Generation of Dendritic Cells from Human Cell Expressed Cytokines

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INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells. Described as "pacemakers" of the immune system, they comprise a system of leukocytes widely distributed in all tissues essential for the initiation and maintenance of adaptive immunity[1]. DC are actively involved in autoimmune diseases, graft rejection, HIV infection and the formation of T-cell dependent antibodies[2]. They are also the principal stimulator of primary mixed leukocyte reactions (MLR)[3-4].

DC represent only 0.1% of all leukocytes[5]. The scarcity of these cells makes it difficult to isolate them in large numbers. Intensive efforts have been made to generate these cells *in vitro* from precursor cells such as $CD34^+$ cells or monocytes, primarily through the use of specific cytokines. These cytokines include GM-CSF in combination with IL-4 or TNF-α[6].

Conventionally, human cytokines have been expressed in non-human cells such as yeast, bacteria and murine expression systems. However, the biological importance of species-specific post-translational modifications, in particular glycosylation, is essential to protein function. We have purified human cell expressed (hcx™) cytokines from modified human 293 cells. Our aim was to use hcx GM-CSF, IL-4 and TNF-α to generate human monocyte-derived DC and compare the resulting DC phenotype and biological function to that of DC generated by cytokines expressed in *E. coli*. The phenotype was characterised by flow cytometry, while functionality was assessed in an allogeneic MLR assay. *In vitro* comparisons of the biological activity of IL-4hcx with IL-4 expressed in *E. coli* revealed that IL-4hcx is more stable.

MATERIALS AND METHODS

Preparation of dendritic cells

Peripheral blood mononuclear cells (PBMC) were purified from buffy coat from 5 different donors (purchased from Australian Red Cross Blood Services, Sydney) by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. Monocytes were purified from the PBMC by adherence to plastic in AIM-V Medium (GIBCO/Life Technologies, Baltimore USA), and non-adherent cells were removed. Dendritic cells (DC) were derived by culture in AIM-V medium containing IL-4 (30 ng/ml) and GM-CSF (50 ng/ml) for 5 days at 37 °C/5 % CO_2, followed by addition of TNF-α (12.5 ng/ml) for a further 2 days. *E. coli* expressed GM-CSF, IL-4 and TNF-α were purchased from either Peprotech (Rehovot, Israel) or R&D Systems (Minneapolis, USA).

Flow cytometry analysis

After 7 days in culture, cells were washed, dislodged and stained with the following panel of fluorochrome-labelled antibodies to dendritic cell markers: CD1a-PE, CD14-FITC, CD209-PE, HLA-DR-FITC, CD80-PE, CD40-FITC, CD83-PE, CD86-FITC, CD123-PE, CD18-FITC, CD11b-PE and appropriate negative control antibodies (IgG1, IgG2b). Antibodies were purchased from BD Pharmingen (USA) and BioLegend (San Diego, USA). 10,000 cells were collected using a FACSCalibur (BD) and analysed with Cell Quest software.

Isolation of $CD4^+$ T cells

Following adherence of monocytes, any non-adherent PBMC were collected and cryopreserved. PBMC from an allogeneic donor were subsequently thawed and $CD4^+$ T cells were purified by negative selection using a MACS separation column (Miltenyi Biotech, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were at least 85% pure and >95 % viable (Figure 3).

Allogeneic mixed leukocyte reaction

Before the assay, dendritic cells were treated for 1 h at 37 °C with 50 µg/ml Mitomycin C (BIOMOL, Germany), washed in AIM-V and dislodged. $CD4^+$ T cells and dendritic cells were plated together in 100 µl of AIM-V medium in 96 well round-bottom plates at a final cell number of 4 x 10^5 allogeneic $CD4^+$ T cells and 8 x 10^3 DC (a ratio of 50:1) per well. Cells were incubated together for 5 days at 37 °C/5 % CO_2, after which cell proliferation was assessed using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay: Promega).

Bioactivity of IL-4hcx

Bioactivity of IL-4 was measured in cell proliferation assays using the human TF-1 cell line. IL-4 was pre-incubated with cell culture medium (RPMI/10% FBS) for 4 days at 37 °C before addition to the assay. N-linked sugars were removed by PNGase F (Roche) digestion of IL-4 for 16 h, and the enzyme was removed by size exclusion chromatography. Cells were subsequently incubated in 96-well tissue culture plates with serial dilutions (0-2.5 ng/ml) of the pre-incubated IL-4 for a further 3 days at 37 °C, after which cell viability was measured using the MTS assay. ED_{50} and p-values were determined from triplicate data points using the statistical software package "R" with the add-on called "drc[7]". Data is representative of at least 3 experiments.

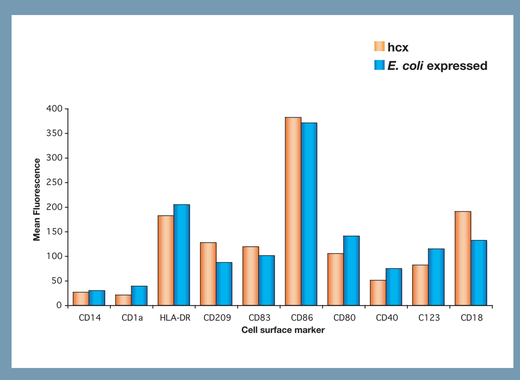

Figure 1. Expression of dendritic cell markers. Data is representative of all experiments.

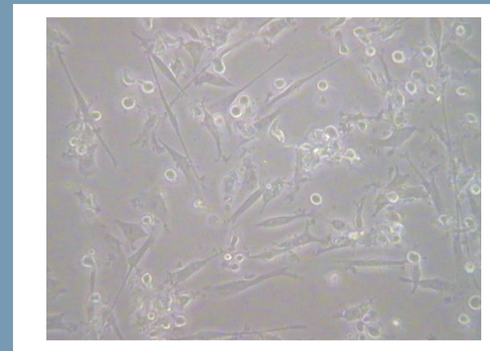

Figure 2. Adherent monocytes cultured with hcx GM-CSF, IL-4 and TNF-α develop dendritic cell morphology as examined by phase-contrast microscopy (20 x magnification).

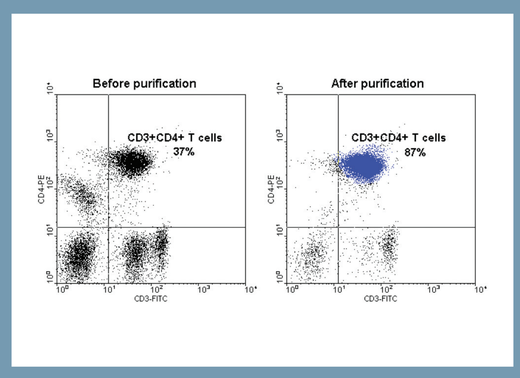

Figure 3. Purification of $CD3^+CD4^+$ T cells by MACS separation.

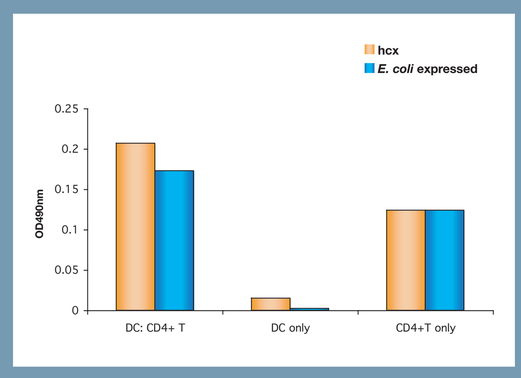

Figure 4. Dendritic cells derived with hcx cytokines are potent mediators of an allogeneic mixed leukocyte reaction.

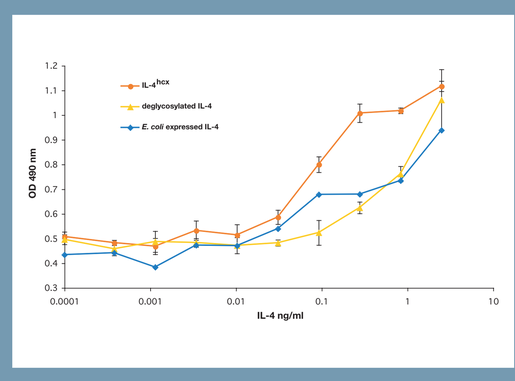

Figure 5. IL-4hcx is more stable in cell culture than *E. coli* expressed IL-4 due to N-linked glycosylation.

RESULTS/DISCUSSION

hcx™ cytokines can be used to derive dendritic cells

The ability of hcx cytokines to induce the differentiation of dendritic cells from peripheral blood monocytes was compared to *E. coli* expressed cytokines. The cells exhibited a mature dendritic cell phenotype, with low expression of CD14 and CD1a (Figure 1), and high expression of CD209, HLA-DR, CD83, CD86 and CD18 amongst other markers. Expression levels were equivalent to those grown with *E. coli* expressed cytokines. The cells also exhibited typical dendritic-cell morphology (Figure 2), with irregular long spindly cytoplasmic projections, prominent nuclei and nucleoli. Results are representative of all donors examined.

Dendritic cells mediate a mixed leukocyte reaction

Dendritic cells derived with hcx™ cytokines were able to stimulate allogeneic T cell proliferation at levels equivalent to *E. coli* expressed cytokines (Figure 4), indicating the DC are functionally active.

IL-4 glycosylation enhances stability

Comparison of IL-4hcx with commercially available rhIL-4 (expressed in *E. coli*) following 4 days pre-incubation in cell culture medium at 37 °C showed that IL-4hcx was 7-fold more active, demonstrating it has greater stability in cell culture medium (Figure 5). Furthermore as *E. coli* does not glycosylate proteins, we investigated whether glycosylation confers increased stability to human cell expressed proteins. The non-glycosylated form of IL-4hcx was 11-fold less active following incubation at 37 °C (Figure 5), indicating that glycosylation confers stability to IL-4hcx and hence enhances the biological activity of IL-4hcx.

CONCLUSION

Human cell expressed cytokines can be used to produce dendritic cells in serum free medium. The dendritic cells are functionally active.
Human cell expressed cytokines are ideally suited to generating dendritic cells *in vitro* as they have:

- Human-specific glycosylation
- No xenogenic material
- Other human-specific post-translational modifications
- Longer half-life in culture

References

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