

## SHORT COMMUNICATION

Cross-presentation of tumour antigens by human induced pluripotent stem cell-derived CD141<sup>+</sup>XCR1<sup>+</sup> dendritic cellsKM Silk<sup>1</sup>, JD Silk<sup>2</sup>, N Ichiryu<sup>1</sup>, TJ Davies<sup>1</sup>, KF Nolan<sup>1</sup>, AJ Leishman<sup>1</sup>, L Carpenter<sup>3,4</sup>, SM Watt<sup>3,4</sup>, V Cerundolo<sup>2</sup> and PJ Fairchild<sup>1</sup>

Monocyte-derived dendritic cells (moDC) have been widely used in cancer immunotherapy but show significant donor-to-donor variability and low capacity for the cross-presentation of tumour-associated antigens (TAA) to CD8<sup>+</sup> T cells, greatly limiting the success of this approach. Given recent developments in induced pluripotency and the relative ease with which induced pluripotent stem (iPS) cell lines may be generated from individuals, we have succeeded in differentiating dendritic cells (DC) from human leukocyte antigen (HLA)-A\*0201<sup>+</sup> iPS cells (iPS cell-derived DC (ipDC)), using protocols compliant with their subsequent clinical application. Unlike moDC, a subset of ipDC was found to coexpress CD141 and XCR1 that have been shown previously to define the human equivalent of mouse CD8 $\alpha$ <sup>+</sup> DC, in which the capacity for cross-presentation has been shown to reside. Accordingly, ipDC were able to cross-present the TAA, Melan A, to a CD8<sup>+</sup> T-cell clone and stimulate primary Melan A-specific responses among naïve T cells from an HLA-A\*0201<sup>+</sup> donor. Given that CD141<sup>+</sup>XCR1<sup>+</sup> DC are present in peripheral blood in trace numbers that preclude their clinical application, the ability to generate a potentially unlimited source from iPS cells offers the possibility of harnessing their capacity for cross-priming of cytotoxic T lymphocytes for the induction of tumour-specific immune responses.

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**Keywords:** dendritic cells; induced pluripotent stem cells; tumour immunotherapy; cross-presentation

## INTRODUCTION

The use of dendritic cells (DC) to prime responses to tumour-associated antigens (TAA) provides a promising approach to cancer immunotherapy,<sup>1</sup> but clinically relevant responses have frequently been disappointing<sup>2–4</sup> partly due to the properties of the DC commonly employed. Monocyte-derived DC (moDC) show significant donor-to-donor variation, frequently compounded by the side effects of ongoing chemotherapy. Furthermore, moDC display a limited capacity for cross-priming of antigen-specific CD8<sup>+</sup> T cells, creating a dependence on exogenous peptides and further restricting the scope of such an approach to those human leukocyte antigen (HLA) haplotypes for which the immunodominant epitopes are known. As CD8 $\alpha$ <sup>+</sup> DC in mice are peculiarly capable of cross-presentation, the recent identification of CD141<sup>+</sup>XCR1<sup>+</sup> DC as their functional equivalent in humans<sup>5,6</sup> has suggested that this subset may be better suited to the induction of antitumor responses.<sup>1,7</sup> Nevertheless, the trace number of such cells present in peripheral blood<sup>8</sup> and the low yields obtained following the *in vitro* culture of cord blood progenitors,<sup>9</sup> poses a substantial obstacle to their clinical application.<sup>10</sup> Given the recent demonstration that human somatic cells may be reprogrammed to pluripotency,<sup>11,12</sup> we have developed protocols for the differentiation of CD141<sup>+</sup>XCR1<sup>+</sup> DC from human-induced pluripotent stem (iPS) cells under conditions compliant with their subsequent clinical use. Our results demonstrate the feasibility of creating a potentially unlimited supply of autologous DC capable of the cross-presentation of TAA for cancer immunotherapy.

## RESULTS AND DISCUSSION

Differentiation of clinical-grade DC from human iPS cells

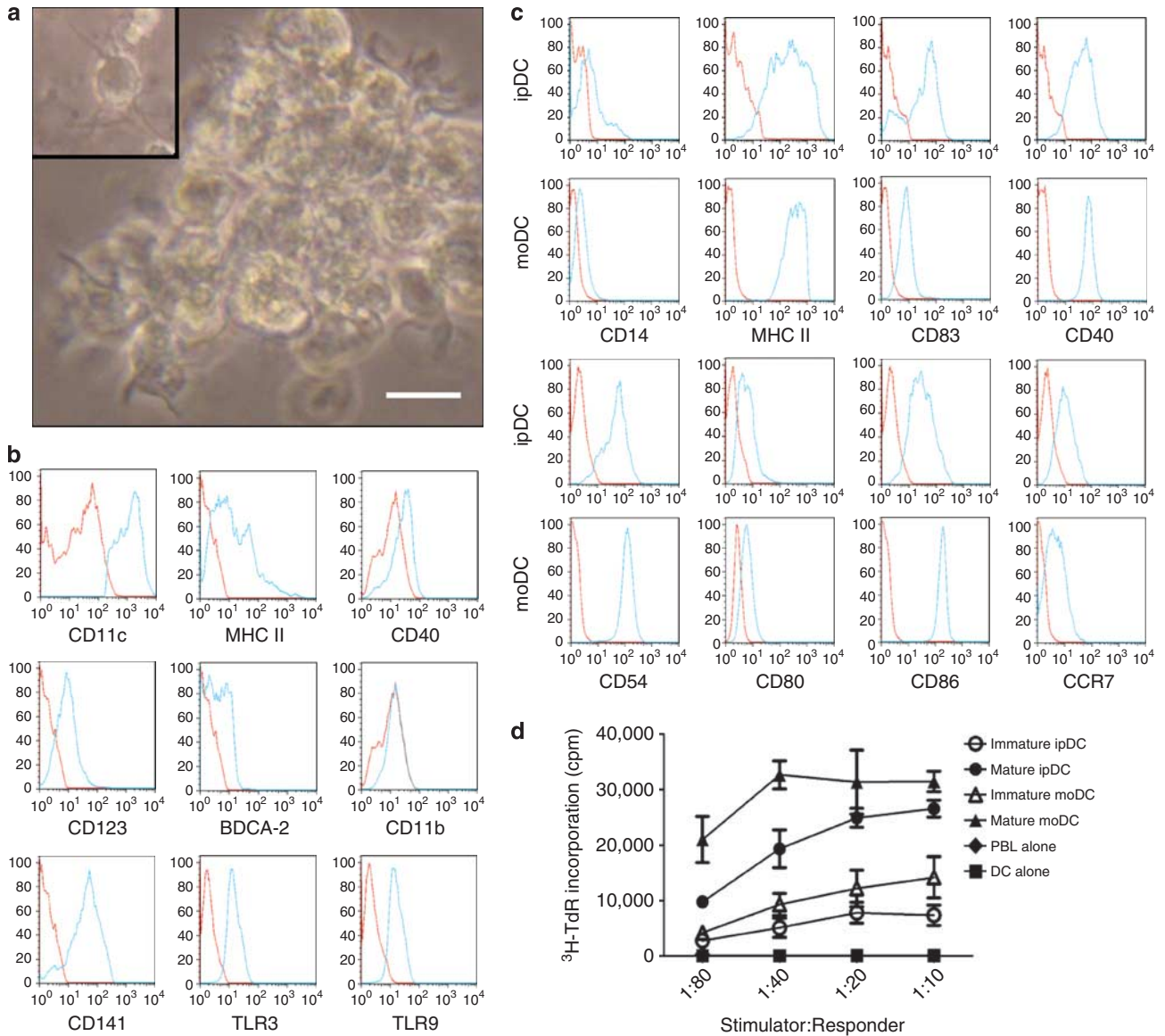
The C15 and C19 iPS cell lines were derived from the dermal fibroblasts of an HLA-A\*0201<sup>+</sup> donor as described<sup>13</sup> and were maintained long-term under feeder- and serum-free conditions. Undifferentiated cells displayed the morphology and high nucleus:cytoplasm ratio characteristic of human embryonic stem (ES) cells (Supplementary Figure 1a) and expressed the pluripotency-associated surface markers SSEA-4 and TRA1-60, together with the transcription factors Oct-4 and Nanog (Supplementary Figure 1b), known to be fundamental to the maintenance of pluripotency among ES cells. Human iPS cells formed conventional teratomas in immune-compromised mice<sup>13</sup> and differentiated *in vitro* into embryoid bodies that, when plated onto tissue culture plastic, differentiated spontaneously into cell types derived from each of the three embryonic germ layers. Staining for the transcription factor SOX17 revealed endoderm commitment, while the presence of CD34<sup>+</sup> hematopoietic cells provided evidence of mesoderm commitment and expression of  $\beta$ III tubulin showed the presence of nascent neurons, consistent with ectoderm differentiation (Supplementary Figure 1c).

Although conventional DC have been differentiated previously from human iPS cells,<sup>14,15</sup> the reliance on animal products and the mouse OP-9 stromal cell line precludes their downstream clinical application. We therefore directed the differentiation of C19 towards the DC lineage by adapting protocols we had established previously for the differentiation of DC from human ES cells in the absence of animal products with the sole exception of matrigel, required for the routine passage of undifferentiated

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**Figure 1.** Characterisation of DC differentiated from human iPS cells. **(a)** Phase contrast micrograph showing a cluster of ipDC and the morphology of individual cells (inset). Scale bar: 40  $\mu$ m. **(b)** Expression of DC-associated markers by ipDC (blue histograms) compared with isotype-matched controls (red histograms). **(c)** Comparison of the phenotype of ipDC and moDC following maturation with a cytokine cocktail. DC were gated as the CD11c<sup>hi</sup> population and dead cells were excluded using 7-AAD. **(d)** ipDC stimulate proliferative responses among naïve allogeneic T cells. DC were mitomycin C (MMC) treated, washed and titrated into cultures of peripheral blood lymphocytes (PBL) from an allogeneic donor at a top stimulator:responder ratio of 1:10. Wells were pulsed with <sup>3</sup>H-thymidine on day 4 and harvested 18 h later. Values of c.p.m. represent the average of triplicate cultures  $\pm$  s.d. Data are representative of three independent experiments.

ES cells and iPS cells.<sup>16</sup> After 19 days, cultures contained cells with distinctive dendritic morphology (Figure 1a), expressing CD11c and low levels of both MHC class II and CD40, suggestive of an immature phenotype (Figure 1b). iPS cell-derived DC (ipDC) could also be differentiated from C15, an independently derived iPS cell line, using the same protocol. Furthermore, although our original experiments were dependent on the use of matrigel for the routine passage of the iPS cell lines, C19 maintained on xeno-free Corning Synthemax culture plates (Corning, Lowell, MA, USA) were found to differentiate efficiently into ipDC, making our protocol fully compliant with clinical applications.

To assess their capacity for maturation, ipDC were cultured with a maturation cocktail consisting of tumour necrosis factor- $\alpha$ , prostaglandin E<sub>2</sub>, interleukin (IL)-1 $\beta$  and interferon (IFN)- $\gamma$ , shown to be effective for the maturation of DC differentiated from human

ES cells.<sup>16</sup> Following exposure to this maturation cocktail, ipDC secreted high concentrations of the pro-inflammatory cytokine IL-6 (Supplementary Figure 2a). Furthermore, upon maturation, ipDC lost expression of CD14 while upregulating MHC class II, CD83, conventional costimulatory molecules and the chemokine receptor, CCR7, yielding a phenotype similar to that of mature moDC (Figure 1c). Indeed, like their counterparts, ipDC stimulated potent proliferative responses among naïve allogeneic T cells in a dose-dependent manner (Figure 1d).

ipDC display the phenotype and function of cross-presenting DC. Despite these similarities, ipDC differed phenotypically from moDC: while they lacked significant expression of the plasmacytoid DC markers CD123 and BDCA-2, ipDC displayed a

CD11b<sup>lo</sup>CD141<sup>hi</sup> phenotype (Figure 1b), reminiscent of the recently described population of cross-presenting DC in human blood and secondary lymphoid organs.<sup>5,6,8,9</sup> Moreover, consistent with initial descriptions of this novel population, ipDC expressed intracellular Toll-like receptor (TLR) 3 (Figure 1b) and secreted high levels of IL-6 in response to poly(I:C), a specific agonist of TLR3, and the TLR7/8 agonist R848 (Supplementary Figures 2b and c). Interestingly, ipDC also expressed TLR9 (Figure 1b) and, unlike moDC, responded to its ligation by the CpG-containing oligodeoxynucleotide ODN2216 (Supplementary Figure 2d); although expression of TLR9 by CD141<sup>+</sup> DC in human blood and lymphoid tissues was not detected, its expression by CD8 $\alpha$ <sup>+</sup> DC in the mouse has been described.<sup>10,17</sup> Furthermore, the ligation of TLR3 and TLR9 has been shown to specifically enhance cross-presentation of antigen by mouse DC.<sup>17-19</sup>

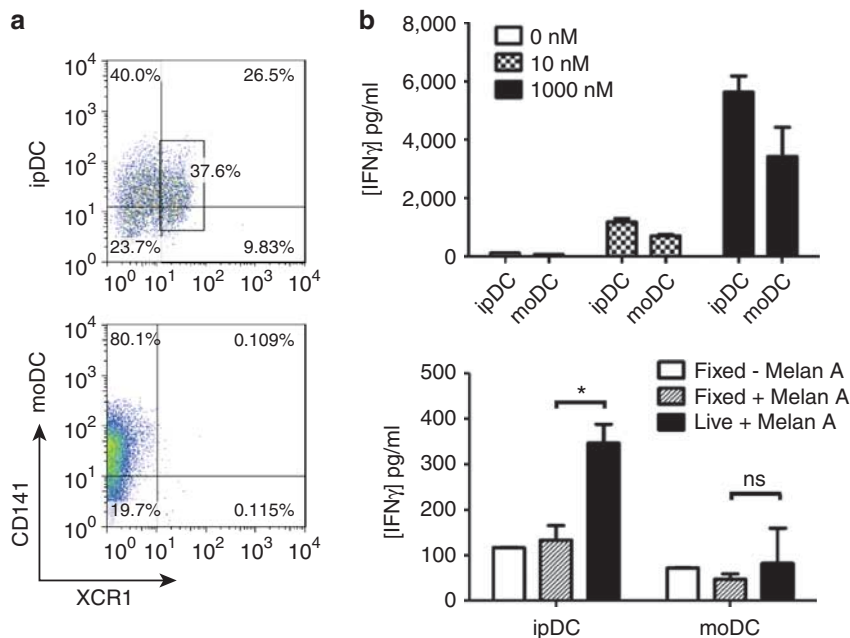
In the light of these findings, we investigated expression of the chemokine receptor XCR1. Although various DC populations are known to upregulate CD141 in culture,<sup>9</sup> XCR1 expression has been shown to be highly specific for cross-presenting DC in both mouse and human, and to augment the antigen-driven expansion of CD8<sup>+</sup> cytotoxic T lymphocytes.<sup>5,6,20</sup> Although moDC failed to express this chemokine receptor, ipDC consistently contained a discrete population of cells coexpressing CD141 and XCR1 (Figure 2a). The CD141<sup>+</sup>XCR1<sup>-</sup> subset is highly reminiscent of DC differentiated from human ES cells,<sup>16</sup> which, like moDC, upregulate CD141 in culture but consistently fail to express XCR1. Given that a subset of ipDC differs phenotypically from their human ES cell-derived counterparts, we investigated the capacity of ipDC to cross-present antigen using the melanoma-specific TAA, Melan A. When pulsed with the HLA-A\*0201-restricted peptide from Melan A (Melan A<sub>26-35</sub>), both moDC and ipDC stimulated IFN- $\gamma$  production by the CD8<sup>+</sup> T-cell clone, 2D10, in a peptide concentration-dependent manner. However, when pulsed with recombinant Melan A protein as a source of unprocessed antigen, only ipDC were able to cross-present the Melan A<sub>26-35</sub> epitope (Figure 2b) to the T-cell clone. Although the capacity for

cross-presentation is most likely to reside within the XCR1<sup>+</sup> subset of ipDC, our attempts to sort the population according to XCR1 expression consistently induced their apoptosis, making it impossible for us to formally exclude any contribution from the CD141<sup>+</sup>XCR1<sup>-</sup> subset. Nevertheless, although the exact identity of cross-presenting ipDC remains to be confirmed, our results strongly suggest the presence within our cultures of the human counterparts of murine CD8 $\alpha$ <sup>+</sup> DC.

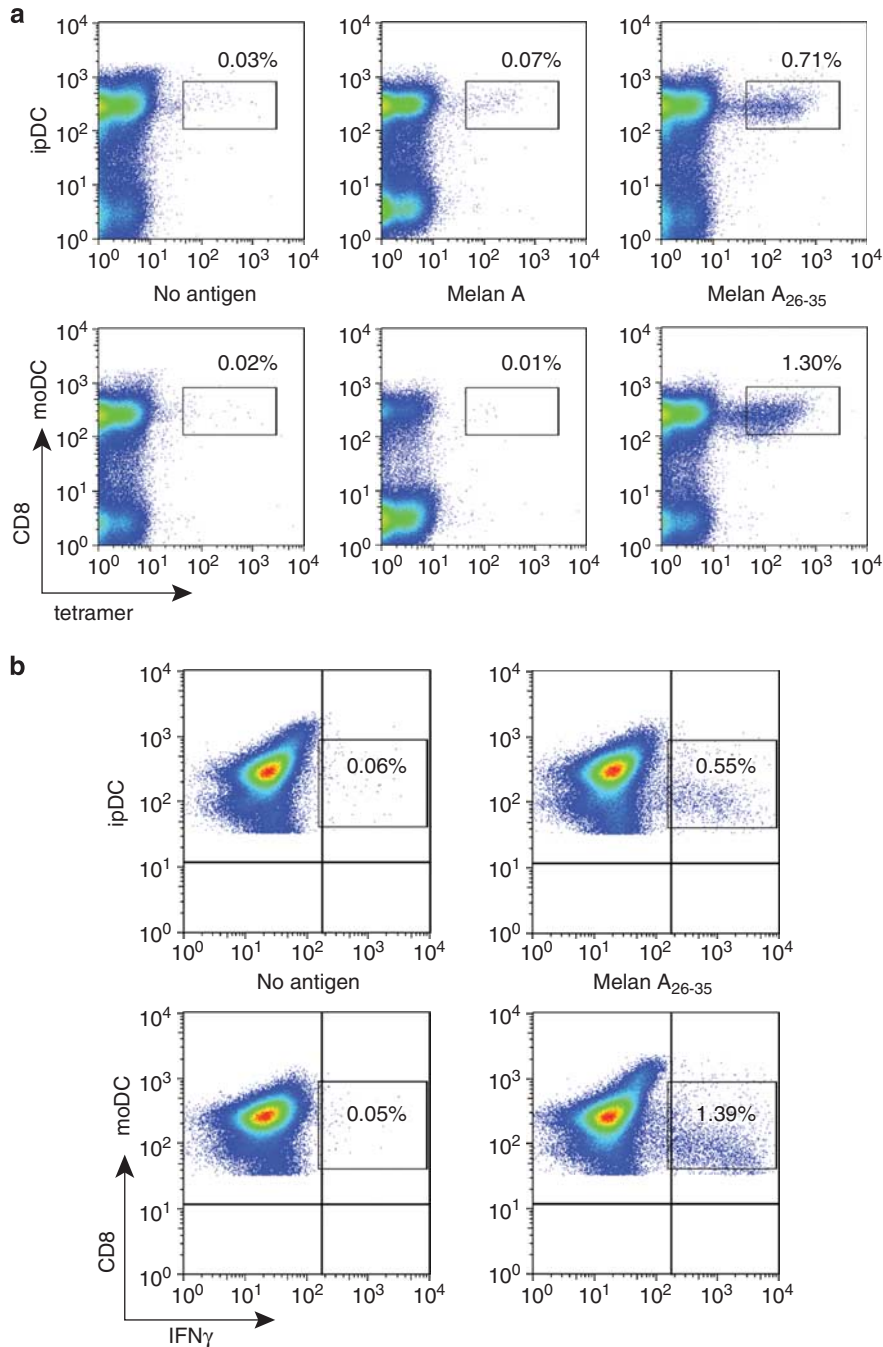
#### Stimulation of primary TAA-specific responses by ipDC

To determine whether ipDC could prime TAA-specific responses among naive T cells, we pulsed moDC and ipDC with Melan A<sub>26-35</sub> peptide and cultured them with T cells depleted of CD45RO<sup>+</sup> memory cells, purified from an HLA-A\*0201<sup>+</sup> donor. After 14 days, T cells were stained for CD8 and with HLA-A\*0201-Melan A<sub>26-35</sub> tetramers to detect antigen-specific T cells.<sup>21</sup> Although both moDC and ipDC primed Melan A-specific CD8<sup>+</sup> T cells when pulsed with the immunodominant epitope, only ipDC elicited a small but reproducible response when pulsed with whole Melan A protein, consistent with their capacity to cross-prime (Figure 3a). Importantly, T cells stimulated with antigen-pulsed moDC or ipDC upregulated IFN- $\gamma$ , consistent with their activation (Figure 3b). Although we consistently showed the appearance of a modest population of antigen-specific T cells in co-cultures pulsed with whole Melan A, our data narrowly failed to reach statistical significance, most likely due to the expansion of residual alloreactive T cells and those specific for other Melan A-derived epitopes, thereby diluting the antigen-specific response.

While identification of the human equivalent of murine CD8 $\alpha$ <sup>+</sup> DC offers promise for their use in cancer immunotherapy, their presence in peripheral blood at a frequency of 1:10 000 mononuclear cells<sup>9</sup> has so far posed an insurmountable barrier to their clinical application.<sup>10</sup> We have employed human iPS cells, capable of indefinite self-renewal, to generate potentially unlimited numbers of such cells *in vitro*, using protocols compatible with



**Figure 2.** Cross-presentation of TAA by ipDC. (a) Cultures of ipDC, but not moDC, contain a discrete population of cells coexpressing CD141 and XCR1. DC were gated on the CD11c<sup>hi</sup> population. Data are representative of four independent experiments. (b) Whereas both HLA-A\*0201<sup>+</sup> ipDC and moDC pulsed with the Melan A<sub>26-35</sub> peptide elicited IFN- $\gamma$  production from the CD8<sup>+</sup> T-cell clone 2D10 (top panel), when incubated with 1  $\mu$ M Melan A protein only ipDC were able to cross-present the epitope to 2D10 (bottom panel). Bars represent the mean of replicate cultures  $\pm$  s.d. Statistical significance was calculated using an unpaired, two-tailed Student's *t*-test. \**P* < 0.03; ns: not significant. Data are representative of three independent experiments.



**Figure 3.** Priming of Melan A-specific T-cell responses by ipDC. **(a)** ipDC cross-prime naïve Melan A-specific T cells from an HLA-A\*0201<sup>+</sup> donor. T cells were costained for CD8 and the relevant TCR using a HLA-A\*0201-Melan A<sub>26-35</sub> tetramer. **(b)** Priming of CD8<sup>+</sup> T cells by ipDC elicits IFN- $\gamma$  production. Following *in vitro* culture of naïve T cells with either unpulsed or Melan A<sub>26-35</sub> pulsed DC, cells were restimulated with tetramer and peptide, and stained for surface CD8 and intracellular IFN- $\gamma$ . Dead cells were excluded using 7-AAD staining. Data are representative of three independent experiments.

their future clinical use.<sup>16</sup> Although significant progress has been made towards the establishment of a xeno-free culture system for the differentiation of conventional DC from human iPS cells, only certain iPS cell lines proved permissive, and viability was reported to be low.<sup>22</sup> In contrast, our own protocols show no compromise of viability and are fully compatible with the generation of a subset of DC normally found *in vivo* in trace numbers. Although the preferred use of genetic modification for reprogramming of somatic cells to pluripotency currently remains an obstacle to the

therapeutic use of ipDC, the advent of more efficient non-genetic approaches to reprogramming suggests that they may offer important clinical applications in the future.

The ongoing construction of extensive banks of clinically approved iPS cell lines, covering the common HLA haplotypes, may permit the matching of individual patients with lines expressing appropriate MHC restriction elements for the relevant TAA. Semi-allogeneic DC differentiated from mouse ES cells have been shown to stimulate potent antitumour responses in mice,<sup>23</sup>

indeed the stimulation of alloreactive T cells may provide a cytokine milieu conducive to the priming of naïve T cells specific for TAA.<sup>24</sup> In this respect, the secretion of high levels of IL-6 by ipDC in response to the maturation cocktail, augurs well for breaking the regulatory T-cell barrier<sup>25–27</sup> that constitutes one of the greatest obstacles to the induction of antitumour responses.<sup>1,28</sup> Although the selection of iPS cell lines from a pre-existing bank may prove to be the most pragmatic approach to their clinical application, where appropriate lines are not available, iPS cell technology offers the additional prospect of generating CD141<sup>+</sup>XCR1<sup>+</sup> DC in a fully autologous manner.<sup>29</sup>

## MATERIALS AND METHODS

### Derivation and culture of human iPS cells

The C15 and C19 human iPS cell lines were derived and maintained, and their pluripotency assessed as described.<sup>13</sup> Ethical approval was granted by the NHS National Research Ethics Service. Cells were cultured in six-well plates in a volume of 4 ml per well and the medium was replaced daily, except on the day following their passage. Before use, plates were coated with matrigel (BD Biosciences, Oxford, UK) diluted 1:30 and stored at 4 °C until use. Alternatively, Corning Synthemax Surface six-well plates were used for maintenance of the iPS cell lines, thereby avoiding the requirement for matrigel. Human iPS cells cultured in mTeSR1 (StemCell Technologies, Genoble, France) were routinely passaged every 6–7 days at a 1:12 dilution, while human iPS cells maintained in TeSR2 (StemCell Technologies) were passaged every 5–6 days at a dilution of 1:12–1:15.

### Directed differentiation of DC

DC were differentiated from human iPS cells by adapting protocols described for human ES cells.<sup>16</sup> Briefly, the differentiation medium consisted of XVIVO-15 (Lonza, Slough, UK) supplemented with non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate (PAA Laboratories GmbH, Yeovil, UK),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma, Gillingham, UK), granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng ml<sup>-1</sup>; Peprotech, London, UK), stem cell factor (20 ng ml<sup>-1</sup>; R&D Systems, Abingdon, UK), vascular endothelial growth factor (50 ng ml<sup>-1</sup>; Peprotech) and bone morphogenetic protein-4 (50 ng ml<sup>-1</sup>; R&D Systems) that were successively removed from cultures until only GM-CSF remained. Bone morphogenetic protein-4 was removed from day 5 onwards, followed by VEGF (day 14) and stem cell factor (day 19). On days 13–17 of culture, the medium was supplemented with 25 ng ml<sup>-1</sup> of recombinant human IL-4 (Peprotech). The concentration of IL-4 in the medium was increased to 100 ng ml<sup>-1</sup> as DC accumulated in the cultures (days 20–24). DC were harvested on days 24–28 using gentle pipetting. Each six-well plate yielded, on average,  $7.2 \pm 0.3 \times 10^5$  (s.e.m.) ipDC that were passed through a 70- $\mu$ m cell strainer (BD Falcon, Oxford, UK) and plated at  $5 \times 10^5$ – $1 \times 10^6$  per well of a six-well CellBind plate (Corning) in complete XVIVO-15 medium containing 50 ng ml<sup>-1</sup> GM-CSF and 100 ng ml<sup>-1</sup> IL-4. After 2–4 days, DC were matured for the final 48 h of culture as described,<sup>16</sup> with a cocktail of cytokines consisting of tumour necrosis factor- $\alpha$  (50 ng ml<sup>-1</sup>; R&D Systems), prostaglandin E<sub>2</sub> (1  $\mu$ g ml<sup>-1</sup>; Sigma), IL-1 $\beta$  (10 ng ml<sup>-1</sup>; R&D Systems) and IFN- $\gamma$  (20 ng ml<sup>-1</sup>; R&D Systems).

### Isolation and culture of primary cells

Monocytes and naïve T cells were isolated from the peripheral blood mononuclear cells of buffy coats (NHS blood transfusion service) or the fresh blood of volunteers, under informed consent, using CD14-coated beads or a pan T-cell selection kit followed by depletion of CD45RO<sup>+</sup> memory cells using the relevant selection kits/beads (Miltenyi Biotech, Woking, UK). CD4<sup>+</sup> T cells were not excluded from the responder population in order to provide a potential source of T-cell help. AutoMACs separation was used to either positively select or deplete labelled populations of peripheral blood mononuclear cells, according to the manufacturer's instructions. Following removal of CD14<sup>+</sup> monocytes, peripheral blood lymphocytes were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal calf serum,

50 U ml<sup>-1</sup> penicillin, 50  $\mu$ g ml<sup>-1</sup> streptomycin and 2 mM L-glutamine (PAA Laboratories GmbH) (R10). MoDC were differentiated by culturing CD14<sup>+</sup> monocytes in R10 containing 50 ng ml<sup>-1</sup> GM-CSF and 100 ng ml<sup>-1</sup> IL-4 for 6–8 days. Although the resulting moDC upregulated CD141 in culture, we were consistently unable to detect XCR1 expression at either the mRNA or protein level, even after prolonged culture for 14 days. This phenotype is consistent with the inability of moDC to cross-present antigen and endorses their use as a control population.

### *In vitro* priming of naïve antigen-specific T cells

Harvested HLA-A\*0201<sup>+</sup> moDC and ipDC were either untreated or pulsed with 1  $\mu$ M Melan A<sub>26–35</sub> peptide for 2½–3 h and washed. DC were plated at  $4 \times 10^4$  per well of a 48-well plate or  $1 \times 10^5$  per well of a 24-well plate, and unpulsed cells were either treated with 1  $\mu$ M whole Melan A protein or left untreated. DC were cultured with HLA-A\*0201-restricted naïve T cells from a different donor to the moDC to yield a ratio of 1:10 DC:T cells. Cells were cultured at 37 °C, 5% CO<sub>2</sub> for 13–14 days in RPMI containing 5% human male AB serum (Sigma), 50 U ml<sup>-1</sup> penicillin, 50  $\mu$ g ml<sup>-1</sup> streptomycin and 2 mM L-glutamine. Recombinant human IL-2 was added at 10 U ml<sup>-1</sup> from days 4–7, and T cells were expanded using 500 U ml<sup>-1</sup> recombinant human IL-2 for the remainder of the culture period.

### Flow cytometry

DC or human iPS cells were incubated for 15 min on ice in blocking buffer (phosphate-buffered saline, 5% normal rabbit serum 0.5% bovine serum albumin, 0.1% NaN<sub>3</sub>) and washed twice. Cells were stained on ice in phosphate-buffered saline/2% fetal calf serum for 30 min with one of the following antibodies: TRA1-60 (Millipore, Watford, UK), SSEA-4 (clone: MC-813-70, R&D Systems), HLA-A2 (BB7.2), CD11c (BU15), CD11b (ICRF44), HLA-DR/DQ/DP (WR18), CD40 (LOB7/6) (AbD Serotec, Kidlington, UK), CD123 (9F5, BD Pharmingen, Oxford, UK), BDCA-4 (446921), BDCA-2 (polyclonal goat IgG), BDCA-3 (501733) (R&D Systems), CD83 (HB15e), CD80 (MEM-233), CD86 (BU63), CD14 (MEM18), CD54 (15.2, AbD Serotec), CCR7 (3D12, eBioscience, Hatfield, UK), XCR1 (polyclonal goat IgG; R&D Systems). For the final 10 min, 7-AAD was added at a concentration of 250 ng ml<sup>-1</sup>. Cells were washed twice and fixed in 2% formaldehyde.

Intranuclear staining was performed using commercial permeabilisation and fixation buffers (eBioscience), according to the manufacturer's instructions together with antibodies specific for the transcription factors Oct-4 (240408) or Nanog (polyclonal goat IgG; R&D Systems). T cells were labelled with tetramer as described<sup>21</sup> and stained for intracellular cytokines. Briefly, T cells were first labelled with HLA-A\*0201-MelanA<sub>26–35</sub> tetramer and were untreated or stimulated with either 20  $\mu$ M Melan A<sub>26–35</sub> peptide or 50 ng ml<sup>-1</sup> PMA and 500  $\mu$ g ml<sup>-1</sup> ionomycin (Sigma) for 6 h, as a positive control. For the final 4 h of culture, 10  $\mu$ g ml<sup>-1</sup> Brefeldin A (Sigma) was added. Cells were washed, surface stained for CD8 and fixed in 2% paraformaldehyde. Buffer containing saponin (phosphate-buffered saline, 0.5% saponin, 0.5% bovine serum albumin, NaN<sub>3</sub>; Sigma) was used to wash cells twice before staining for IFN- $\gamma$  (25723, R&D Systems). Cells were analysed on a Beckton Dickinson FACScalibur (BD Biosciences).

### Antigen processing and presentation

DC were harvested and either untreated or fixed with 0.5% paraformaldehyde for 10 min at room temperature. DC were plated at  $1$ – $1.5 \times 10^4$  cells per well in a 96-well flat-bottomed plate. Fixed or unfixed cells were left untreated or incubated with either 1  $\mu$ M Melan A (AMS Biotechnology, Abingdon, UK) or, as a positive control for antigen presentation, pulsed with either 10 nM or 1  $\mu$ M Melan A<sub>26–35</sub> peptide (ELAGIGILTV).<sup>21</sup> The CD8<sup>+</sup> T-cell clone, 2D10, which is specific for the HLA-A\*0201-Melan A<sub>26–35</sub> complex was plated at a 1:5 ratio of DC:T cells. Co-cultures were incubated at 37 °C, 5% CO<sub>2</sub> for 40 h, before supernatants were harvested.

### Enzyme-linked immunosorbent assays

IFN- $\gamma$  and IL-6 ready-SET-go enzyme-linked immunosorbent assay (ELISA) kits and immunosorb plates were purchased from eBioscience and ELISAs

were performed according to the manufacturer's instructions. ELISAs were read at 450 nm using a BioTek ELx808 plate reader (BioTek, Potton, UK).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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