HUMAN CELL-EXPRESSED IL-12 HAS ENHANCED PRO-INFLAMMATORY ACTIVITY

Rosie Newman, Hui Jiang, Kate Liddell, Linda Crofts, Raina Simpson, and Denese Marks

Apollo Cytokine Research Pty Ltd, Sydney, NSW, Australia
Email: info@apollocytokineresearch.com

Human Proteins, Human Source™

hcx™ Human Cell Expressed

www.apollocytokineresearch.com

INTRODUCTION

- IL-12 is a potent pro-inflammatory cytokine that consists of a heterodimer composed of two disulphide-linked subunits, p35 and p40[1].
- Produced by peripheral blood mononuclear cells, IL-12 plays a central role in the initiation and control of cell-mediated immune responses through its effects on NK cells and T lymphocytes[2].
- IL-12 activates the Jak/STAT pathway via the IL-12 receptor, inducing IFN-γ production. IL-12 enhances the lytic activity of NK and lymphokine-activated killer cells, and induces the proliferation of activated T and NK- cells p40[1].
- Clinically, recombinant human (rh)IL-12 has been evaluated for its therapeutic efficacy in multiple clinical trials in cancer and chronic infections[3].
- Currently, rhIL-12 is produced in non-human cell systems including insect and CHO. However, it is becoming apparent that human-specific post-translational modifications, in particular glycosylation, are important to human protein function.

Aim

We have purified human cell expressed rhIL-12 (IL-12hcx) from modified human 293 cells. Our aim was to compare the glycan structures and *in vitro* biological activities of IL-12hcx to that of CHO-expressed IL-12 (CHO IL-12) in human peripheral mononuclear cells.

METHODS

Characterisation of IL-12hcx

Purified IL-12hcx and CHO IL-12 were subjected to enzymatic treatment for the analysis of glycan structures using LC-MS. N- and O-linked structures were assigned from the acquired data using GlycosidIQ (www.glycosuite.com). The C-mannosylated tryptophan was identified by MS/MS of the peptides in a trypsin-V8 digest of IL-12.

PBMC isolation and activation

Peripheral blood mononuclear cells (PBMC) were purified from healthy donors by density centrifugation using Lymphoprep (Axis-Shield). Cells were activated with 10 μg/ml phytohemagglutin (PHA; Sigma-Aldrich) for a total of 4 days, during which time the cells became lymphoblasts. On day 3, the cultures were split and 10 ng/ml rhIL-2 (R&D Systems) was added to promote lymphoblast proliferation. On day 4, the lymphoblasts were harvested, washed and prepared for IL-12 treatment.

Phosphorylated STAT4 and STAT5 immunoblotting

Lymphoblasts were serum starved for 4 hrs prior to stimulation with 50 ng/ml IL-12 at 37ºC/5% CO_2. Lysates were prepared with 1x passive lysis buffer (Promega), subjected to western blotting and probed with antibodies to phospho-STAT4 (pY693) and total STAT4 (BD Biosciences, Santa Cruz Biotechnologies) or phospho-STAT5 (pY694) and total STAT5 (Cell Signalling). Western blots were analysed with Fuji Film LAS-3000 and quantification of bands by densitometry was performed with MultiGauge software.

IFN-γ detection by ELISA

$2x10^5$ lymphoblasts were treated with IL-12 (0 –10 ng/ml) for 2 days at 37ºC/5% CO_2. Supernatants were collected and IFN-γ concentrations measured using the IFN-γ DuoSet ELISA Development kit (R&D Systems). Data is expressed as mean ± s.d. Statistical significance was evaluated using one-way ANOVA using GraphPad Prism 5.

Proliferation assays

$4x10^4$ lymphoblasts were treated with serial dilutions (0 – 50 ng/ml) of IL-12 for 2 days at 37ºC/5% CO_2. Proliferation was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) and ED_{50}s were determined.

Cytolytic assay: Calcein-AM release

PBMC were stimulated with 1 ng/ml IL-12 for 3 days at 37ºC/5% CO_2 for use as effector cells. K562 cells were labelled with 15 μM Calcein-AM (Molecular Probes) and used as target cells. Effector and target cells were added together in 96-well u-bottom plates and incubated for 4 hrs at 37ºC/5% CO_2 at various effector (E):target (T) ratios. 75 μl of supernatant was then transferred into a new plate and Calcein-AM release was measured using a FLUOstar OPTIMA plate reader at 485nm/520nm (BMG LABTECH). Specific lysis was calculated as: [(test release – spontaneous release)/ (maximum release – spontaneous release)] x 100%.

SUMMARY AND CONCLUSIONS

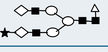
- IL-12hcx has greater biological activity compared to CHO-expressed IL-12. This has been demonstrated by:
  - increased activation of STAT molecules
  - increased production of IFN-γ
  - increased cell proliferation
  - enhanced cytolytic activity of PBMC

These effects may be attributed to the structural differences observed between human and non-human cell expressed IL-12.

- IL-12hcx may provide unique benefits for the study of the role of IL-12 in disease and normal immunity.

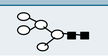
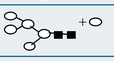
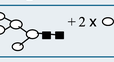
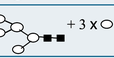
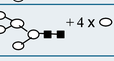
RESULTS AND DISCUSSION

Table 1a. IL-12 N-linked Oligosaccharides (complex and sialylated types)

N-linked Oligosaccharides	% of Total N-oligosaccharides estimated by LC-MS	
	IL-12hcx	CHO IL-12
	0	7
	0	7
	0	4
	0	19
	35	19

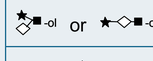
○ Mannose ■ N-Acetylglucosamine
◇ Galactose ★ Sialic acid △ Fucose

Table 1b. IL-12 N-linked Oligosaccharides (high mannose type)

N-linked Oligosaccharides	% of Total N-oligosaccharides estimated by LC-MS	
	IL-12hcx	CHO IL-12
	0	11
	0	9
	22	9
	16	8
	27	7

○ Mannose ■ N-Acetylglucosamine

Table 2. IL-12 O-linked Oligosaccharides

O-linked Oligosaccharides	% of Total O-oligosaccharides estimated by LC-MS	
	IL-12hcx	CHO IL-12
	60	47
	40	53

■-ol N-Acetylglucosamine alditol ◇ Galactose ★ Sialic acid

Glycan strutures of IL-12hcx

Both IL-12hcx and CHO IL-12 contain N and O-linked glycan structures. For N-linked structures, IL-12hcx contains more sialylated and high mannose structures when compared to CHO IL-12 (Table 1a and b). No differences were observed in O-linked structures (Table 2). Both IL-12hcx and CHO IL-12 express C-linked mannosylation (data not shown).

IL-12hcx induces more STAT4 and STAT5 activation than CHO-expressed IL-12

IL-12hcx induced up to 3-fold more STAT4 and 2-fold more STAT5 activation than CHO IL-12 (Figure 1a and b).

IL-12hcx induces more IFN-γ production by lymphoblasts than CHO-expressed IL-12

IL-12 induced dose-dependent IFN-γ production by lymphoblasts. IL-12hcx induced IFN-γ production at concentrations as low as 0.5 ng/ml, whereas CHO IL-12 did not induce IFN-γ below 5 ng/ml. IFN-γ induction by IL-12hcx was significantly higher than CHO IL-12 at 0.5 – 7.5 ng/ml ($p<0.001$; Figure 2).

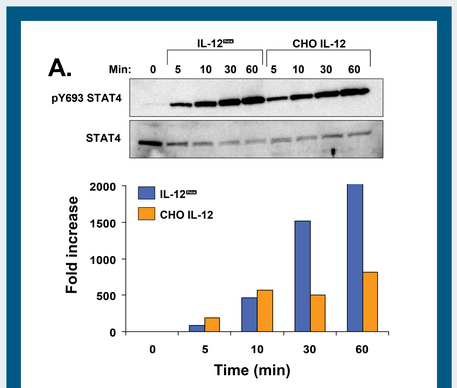


Figure 1. IL-12hcx is a more potent activator of STAT4 (a) and STAT5 (b) signalling than CHO-expressed IL-12 in lymphoblasts. Data are representative of 3 experiments.

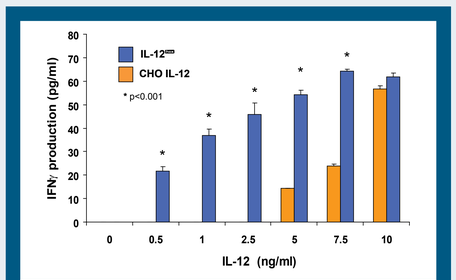


Figure 2. IFN-γ secretion is significantly induced upon IL-12hcx treatment of lymphoblasts. Data are representative of 3 experiments and expressed as mean ± s.d .

IL-12hcx induces more proliferation of lymphoblasts than CHO- expressed IL-12

IL-12hcx was 6-fold more active at inducing lymphoblast proliferation compared to CHO IL-12; ED_{50}: 80 ng/ml v 500 ng/ml (Figure 3).

IL-12hcx enhances lytic activity of PBMC against K562 cells more than CHO-expressed IL-12

IL-12 enhances the lytic activity of PMBC against K562 cells. We demonstrated that this effect was more pronounced in PBMC stimulated with IL-12hcx compared to CHO IL-12 for all E:T ratios (Figure 4).

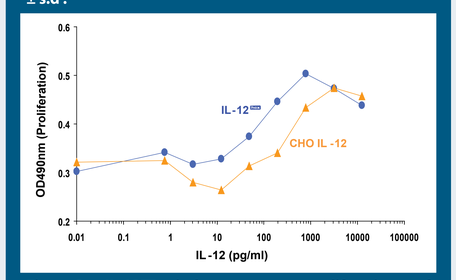


Figure 3. IL-12hcx induces more proliferation of lymphoblasts compared to CHO-expressed IL-12. Data are representative of 3 experiments.

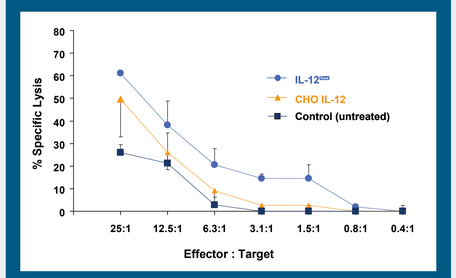


Figure 4. Increased lytic activity of IL-12hcx treated lymphoblasts against K562 cells. Data are representative of 3 experiments and expressed as mean ± s.d.

References

1. Gately *et al.* (1998) Ann.Rev. Immunology, 16, 495-521
2. Chehimi and Trinchieri (1994) J Clin. Immunology, 14, 149-161
3. Del Vecchio *et al.* (2007) Clin. Cancer Res., 16, 4677-4685