ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 6 (2): 225-231, 2007
ISSN 1682-296X

Functions of Human Cytosolic Domain of β-common Subunit Receptor GM-CSF in FDCP-MIX Stem Cells

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Abstract: The mechanisms of cytokines or growth factors mediate the regulation of cell survival, proliferation and differentiation into mature cells remain unclear. The cytokine interleukin-3 and Granulocyte-macrophage Colony Stimulating Factor (GM-CSF) play important roles in the survival, growth and development of haemopoeitic stem cells. Activation of human IL-3 or GM-CSF receptor ectopically expressed in polyclonal murine FDCP-Mix multipotent cell will stimulate self-renewal or myeloid differentiation respectively. Each of the GM-CSF and IL-3 receptors comprises a unique ligand-specific α subunit and a common β subunit (βc). The common βc subunit has a relatively large cytosolic domain compared to α subunit thus considered to play a pivotal role in receptor's signal transduction. In these studies, the chimeric receptor composed of the extracellular domain of GM-CSF and cytosolic domain of βc was generated, i.e., hGM-CSFR/βc. The respective receptor was expressed in the FDCP-Mix stem cells, showed that the receptor was capable in promoting maintenance of primitive phenotype in the absence of α subunit. This result showed that the βc subunit alone could contribute signals principally for proliferation and self-renewal.

Key words: Cytosolic domain, haemopoietic stem cells, human βc subunit, cell's signals

INTRODUCTION

The Factor Dependent Cell Paterson-Mix (FDCP-Mix) cell lines were cloned and isolated from long-term bone marrow cultures infected with a molecular recombinant Rous sarcoma virus and Moloney leukaemia virus (src-MoMuLV) (Spooncer and Dexter, 1997). Unlike any other cell lines, FDCP-Mix cells do not contain the src oncogene sequences nor do they possess any leukaemic characteristics following infection with src-MoMuLV (Mladenovic and Anderson, 1992; Spooncer and Dexter, 1997). *In vitro*, they are also capable of forming spleen colonies in irradiated and reconstitute haemopoiesis in lethally irradiated mice in the absence of IL-3. Unfortunately, these two characteristic are lost within 15 weeks of establishing the cell line but the cell still retain characteristic of primitive progenitor cells after 15 weeks, i.e., the ability to self-renewal and differentiate into multiple lineages (Spooncer and Dexter, 1997).

FDCP-Mix cell lines share many characteristics with normal stem cells, since they are karyotipically stable and maintain a diploid chromosome complement. The respective cells also cytokine dependent and are totally dependent on IL-3 for survival and proliferation. In the presence of IL-3, FDCP-Mix cells proliferate, exhibit immature blast cell morphology and express the cell surface murine stem cell marker that is Sca-1 (Spooncer and Dexter, 1997). Removal of IL-3 will result in cell death via apoptosis (Spooncer and Dexter, 1997; Ekert *et al*., 2006). FDCP-Mix can also be induced to undergo terminal differentiation and clonal extinction in modified culture conditions by manipulating the cytokine combination in which they are cultured. This multipotential cell line is capable of differentiation into granulocytes, macrophages (Spooncer and Dexter, 1997), erythrocytes (Heyworth *et al*., 1995), osteoclast (Petrovic *et al*., 2004), dendritic cells (Schroeder *et al*., 2000) and early B lymphocytes (Spooncer and Dexter, 1997). The capabilities of FDCP-Mix to proliferate and differentiate into various mature cells when culture conditions are modified, means that they represent a suitable and valuable model for the biochemical and molecular analysis of self-renewal and differentiation signals of primitive cells. In our study, we used murine FDCP-Mix cell line as a host model to study the function of human βc cytosolic domain in the absence of β-subunit.

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The GM-CSF, IL-3 and IL-5 receptors are multisubunit receptors in which each subunit is a single spanning transmembrane polypeptide. These receptors comprise a unique ligand specific α subunit and a common β chain referred to as βc. This explain why, that although GM-CSF, IL-3 and IL-5 cytokines have distinct effects on different target cells, all three cytokines still exhibit similar cellular responses and sometimes compete for binding site on the same cells (Lopez *et al*., 1991). The cytoplasmic domain of the receptor subunits that activate signal transduction have limited homology in the membrane-proximal region and referred to as box 1 or box 2 motif (Shahrul Hisham *et al*., 2005).

The βc subunit is considered to play a major role in signal transduction. This is due to longer cytoplasmic domain as compared to α subunit. The βc cytoplasmic domain consisted 420 amino acids, whereas 38-57 amino acids of α-subunit cytoplasmic domain. Like all cytoplasmic domain of Cytokine Receptor Superfamily Class I, the βc subunit does not posses any known intrinsic enzymatic activities. Therefore, association with cytoplasmic tyrosine kinase in the cytoplasmic region are important in triggering cell signals (Shahrul Hisham *et al*., 2005). The main cytoplasmic kinase family is the Janus Kinases Family (JAKs). The JAK member that is activated in response to IL-3, GM-CSF and IL-5 is JAK2 (de Groot *et al*., 1998).

The activation of IL-3, GM-CSF and IL-5 receptors complexes occurs through heterodimerisation of receptor α and βc subunits through non-covalent and covalent process (Stomski *et al*., 1996; Bagley *et al*., 1997). However, the exact subunit stoichiometry of the receptor complexes remains unclear but studies suggest that the active receptor complex consist of heterodimer that are two α-subunits and two β-subunits ($\alpha_2\beta_2$) (Lia *et al*., 1996; D'Andrea and Gonda, 2000). Studies had showed that IL-3, GM-CSF and IL-5 receptor complexes can only phosphorylated in the presence of disulphide-linkage between α and βc subunit (Stomski *et al*., 1996; 1998). Indications that both subunits important in producing cell's signals. Mutation of the βc subunit of either Cysteine 86 or Cysteine 96 resudues located at the extracellular domain to alanine abolished tyrosine phosphorylation of the βc cytoplasmic domain. This is due to abolishment of disulphide cross-linkage formation between the α and β subunits.

In order to determine which subunit is important in producing cell's signals, we generate the chimeric receptor consisting GM-CSF of α subunit portion at the extracelluar domain and the βc portion at the cytoplasmic region, i.e., hGM-CSFR/βc. This chimeric receptor was expressed in the primitive cells; FDCP-Mix cell lines. The observation showed that expression of hGM-CSF/βc clone, i.e., homodimerisation of the cytosolic region of the βc subunits in the presents of its cognate cytokine was able to activate the receptor to trigger cell's signals similar to cells that co-expressed the wild type hGM-CSF α and βc subunit. The absent of cytosolic domain of α-subunit as represented by the chimeric has showed that the cytosolic domain of βc subunit is essential in cell's functions compared to the α-subunit.

MATERIALS AND METHODS

Cells: FDCP-Mix cell cultures were maintained at 37°C in 5% CO_2 with FSS (Gibco, BRL) supplemented with 5% (v/v) murine IL-3, 20% (v/v) Fetal calf serum.

Transfection: FDCP-Mix cell were grown to 90% confluence in culture plates. The cells were rinsed two times with 5 mL Optimem prior to lipofection. Each plate was transfected with plasmid pM5-Neo followed by pM5-Hyg by the lipofections' procedure. Lipofectamine (Gibco, BRL) in Optimem of 11 μL was added to a polystyrene tube containing 4 ug DNA containing plasmids in 200 μL Optimem (Gibco, BRL). After gentle mixing, the mixture was incubated at room temperature for 30 min. Then, an extra 1600 μL Optimem was added to the flask containing 50% confluent cells. The flask was gassed with 5% CO_2 in air and incubated at 37°C for 6 h. The lipofection solution was then replaced with 10 mL of complete fibroblast medium, gassed with 5% CO_2 in air and incubated at 37°C overnight. Selection of transfected cells by G418 or Hygromycin B were done on the following day. Successfully transfected cells were obtained by antibiotic selections over a period of several weeks.

Detection and isolation of cell surface receptors subunits expression: The cell population after the antibiotic selection was analyzed for receptor subunit expression by flow-cytometry on a FACS Vantage flow cytometer. A two-step antibody labeling procedure was used (Evans *et al*., 2002). The cell population expressing the chimeric of hGM-CSFR/βc specifically identified with monoclonal antibodies hGM-CSFRα (0.5 mg mL^{-1}). The monoclonal antibodies were detected by incubating with respective secondary FITC labeled antibody. Population of cells expressing the respective subunits was isolated from the non-expressing cells using the same device, i.e., flow-cytometer.

Generation of clonal cell expressing single chimeric hgm-csfr/βc receptor: Each cell from the receptor subunits expressed populations were sorted out from the rest of cells population by the FACS Vantage flow cytometer into a 96 well plates. The expressed cells were

incubated on 37°C in 5% CO_2 for approximately three weeks with continuous complete media when necessary. Three clonal cells populations were then subjected into receptor surface detections prior to Functional Assay.

Functional assay

Determination of cells viability: The viable cells were assessed with trypan blue (Low Laboratories) and viable cells were determined using an improved Neubauer Haemocytometer.

Differentiation assays or g-Diff condition: Cells in logarithmic growth phase were washed with FSS and resuspended in IMDM supplemented with 20% (v/v) FCS, 0.01 ng mL^{-1} IL-3, 5000 unit mL^{-1} G-CSF and 300 U mL^{-1} GM-CSF. The cells were seeded at 1×10^5 cells mL^{-1} and then incubated at 37°C in 5% CO_2, 5% O_2 (Evans *et al*., 2002). On day 7, a volume containing approximately 1×10^5 cells was removed and cytocentrifuged onto glass slides at 1000 rpm for 5 min. After air dried, slides were subjected into staining using May-Grunwald-Giemsa staining procedures.

RESULTS AND DISCUSSION

Construction of chimeric human βc subunit: Both hGM-CSFRα and hβc initially were cloned at the BamHI sites of multiple cloning sites pUC19. The restriction enzyme site of NheI was introduced at the transmembrane domain of both hGM-CSFR α and hβc via site directed mutagenesis. This based on the hypothesis that transmembrane domain was primarily involved as a passive lipid anchors that holds the receptor in its respective position, i.e., the amino-terminus outside and the carboxyl-terminus inside the cell. The results of the mutagenised subunits are shown as representative of mutagenised region in Fig. 1A. The mutated product was subjected to DNA sequencing for conformation. Both the extracellular domain of human GM-CSF α subunit and the cytosolic domain of human βc were digested using Nhe-1 and Sca-1 followed by purification of interested DNA fragment from the agarose gel, i.e., the fragment consisting extracellular domain of GM-SCF-α-subunit and βc cytosolic domain. The respective fragments were ligated to the cloning vector, pUC19. After that, the recombinant DNA were propagated in *E. coli*. The respective plasmid was digested using BamHI and ligated into retroviral vector pM5-Neo and propagated in *E. coli*. The chimeric product that has been generated was subjected to DNA sequencing for conformation prior to transfection and expression of the respective chimeric into FDCP-Mix cell line. The result representing generated chimeric is shown in Fig. 1B.

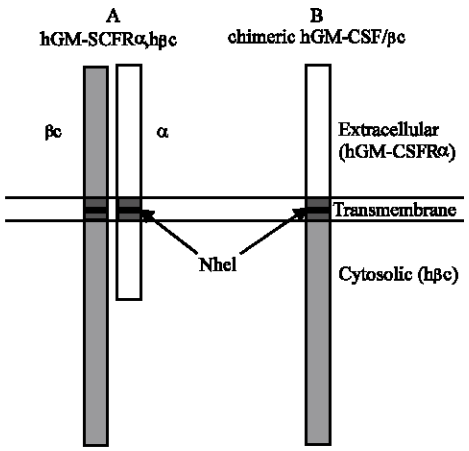

Fig. 1: Schematic representation of mutated and chimeric receptors that were generated. (A) Mutagenated human GM-CSF α-and βc subunits at the transmembrane domain. Both subunits were expressed in the FDCP-Mix cell line. (B) Chimeric subunit generated from the human GM-CSF α (extracellular domain) and human βc (cytosolic domain) subunits. This generated chimeric was transfected and expressed in the FDCP-Mix cell line

Expressions and generations of clonal FDCP-Mix cells: The FDCP-Mix cells that expressed single transfected hGM-CSF/βc polyclonal cells were analysed for receptors expression using flow cytometer. The transfected cells were selected with the antibiotic G418 and analysed using a two-step procedure. The primary antibody was specific to the extracellular domain of hGM-CSF α subunit and was used to label the cells, which were then labelled with rabit anti-mouse IgG conjugated with FITC as secondary antibody. This is followed by generation of clonal cells using the Automated Cell Deposition Unit (ACDU) facility of the flow cytometer. The recovered clonal cell populations were again analysed for extracellular domain expression of the hGM-CSF receptor α-subunit. Three clones were generated and used for further investigation, as representative, one of the three clones is shown in Fig. 2. The parental FDCP-Mix cells showed no increased in fluorescence compared to the presence of labelled antibodies, demonstrating that the hGM-CSF α subunit antibodies and secondary labelling antibodies (anti-mouse FITC) were specific. The hGM-CSFR/βc transfected FDCP-Mix cells (Fig. 2A) showed no significant increase in fluorescence with the secondary anti-mouse FITC antibodies alone (Gray line). The expression of the extracellular domain human α subunit has shown a significant increase in fluorescence (Black

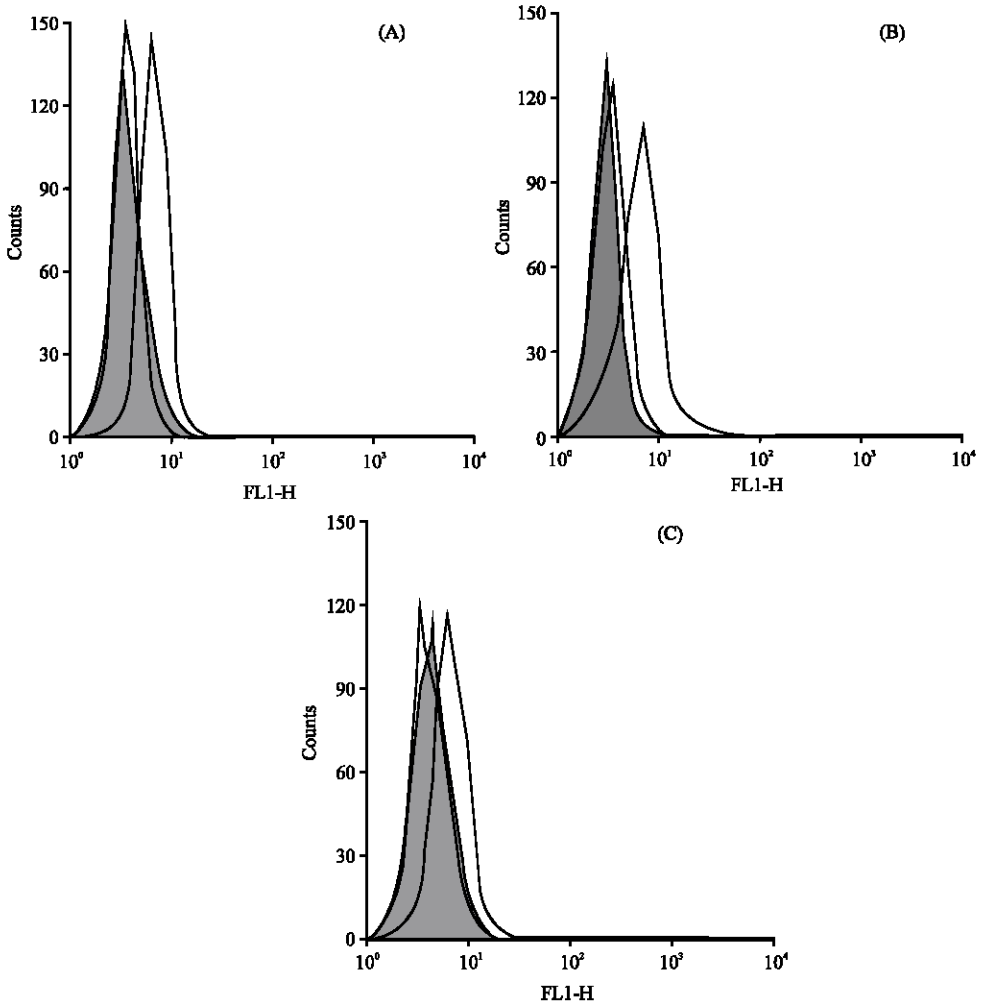

Fig. 2: Flow cytometer profiles of generated clones. The FACS profiles of represented single expressed hGM-CSF/βc clones (A). The FDCP-Mix expressing hβc polyclone (B) was generated prior to transfection of hGM-CSF α subunit. The hGM-CSFRα, hβc polyclones were subjected to FACS vantage flow cytometer to generate clones. Representation of generated hGM-CSFRα, hβc clone was shown in Fig. C.

line) relative to fluorescence observed in the absence of antibody (Full grey) and in the presence of the secondary antibody alone (Grey line). As for comparison, the FDCP-Mix transfected with hβc were analysed using hβc subunit antibodies and secondary antibodies (anti-mouse FITC) had showed significant increase in fluorescence (Fig. 2C; Black line). Indications that the hβc subunit was expressed in the FDCP-mix cell line. The hβc polyclonal cells were transfected again with pM5-Neo carrying hGM-CSF receptor α subunit gene followed by similar selection and isolation procedure of the chimeric subunit. The results representing one of three clones was showed an increased in fluorescence (Black line; Fig. 2A and B) when analysed using hGM-CSF receptor α-subunit and specific secondary antibodies. Indications that both transfected FDCP-Mix cells were expressed the extracellular portion human of α-subunit genes.

Cells Survival and proliferation: The transfected of both clones; hGM-CSF/βc and hGM-CSFRα, hβc (act as control) were unable to survive for more than 2 days in the absent of hGM-CSF; 0 ng mL^{-1} hGM-CSF (Fig. 3). Indication that the FDCP-Mix cloned cells were depended on growth factors, similar to all recent transfected polyclones and clones that have been generated by Evans *et al.* (2002). On the other hand, transfected clone with chimeric receptor's subunit were able to survive for 7 days of observation in the present of hGM-CSF cytokine (Fig. 3A). As for control; hGM-CSFRα, hβc clones, these type of cells were also able to survive until

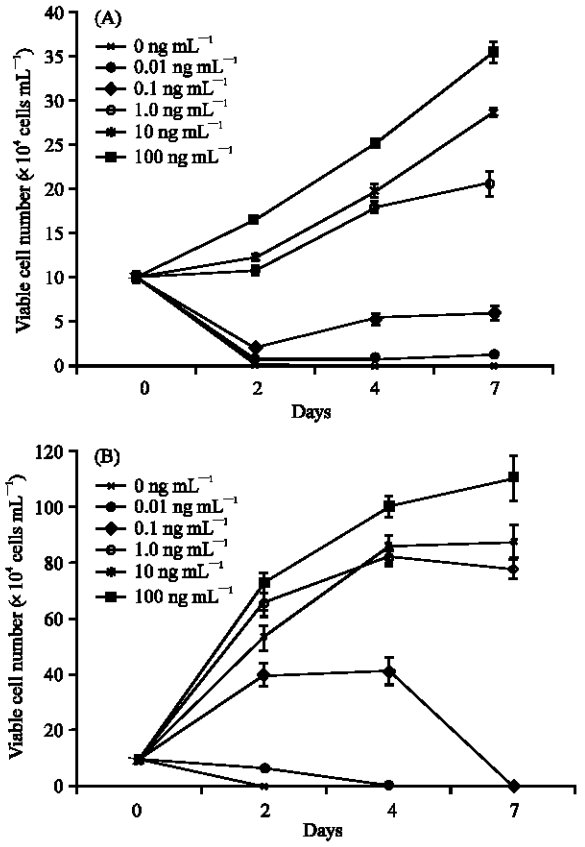

Fig. 3: The cells viability of singly transfected (A) hGM-CSF/βc chimeric (B) hGM-CSFRα, hβc clones were assayed in following conditions from 0-100 ng mL^{-1} of hGM-CSF or 10 ng mL^{-1} recombinant murine IL-3 (rmIL-3). Viability was assessed by trypan blue exclusion and the results shown are the mean of total viable cell numbers from 3 experiments±SEM

7 days with higher cells' viability (Fig. 3B). However, the proliferation rate of the respective clonal cell's populations were reduced compared to the hGM-CSFR/βc clones as gradient of the graph reduced after 4 days in various concentrations of hGM-CSF as showed by the graph's curve of Fig. 3. This shows that, the cell that expressed chimeric receptor's subunit were able to produce cell survival and proliferation signals after 7 days in each concentration of hGM-CSF cytokines (Fig. 3A) whereas the wild type receptors could only produced cells survival at higher concentrations of hGM-CSF, i.e., 1-100 ng mL^{-1} (Fig. 3B). This indicates that a change of cell's fate is due to the changes of the cytosolic domain. These results also showed that in the presence of homogenous hβc cytosolic domain the cells will produce stronger proliferation signals compared to heterogenous α and hβc cytosolic interaction. An indication that the cytosolic domain of βc-subunit plays a major role in cell growth, i.e., cell's proliferation signals.

Cells morphology: Cell morphology was categorised into 4 cell types, based on their characteristic morphological features, i.e., Blast, Early Granulocytes, Late Granulocytes and Macrophages. Cells expressing hGM-CSF/βc and hGM-CSFRα, hβc-subunits that were cultured in 10 ng mL^{-1} murine IL-3 (mIL-3) produced a majority of more than 90% of blast cells. The photomicrograph of hGM-CSF/βc and hGM-CSFRα, hβc cultured in recombinant murine IL-3 showed that the cell population were also primitives (Fig. 4A and C). As comparison, photomicrograph of hGM-CSF/βc and hGM-CSFRα, hβc cultured in 10 ng mL^{-1} of hGM-CSF cytokine showed that the cell population were slightly differentiated (Fig. 4B and C).

Table 1: The effect of cytokine and comparison of the morphology of hGM-CSF/βc pooled from 3 clones. Morphology was scored after cytospin preparation and May-Grunwald-Giemsa stain. The results are represented as mean values of the percentage of total cells pooled froem 3 clones±SEM. Prior to culture in hGM-CSF, i.e., on day 0, the clones' morphology was >95% blast

		Percentage of hGM-CSF/βc clones morphology (%)			
Dose	Day	BL	EG	LG	Macrophages
1 ng mL^{-1}	4	89.0±2.1	11.0±2.1	0	0
	7	70.3±0.3	21.0±0.6	8.7±0.9	0
10 ng mL^{-1}	4	87.7±3.9	12.3±3.9	0	0
	7	75.7±0.3	16.6±0.7	7.7±0.3	0
30 ng mL^{-1}	4	87.0±8.7	13.0±6.8	0	0
	7	71.3±0.3	22.7±0.3	6.0±0.6	0
100 ng mL^{-1}	4	87.3±1.9	12.7±1.9	0	0
	7	79.3±0.9	15.7±0.3	5.0±0.6	0
G-Diff conditions	7	0	1.7±0.7	5.6±0.3	92.7±0.9

BL; Blast Cells, EG; Early Granulocytes, LG; Late Granulocytes. The results are represented as mean values of the percentage of total cells pooled from 3 clones±SEM

Table 2: The effect of cytokine and comparison of the morphology of hGM-CSFRα,hβc_pooled from 3 clones. Morphology was scored after cytospin preparation and May-Grunwald-Giemsa stain. The results are represented as mean values of the percentage of total cells pooled from 3 clones±SEM. Prior to culture in hGM-CSF, i.e., on day 0, the clones' morphology was >90% blast

		Percentage of hGM-CSFRα,hβc clones morphology (%)			
Dose	Day	BL	EG	LG	Macrophages
1 ng mL^{-1}	4	82.5±3.0	17.5±3.1	0	0
	7	18.3±0.3	44.2±2.1	14.4±0.2	23.1±0.5
10 ng mL^{-1}	4	80.6±3.2	19.4±0.8	0	0
	7	17.5±0.7	40.4±2.0	21.1±0.9	21.0±0.8
30 ng mL^{-1}	4	81.5±2.9	18.5±0.2	0	0
	7	11.8±0.3	43.9±1.9	24.2±0.7	20.1±0.5
100 ng mL^{-1}	4	80.3±3.1	19.7±0.8	0	0
	7	9.5±0.1	46.2±1.2	25.3±0.7	19.0±0.3
G-Diff conditions	7	0	7.9±0.1	17.9±1.2	74.2±2.7

BL; Blast Cells, EG; Early Granulocytes, LG; Late Granulocytes. The results are represented as mean values of the percentage of total cells pooled from 3 clones±SEM

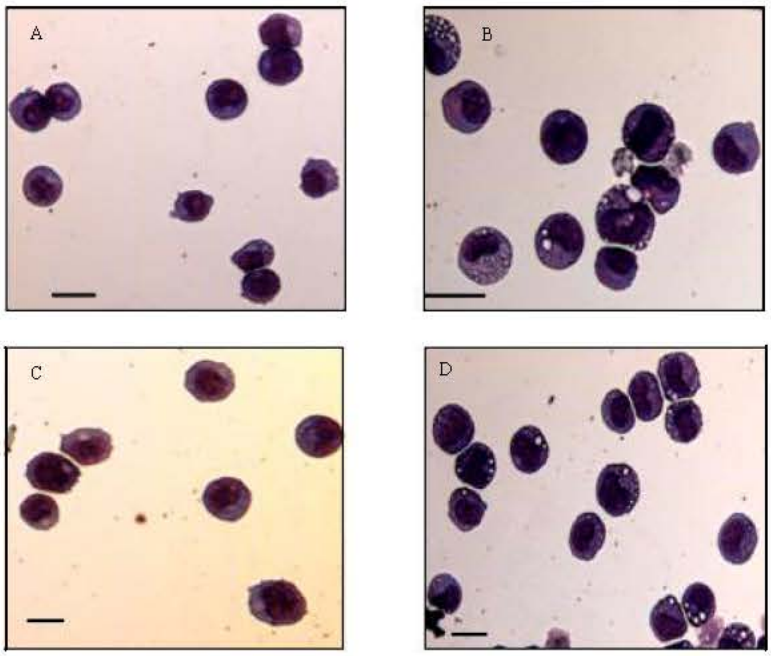

Fig. 4: Cells morphology. Photomicrographs of FDCP-Mix cells expressing chimeric hGM-CSF/βc subunit cultured in 10 ng mL^{-1} murine IL-3 for 7 days (A) and 10 ng mL^{-1} of hGM-CSF cytokine for 7 days (B). The photomicrograph of FDCP-Mix cells co-expressing hGM-CSF α and hβc subunits (hGM-CSFRα, hβc cells) cultured in 10 ng mL^{-1} murine IL-3 (C) and 10 ng mL^{-1} of hGM-CSF cytokine for 7 days (D) are shown for comparison. The photomicrographs were prepared by cytospin followed by May-Grunwald-Giemsa staining. The bar in each photomicrograph represented 10 μm

Both cells are capable of differentiated into granulocytes and macrophages when cultured in G-Diff (hGM-CSF/βc; Table 1 and hGM-CSF, hβc; Table 2), i.e., a combination of IL-3 (0.01 ng mL^{-1}), G-CSF (5,000 U mL^{-1}) and GM-CSF (300 U mL^{-1}). These cells are similar to untransfect FDCP-Mix cells (Heyworth *et al*., 1995; Spooncer and Dexter, 1997). After 4 days cultured under various concentrations of hGM-CSF cytokine (1-100 ng mL^{-1}), more than 85% of hGM-CSF/βc clones were shown to remain as blast cells with others has differentiated into early granulocytes (Table 1). After 7 days, the hGM-CSF/βc clones cultured in hGM-CSF (1-100 ng mL^{-1}) were slightly more differentiated compared to day 4. The respective cells produced increasing percentages in early granulocytes (more than 15%) and late granulocytes, i.e., less than 10% (Table 1). As comparison, FDP-Mix cell line co-expressed with hGM-CSFRα and hβc were subjected to similar conditions as the chimeric clones. After 4 days cultured in hGM-CSF cytokine (1-100 ng mL^{-1}), the respective cells showed slightly more differentiated status as the chimeric clones. The hGM-CSFRα, hβc cells produced partially differentiated status. The hGM-CSFRα,hβc cells produced less than 83% of blast cells (Table 2) as compared to less than 90% for the chimeric clones (Table 1). After 7 days in 1-100 ng mL^{-1} of hGM-CSF, the clones expressing hGM-CSFRα,hβc produced more differentiated status compared to the same cells cultured for 4 day (Table 2). The respective cells also produced more differentiated cells compared to the chimeric clones cultured in 7 days of the same concentrations of hGM-CSF. Indications that cells expressing chimeric subunit were less differentiated in the presence of cognate cytokine (human GM-CSF) compared to cells expressing human wild type α and βc receptor subunits.

CONCLUSIONS

These results showed that the chimeric receptor consisted of homogenus human βc cytosolic domain produced stronger proliferation and survival signals as compared to heterogenus cytosolic domain, i.e., cytosolic domain consisting human βc and GM-CSF α-subunit. The chimeric expressing cells also produced less differentiated state compared to wild type heterogenous subunits in the presence of their respective cytokine. An indication that

the human βc subunit plays a central role in producing stronger cell's proliferation and survival signals compared to differentiation signals.

ACKNOWLEDGMENTS

The authors would like to thank Intan Zarina Zainol Abidin for organising references and helping us in submitting the manuscript. We also would like to thank Department of Biomolecular Sciences, UMIST where this study took place under supervision Prof. A.D. Whetton and colleagues.

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