the mechanism of tumour targeting has not been previously explored.

Methods: Here, human CIK cell expansions were examined for the level, profile and kinetics of CKR expression.

Results: It was found that CIK cells express a panel of CKRs, with considerable variation between donors. Importantly, CKR levels dropped considerably beyond 14 days in culture, being significantly reduced by day 28 (the time at which cytolytic activity peaked). As such, CIK preparations that are used clinically may not have optimal CKR expression. Several approaches were found to re-stimulate CKR cell-surface levels at these later time points. These approaches also enhanced cytolytic activity in vitro and were demonstrated to increase both in vivo tumour trafficking and anti-tumour activity in mouse models.

Conclusions: Simple modifications of the CIK expansion protocol could therefore be used to significantly enhance the anti-tumour effects of this therapy.

An expansion protocol for the production of cytokine-induced Killer (CIK) cells was initially reported 20 years ago (Schmidt-Wolf et al, 1993; Lu and Negrin, 1994). However, despite widespread use in Asia and some promising clinical data targeting haematopoietic malignancies in the United States (Leemhuis et al, 2005), the treatment of solid tumours with this immune cell therapy has, to date, been disappointing (Belin et al, 2013; Zhao et al, 2013). This is primarily because of the limited cytolytic potential of this population. However, one of the greatest strengths of CIK-based therapies over similar cell types, such as LAK cells, is their capacity to traffic systemically to solid tumours, including residual disease (Sasaki et al, 1991; Scheffold et al, 2002). This tumour-targeting potential has been used in several recent pre-clinical reports where CIK cells have been used in combination with other immunotherapies or biological therapies to act as delivery vehicles or targeting agents, and this may lead to significant improvement in clinical responses (Thorne et al, 2006; Liu et al, 2013).

However, the basis for the tumour-directed trafficking capacity of CIK cells is poorly understood at present. It is likely that one important factor directing CIK cell trafficking would be the interaction of chemokines released from the tumour microenvironment with chemokine receptors (CKRs) on the CIK cell surface (Baker et al, 2001). However, a detailed analysis of CKR profiles on CIK cells has not been reported previously, and the effects of the
Expansion protocol on CKR expression are not known. It is predicted that a better understanding of the mechanisms used by CIK cells to traffic to a broad range of tumour types could ultimately be applied to other adoptive immune cell therapies.

It was discovered that, although a variety of CKRs are expressed on the surface of CIK cells and considerable donor-to-donor variability existed, one consistent factor was that CKR levels dropped during the later stages of the ex vivo expansion protocol. As a result, the levels of many CKRs were significantly reduced by the time the cytolytic activity peaked (the time at which CIK cells were typically reinfused back to patients in clinical settings). This observation might at least partially explain why CIK cell therapies have been less effective in clinic than predicted from pre-clinical models.

Approaches to boost cell-surface CKR levels at these later times were examined, and the effects of these on cytolytic activity and on in vivo tumour trafficking, as well as anti-tumour effects in mouse models, were determined.

**RESULTS**

CIK cells express multiple chemokine receptors on their cell surface, and there is significant donor-to-donor variation. In initial studies, multiple CIK preparations were expanded fromuffy coats obtained from healthy donors \((n=28)\) and colorectal cancer (CRC) patients \((n=11)\). Our previous studies and those of
other groups (Baker et al., 2001; Marin et al., 2006) have determined that 6 chemokine receptors are expressed on CIK cells (CCR4, CCR5, CCR6, CCR7, CXCR3 and CXCR4). In addition, because CIK cells represent a mix of NK (CD3−/CD56+), NK-T (CD3+CD56+), and T cells (CD3+/CD56−), it was important to determine the relative abundance of CKR on the different cell lineages.

It was seen (Figure 1A) that, in general, chemokine receptor levels were higher in T-cell and NK-T-cell populations relative to NK cells (which is in agreement with previous studies showing that these populations are more effective at trafficking to the tumour target (Schmidt-Wolf et al., 1993; Nishimura et al., 2008)). In addition, CCR5, CXCR3 and CXCR4 were the most highly expressed chemokines in the CD3+/CD56+ population (with 55, 52 and 62% of CD3+/CD56+ cells positive for these CKRs, and 12, 27 and 17% positive for CCR4, CCR6 and CCR7, respectively). However, differences between the cell lineages and between healthy and CRC patient donors were rarely significant.

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**Figure 1. CKR profiles on human CIK cell expansions.** (A) Healthy donor (HD; n = 28) and colorectal cancer patient (CRC; n = 11) CIK cells at day 14 were stained for NK (CD56) and T-cell (CD3) markers, as well as the 6 chemokine receptors known to be expressed on CIK cells (CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4). Percentages of cells of different cell types (CD3−/CD56+, CD3+/CD56+, CD3+/CD56−) positive for different CKRs are shown. (B) Comparisons of CKRs on CD3+/CD56+ CIK cells from HD and CRC (no significant differences seen for any CKR; N.B. Other differences between these groups include average age (HD = 39 years; CRC = 53 years) and sex (HD = 29% female; CRC = 45% female). (C) Differences in CKR profiles in CD3 + CD56+ CIK cell populations between donors (for 4 donors) and at different times after expansion are shown. Individual donors show distinct patterns of CKR profiles, which are gradually reduced over time in culture.
any of these treatments resulted in some level of re-stimulation of coated beads to the culture for 24 h. It was observed (Figure 4) that dose of IL-2 for 24 h, or the addition of anti-CD3/anti-CD28-this time point. These treatments included adding IL-12 or a high expression levels at the later time points (28 day expansion), a

to determine whether it is possible to boost the CKR cell-surface expression on CIK cells can be boosted through the addition of different immunostimulants. To determine whether the observed loss of CKR after 14 days in culture. This observation may help explain why CIK cells used against solid tumours in the clinic have been less effective than expected based on pre-clinical results.

This was again found to be reproducible for multiple donors (Figures 1C and 2). It was apparent that variability in CKR profiles primarily existed between donors, and that the relative expression of different CKRs for any individual donor remained relatively constant during the expansion process (Figure 1C).

It was also observed that the level of cell-surface expression of the majority of the CKRs examined dropped during the course of CIK expansion, peaking at about day 14 in culture and demonstrating considerable subsequent reduction by day 28. This was again found to be reproducible for multiple donors (Figures 1C and 2). This apparent exhaustion of CKR expression may be important, as CIK reinfusions to patients are typically not performed until 28 days in culture. This observation may help explain why CIK cells used against solid tumours in the clinic have been less effective than expected based on pre-clinical results.

Cytolytic activity of CIK cells peaked at 28 days in culture. To determine whether the observed loss of CKR after 14 days in culture correlated with a loss of overall cytolytic activity of the CIK cells, the same preparations were used in basic CTL assays against several human tumour cell lines at different times during the expansion process (Figure 3). It was seen that although CKR cell-surface levels peaked at day 14, the cytotoxic capability of the cells did not reach maximum levels until day 28. This disconnect means that CIK cells used against solid tumours where trafficking of the cells to the tumour is crucial might be better used after a shorter expansion period, especially if the CIK cells are being primarily incorporated as a viral or gene delivery vehicle.

Figure 2. CKR expression is lost over time in culture. The levels of each CKR (CCR4, CCR5, CCR7, CXCR3 and CXCR4 shown as the percentage of cells positive in the preparation relative to isotype control) are shown at days 14 and 28 for each expansion. A trend towards reduced CKR expression is seen for all CKRs studied; this is significant (P<0.05, paired t-test) for CCR5 (P = 0.0003), CCR7 (0.013) and CXCR3 (0.0024).
Chemokine receptor expression on CIK cells

CIK cells represent an important model system for studying the trafficking of T cells to tumours as, unlike many vaccine induced...
T-cell populations, they efficiently traffic to both large and small solid tumours in mouse models (Baker et al., 2001; Thorne et al., 2006). A better understanding of how this trafficking is mediated will be important for establishing how to optimise CIK therapy, as well as how to enhance other immunotherapies such as adoptive T-cell transfer or cancer vaccines. However, such studies are complicated by the autologous nature of CIK cells, meaning that no immortalised cell line exists for study.

The importance of cell-signaling pathways induced by binding of chemokines to chemokine receptors in the directed trafficking of immune cell populations is well known (Lippitz, 2013), and thus it is likely that these pathways are involved in CIK cell trafficking, although there are only limited reports of CKR expression profiles on CIK cells, especially in a human setting.

Here, we initially incorporated multiple expansions from different human donors to try to identify specific CKR patterns in human CIK cell populations. This determined (i) that hCIK cells express an impressive number of different CKRs. This might be a necessary requirement for trafficking to multiple different tumour types that may produce different chemokine profiles, and (ii) that there is a high degree of variability in the relative numbers of cells positive for these different CKRs. This appears to be determined by the donor, as relative levels of CKRs did not vary significantly during the expansion process. The importance of differences in the particular chemokine receptor patterns remains to be established. More in-depth profiling of the chemokines produced by different

Figure 4. Effects of different immune stimulants at day 28 in culture on CKR levels. (A) Several of the expansions were treated with different immune stimulants (IL-12, high-dose IL-2 or exposure to CD3/CD28-coated beads for 24 h), and the effects on the overall levels (percentage of positive cell) of the three different CKR that displayed significant reductions between days 14 and 28 (CCR5, CCR7, CXCR3) were re-determined by flow cytometry, as described earlier. Fold increase in the percentages of positive cells relative to untreated controls at the same time are shown. In all cases, CKR levels were increased, with CD3/CD28-coated beads producing the most consistent and reliable increases in all three CKR levels. (B) Cell migration assay. In additional experiments, CIK expansions (day 28) were treated with CD3/CD28 beads for 24 h before addition to the top well of transwell plates. The indicated chemokines were applied in the lower wells, and migration (number of cells in lower plate) was determined by flow cytometry after 24 h (*P < 0.05 for paired t-test comparing percentage positive with or without treatments).

Figure 5. Cytotoxicity assay for CIK expansions (28 days) treated with different immune stimulants as before. No treatment had a negative effect on CIK cellular cytotoxicity, with CD3/CD28-coated beads producing an increase in CTL cytotoxicity for 2 of the 3 CIK expansions shown (*P < 0.05 relative to PBS control).
tumours and how this correlates with chemokine receptor profiles on CIK cells and subsequent therapeutic effects will be needed to determine the clinical importance of different CKR expression; (iii) the level of CKR expression on hCIK cells peaks around day 14, and subsequently declines. This is reproducible among different donors and may be especially important, as CIK cells are usually infused back into a patient after about 28 days. This time was shown to correlate with maximum cytotoxic capability of the CIK cells, but it is clearly sub-optimal for trafficking. As a result, it is likely that CIK cell therapies could be tailored in the clinic, with, for example, earlier (day 14) expansions used when cell trafficking is more important (such as in combination studies where CIK cells may be used primarily as a delivery vehicle) and later (day 28) expansions used when cytolytic activity is critical (such as when treating leukaemia).

The importance of trafficking in the treatment of solid tumours was verified in mouse models. It was confirmed that mouse CIK cells also displayed a reduction in CKR levels, only over a shorter time in culture (day 10 to day 14), and for a smaller range of CKR (CXCR3 appears critical); data shown are for percentages of CXCR3-positive cells in mCIK cultures expanded from C57/BL6 mice at days 7 and 14 (n = 7, reduction is significant, P = 0.0018). CTL assay-determined cellular cytotoxicity also increased over the same time period (MC38-luc cells used as target) (data is average from n = 3 preparations). (B) Loss of CXCR3 reduces trafficking of CIK cells to their tumour targets and reduced anti-tumour effects in vivo. Mice (C57/BL6) bearing subcutaneous tumours (MC38-luc of 50–100 mm³) were treated with intravenous (tail vein) injection of CIK cells (1 × 10⁷ cells) expanded from a C57BL6 CXCR3–/– and GFP + transgenic mouse or from a GFP + strain (n = 4 per group). Subsequent tumour trafficking was determined both by flow cytometry on dissociated tumours (left) and through examination of GFP staining in tumour sections post mortem. Significantly less CXCR3–/– CIK cells were found in the tumours, P = 0.0014); (C) Anti-tumour effects as determined by bioluminescence imaging of MC38-luc cells were determined in the same model (n = 6 per group). *CIK cells produced significantly enhanced therapeutic effects relative to all other groups on days 11, 14 and 18 (P<0.05).
Chemokine receptor expression on CIK cells

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Figure 7. Treatment of mouse CIK cells with CD3/CD28-coated beads results in increased CKR expression, enhanced trafficking and enhanced anti-tumour effect. (A) Levels of the CRX CXCR3 are increased after exposure to beads for 24 h in a day 14 culture (n.B. cellular cytotoxicity was unchanged, data not shown). (B) Transwell assay was used to follow movement of CIK cells (day 14, with or without bead treatment) towards lower wells containing recombinant IP-10 (Biolegend). Numbers of cells in the lower wells were determined by flow cytometry after 3 h. (C) Trafficking of bead-treated mCIK cells is significantly improved for in vivo delivery to subcutaneous MC38 tumours following tail-vein delivery of mCIK cells bound to NHS Cy5.5. CIK delivery to the tumour at 24 h after injection was determined by fluorescence imaging (using the FMT2500, Perkin Elmer) (n = 4). Significantly greater CIK cell fluorescence was found in the tumour for CIK cell preparations that had been pretreated with CD3/CD25-coated beads (P = 0.01). (D) Tumour volumes were also measured at the time of killing for these same groups (at day 7 post treatment), and an increased therapeutic effect was seen for bead-treated CIK cells (n = 8, P = 0.003).

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