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To cite this version:

Michel Jourdan, Karène Mahtouk, Jean-Luc Veyrune, Guilhem Couderc, Geneviève Fiol, et al.. Delineation of the roles of paracrine and autocrine interleukin-6 (IL-6) in myeloma cell lines in survival versus cell cycle. A possible model for the cooperation of myeloma cell growth factors.. European Cytokine Network, 2005, 16 (1), pp.57-64. inserm-00130868

HAL Id: inserm-00130868

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Submitted on 6 Mar 2007

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Eur Cytokine Netw 2005; 16(1): 57-64

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Delineation of the roles of paracrine and autocrine interleukin-6 (IL-6) in myeloma cell lines in survival versus cell cycle. A possible model for the cooperation of myeloma cell growth factors

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Key words: myeloma; IL-6; Mcl-1; apoptosis; cell cycle.

Running title: Dual concentration-dependent role of IL-6 on myeloma cells

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ABSTRACT

Primary myeloma cells rapidly apoptose as soon as they are removed from their bone-marrow environment. A likely explanation is that the tumor environment produces survival factors that may counteract a spontaneous activation of pro-apoptotic program. Additional factors may trigger cell cycling in surviving myeloma cells. Interleukin-6 (IL-6) is a well recognized myeloma cell growth factor produced mainly by the tumor environment. However, myeloma cells themselves may produce low level of autocrine IL-6. The respective roles of paracrine versus autocrine IL-6 are a matter of debate. We investigated these roles using the XG-6 myeloma cell line whose growth is dependent on addition of exogenous IL-6, despite its weak IL-6 mRNA and protein expression. The apoptosis induced by exogenous IL-6 deprivation was blocked by transferring *Mcl-1* gene coding for an anti-apoptotic protein in XG-6 cells. A XG-6Mcl-1 cell line which can survive and grow without adding IL-6 was obtained. We show that anti-IL-6 or anti-gp130 antibodies abrogated the cell cycling whereas they did not affect the cell survival.

These data indicate that the weak autocrine IL-6 produced by myeloma cells is sufficient to trigger cell cycling whereas their survival requires large exogenous IL-6 concentration. This important role of autocrine IL-6 has to be considered when evaluating the mechanism of action of myeloma cell growth factors. These factors may actually block an activated pro-apoptotic program, making it possible a weak production of autocrine IL-6 to promote cell cycling.

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INTRODUCTION

Myeloma cells develop in close contact with their bone-marrow environment. A major role of this environment is to counteract a spontaneous pro-apoptotic program in myeloma cells which rapidly dye in vitro as soon as they are purified. Primary myeloma cells express at least four pro-apoptotic proteins that may account for this spontaneous pro-apoptotic program: Bax, Bak, Bid and Bim [1, 2]. The myeloma cell growth factors induce expression of anti-apoptotic proteins, namely Mcl-1, Bcl-2 and Bcl-xL that counteract these pro-apoptotic members. IL-6 is a major survival and proliferation factor for human myeloma cells, mainly produced by the tumour environment [3]. Whether IL-6 is an autocrine or paracrine myeloma cell growth factor is still a challenging issue [4, 5]. The tumour environment produces a very large amount of IL-6 (in the range of several ng/10^6 tumour environment cells), whereas myeloma cells weakly express IL-6 mRNA or protein when detectable [4-10].

The biological role of this weak autocrine IL-6 is unclear. Indeed, we and others have reproducibly obtained myeloma cell lines whose growth is strictly dependent on adding exogenous IL-6, despite their weak *IL-6* mRNA expression [11-13]. In addition, we found that interferon-α (IFN-α) is a survival and proliferation factor for these cell lines [14, 15]. Whereas the survival activity of IFN-α was independent on autocrine IL-6, its proliferation activity was blocked by anti-IL-6 monoclonal antibodies [15]. The survival activities of both IL-6 and IFN-α are linked to their ability to up-regulate Mcl-1 only, among 10 anti- and pro-apoptotic Bcl-2 family proteins [1]. Thus, we can postulate that myeloma cell survival requires a high IL-6 concentration to up-regulate Mcl-1 expression, whereas the cell cycle can be triggered by low autocrine IL-6 production. A major difficulty in addressing this question is that these myeloma cell lines apoptose in the absence of exogenous IL-6. To bypass this problem, we

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blocked myeloma cell apoptosis induced by IL-6 deprivation using *Mcl-1* retroviral transduction. Indeed, we previously showed that *Mcl-1*-transduced myeloma cell lines could survive and progressively proliferate autonomously without the requirement of exogenous IL-6 [16]. Using this attractive model, we demonstrate here that the survival of the Mcl-1 myeloma cell line is independent of autocrine IL-6, whereas neutralization of this autocrine IL-6 completely abrogated the proliferation.

These data clearly demonstrate for the first time the dual concentration–dependency role of IL-6, which may clarify the previous challenging data. Large IL-6 concentrations are necessary to trigger myeloma cell survival, whereas a weak IL-6 concentration, produced by some myeloma cells themselves, is sufficient to trigger cell proliferation.

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MATERIALS AND METHODS

Myeloma cell lines

The XG-6 IL-6-dependent human myeloma cell line (HMCL) was previously established in our laboratory [11]. XG-6GFP and XG-6Mcl-1 cell lines were obtained by transducing XG-6 cells with, respectively, a control green fluorescent protein (GFP) retrovirus or an Mcl-1-GFP retrovirus, as previously described [16]. The autonomously growing myeloma cell line U266 was obtained from ATCC (Rockville, MD, USA).

Cytokines and antibodies

Purified human recombinant IL-6 was purchased from AbCys SA (Paris, France). The B-E8 anti-IL-6 monoclonal antibody (mouse IgG1) was kindly provided by Dr Wijdenes (CRTS, Besançon, France). The neutralizing A1 anti-gp130 monoclonal antibody and the G4 nonneutralizing anti-gp130 monoclonal antibody, used as control antibody, were obtained in our laboratory (both are mouse IgG1) [17].

Myeloma cell culture

XG-6 cells were routinely cultured with 2 ng/mL of IL-6 in RPMI1640 supplemented with 10% fetal calf serum (FCS). For apoptosis and proliferation assays, myeloma cells were starved of IL-6. Therefore, the cells were washed twice, cultured for 1 hour in RPMI1640 with FCS and washed once again. Cells were then cultured at a concentration of 2 x 10^5 cells/mL in RPMI1640, 10% FCS with either no cytokine or IL-6 (2 ng/mL) for 72 hours.

Apoptotic cell detection assay

Apoptotic cells were detected using biotin-conjugated annexin-V (annexin-V-biotin, Boehringer, Mannheim, Germany) and streptavidin-phycoerythrin. Cells were washed and incubated for 20 minutes with annexin-V-biotin according to the manufacturer's

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recommendations. The cells were washed once and incubated for 15 minutes with streptavidin-phycoerythrin. Two-colour immunofluorescence was analyzed with a FACScan flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

Cell proliferation assay

Cells were cultured for 72 hours in 96-well flat-bottomed microtiter plates at 4.10^4 cells/well in 200 µL of RPMI 1640 culture medium and 10% FCS. Various concentrations of cytokines were added at the beginning of the culture in six culture wells per group. At the end of the culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech, Orsay, France) for 12 hours, then harvested and counted, as reported previously [14].

Cell cycle analysis

DNA was stained with propidium iodide (PI), as described by Collins *et al.* [18]. Cells (10^6 per sample) were washed in PBS, suspended in 1 mL of 75% ethanol / 25% water at room temperature for 2 minutes and washed again. We added 500 µL of PBS containing PI (40 µg/mL) and RNAse (100 µg/mL) (both from Sigma, St Louis, MO, USA) per sample. Cells were incubated for 30 minutes at 37°C and stored at 4°C in the dark before analysis with a FACScan flow cytometer using Cell Quest software. The cell cycle was analyzed with the ModFit LT software (Verity Software House, Topsham, ME, USA).

RT-PCR and quantitative RT-PCR

RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA). Total RNA was converted to cDNA using the Superscript II reverse transcriptase (RT; Invitrogen, Cergy-Pontoise, France).

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For quantitative RT-PCR, we used the assays-on-demand primers and probes and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions. Gene expression was measured using the ABI Prism 7000 Sequence Detection System. For each sample, the C_T value for IL-6 was determined and normalized to its respective C_T value for β2-microglobulin ($\Delta C_T = C_T$ IL-6 - C_T β2-microglobulin) and compared to XG-6 cells used as positive control. The formula used was: $1/2^{\Delta CT \text{ sample} - \Delta CT \text{ XG-6}}$. The results were expressed as the relative IL-6 mRNA levels to XG-6 IL-6 mRNA.

Bone marrow stromal cells and monocytes

Bone marrow stromal cells were obtained by culturing adherent bone marrow mononuclear cells in Dulbecco modified Eagle medium (DMEM)/ 20% FCS for 3 weeks. Bone marrow CD14 monocytes were purified (>98% CD14 cells) with anti-CD14 MACS microbeads (Miltenyi Biotech, Paris, France).

IL-6 production measurement

The XG-6 myeloma cells or the bone marrow stromal cells were cultured for 24 hours at a concentration of 10^6 cells/mL in RPMI1640, 10% FCS. Supernatants were harvested and IL-6 was assayed using a commercially available IL-6 ELISA (Beckman-Coulter, Marseilles, France). In our hands, the sensitivity of the ELISA is 10 pg/mL, below that given by the manufacturer.

IL-6 activity was assayed using the IL-6-dependent hybridoma cell line B9 (generous gift from Dr L. Aarden, Amsterdam, The Netherlands) as previously described [5, 19]. In all assays, we verified that we effectively assayed IL-6 activity by inhibiting the B9 growth factor activity with a monoclonal antibody to human IL-6 (B–E8). One unit of IL-6 was defined as the amount inducing half-maximal proliferation of B9 cells and corresponded to about 1 pg recombinant human IL-6.

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Statistical analysis

The mean percentages of apoptotic cells in culture groups with IL-6 or with antibodies were compared to corresponding control groups using a Student *t* test for pairs. For proliferation analysis, data were the means ± SD of thymidine incorporation determined in sextuplate culture wells. The statistical significance of these results was determined using a Student *t* test.

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RESULTS

IL-6 is a myeloma cell survival factor

As illustrated in *Figure 1A*, exogenous IL-6 is able to promote the long-term growth of XG-6 myeloma cells. Without adding recombinant IL-6, myeloma cells progressively died within several days. We previously showed that exogenous IL-6 is a survival factor. Without adding IL-6, a high percentage of apoptotic myeloma cells (*i.e.* annexin-V^+) could be detected within 3 days and addition of recombinant IL-6 prevented this apoptosis. The concentration of IL-6 yielding 50% survival was 36 pg/mL for XG-6 cells (*Figure 1B*) and ranged from 10 to 100 pg/mL for eight different myeloma cell lines [11]. Using quantitative RT-PCR, we found that *IL-6* expression in XG-6 cells was 25- to 100-fold lower than that found in the autonomously growing U266 myeloma cell line or in the tumour environment, in particular bone marrow monocytes and stromal cells (*Figure 2A*). In agreement with this weak *IL-6* expression, no IL-6 protein (< 10 pg/mL) were detected by ELISA in the culture supernatant of XG-6 cells, whereas high IL-6 levels were found in stromal cell culture supernatants (median value, 220 pg/mL; range, 90–4320 pg/mL, *n*=13). However, using the IL-6-sensitive murine B9 cell line, we detected a low IL-6 activity (1.2 U/ml corresponding to 1.2 pg/ml IL-6) in the XG-6 supernatant (*Figure 2C)*.

Autocrine IL-6 can trigger cell cycle

To look for a possible role of autocrine IL-6, we used the XG-6 myeloma cell line transduced with a Mcl-1 retrovirus termed XG-6Mcl-1. XG-6Mcl-1 cells expressed the *IL-6* gene at a low level, but at a level threefold higher than that of parental cells or cells transduced with a control retrovirus (*Figure 2B*). In good agreement, a 3-fold higher IL-6 secretion (3.2 U/ml corresponding to 3.2 pg/ml IL-6) was found in the

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culture supernatant of XG-6Mcl-1 cells compared to XG-6 cells (*Figure 2C*). Again, the IL-6 ELISA was not enough sensitive to detect it.

As indicated in *Figure 3* and published before [16], XG-6Mcl-1 cells failed to apoptose and proliferated in the absence of exogenous IL-6, unlike XG-6 cells transduced with a control retrovirus (XG-6GFP). Adding recombinant IL-6 did not increase the survival that was already maximum and slightly increased the proliferation. It is of interest to note that adding a neutralizing monoclonal antibody to IL-6 completely blocked the spontaneous proliferation of the XG-6Mcl-1 cells, whereas it did not significantly affect their survival. In order to better evaluate the cell cycle in myeloma cells, the non-apoptotic myeloma cells were sorted based on the lack of annexin-V labelling. Fully viable myeloma cells (> 99% annexin V^{-}) could be purified after 2 days of culture without cytokine, with IL-6 or with IL-6 and anti-gp130 antibody. Results of a sorting experiment are shown in *Figure 4A*. The cell cycle of fully viable annexin V^{-} myeloma cells was determined with propidium iodide staining (*Figure 4B*). Twenty-four per cent of the XG-6Mcl-1 cells were in the S phase of the cell cycle without recombinant IL-6. Adding recombinant IL-6 did not promote cell cycling. Adding an anti-gp130 IL-6 transducer antibody blocked cell cycling in the fully viable annexin-V^{-} cells, with 8% of cells in the S phase.

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DISCUSSION

The cell growth requires first survival and then cell cycling. Using the XG-6 myeloma cell line, we show here that IL-6 has this dual role but at different concentrations. A high concentration of IL-6 is necessary to trigger myeloma cell survival, mainly by up-regulating the Mcl-1 anti-apoptotic protein. Using transduction of the *Mcl-1* gene in XG-6 cells in order to block apoptosis in absence of exogenous IL-6, we demonstrated that a weak concentration of IL-6, produced by XG-6 myeloma cells themselves, is sufficient to trigger cell cycling. It should be noted that this artificial model mimics the positive effect of IFN-α on myeloma cell growth reported by several groups [14, 20, 21]. We previously reported that IFN-α is a myeloma cell growth factor mainly because it induces Mcl-1 over-expression and myeloma cell survival [1], thus allowing the low autocrine IL-6 production by myeloma cells to trigger cell cycling.

Autonomously growing myeloma cell lines have been reported and, it should be noted, the often-studied U266 cell line. Several groups have shown that IL-6 is an autocrine growth factor for U266 cells [22, 23]. Indeed, we show here that U266 cells highly express the *IL-6* gene compared to XG-6 cells, to the same extent as stromal cells or purified monocytes from MM patients. This large autocrine IL-6 production may explain why U266 cells can survive without adding growth factors.

How can we extend these data obtained with cell lines to primary myeloma cells? To date, all reports on myeloma growth factors used myeloma cell lines or primary myeloma cells from patients with extramedullary proliferation. In these patients, primary myeloma cells can survive and proliferate substantially, and are close to our XG cell lines. In patients with intramedullary MM, primary myeloma cells rapidly

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apoptose as soon as they are separated from the bone-marrow environment [24]. Actually, primary myeloma cells express pro-apoptotic proteins of the Bcl-2 family that have to be continuously counteracted by signals delivered by the bone-marrow environment. IL-6 is one of the signals but cannot replace alone the bone-marrow environment to trigger survival [24]. However, this role for autocrine IL-6 could be important for a minor population of myeloma stem cells. This population is not fully identified but could be close to plasmablastic cells, in particular expressing CD45 and a low level of syndecan-1. It is noteworthy that Hata *et al* have reported that immature $CD45^+$ myeloma cells express the *IL-6* gene, unlike mature myeloma cells [8]. In this population of myeloma stem cells, autocrine IL-6 could be sufficient to trigger cell cycling, whereas the bone marrow environment could be critical to trigger survival by producing other survival factors, in particular syndecan-1-binding growth factors such as heparin-binding epidermal growth factor-like [25], hepatocyte growth factor [26] or fibroblast growth factor [27].

The present observation invites caution in interpreting the data with growth factors by studying their role in cell survival and cell cycling in detail, taking into account the possible cell cycling role of low-concentration autocrine IL-6.

ACKNOWLEDGMENTS. This work was supported by a grant from La Ligue contre le Cancer (Equipe Labellisée) (Paris, France).

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LEGENDS TO FIGURES

Figure 1. IL-6 is a myeloma cell survival factor

A: IL-6 promotes the long-term growth of XG-6 cells. Cells were cultured at 10^5/mL with or without IL-6 (2 ng/mL). Every 3–4 days, cells were counted and replated in the same culture conditions. Results are the cumulative cell numbers obtained in 10 days of culture and are from one experiment representative of three.

B: IL-6 concentration-dependent protection of XG-6 cells from apoptosis. Cells were starved of IL-6 for 1 hour and cultured with various IL-6 concentrations. At day 3 of culture, cells were harvested and apoptotic cells were detected with annexin-V staining. Results are from one experiment representative of three.

Figure 2. Weak *IL-6* expression in XG-6 cells

A: *IL-6* expression was evaluated by quantitative RT-PCR in myeloma cell lines (XG-6, U266), bone marrow monocytes (CD14) and stromal cells (ST-1 to 4). The relative IL-6 mRNA level was determined as described in Materials and Methods. The "1" value is assigned to the XG-6 parental cell line.

B: XG-6Mcl-1 cells expressed a threefold higher IL-6 mRNA level than the parental XG-6 cell line. The relative IL-6 mRNA level was determined as indicated in **A**.

C: IL-6 activity in culture supernatants of XG-6 cell lines. IL-6 activity was determined using the B9 hybridoma proliferation assay, 1 U/ml corresponded to about 1 pg/ml of human IL-6 recombinant protein.

Results are from one experiment representative of two.

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Figure 3. Neutralizing anti-IL-6 antibody abrogated cell proliferation, unlike cell survival of XG-6Mcl-1 cells

XG-6GFP or XG-6Mcl-1 cells were starved of IL-6 for 1 hour and cultured with or without IL-6 (2 ng/mL) for 3 days in the presence of control antibody (Co) or 10 µg/mL of anti-IL-6 neutralizing antibody.

A: Apoptotic cells were evaluated by annexin-V binding and FACS analysis. Results are means ± SD of the percentages of apoptotic cells determined in three separate experiments.

* Indicates that the mean value is statistically significantly different ($p<0.05$) from that obtained without IL-6, using a Student *t* test for pairs.

** Indicates that the mean value is statistically significantly different ($p<0.05$) from that obtained without adding anti-IL-6 antibody, using a Student *t* test for pairs.

B: Proliferation was assessed by thymidine uptake as described in Materials and Methods. Data are means ± SD of thymidine incorporation determined in sextuplate culture wells.

* Indicates that the mean value is statistically significantly different ($p<0.05$) from that obtained without IL-6, using a Student *t* test.

** Indicates that the mean value is statistically significantly different ($p<0.05$) from that obtained without adding anti-IL-6 antibody, using a Student *t* test.

Figure 4. XG-6 cell cycling is blocked by an anti-gp130 IL-6 transducer antibody

XG-6Mcl-1 cells were starved of IL-6 for 1 hour and cultured with or without IL-6 (2 ng/mL) for 2 days in the presence of control antibody, or with IL-6 and 150 µg/mL of anti-gp130 IL-6 transducer neutralizing antibody. The cells were stained with annexin-V and non-apoptotic cells (annexin-V^-) were sorted using a FACS-Vantage

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cell sorter. Before sorting, the percentage of annexin-V^+ cells was 13% without IL-6, 14% with IL-6 and 26% with IL-6 + anti-gp130. After sorting, it was <1 % in each group.

A: sorting results obtained with myeloma cells cultured with IL-6 and anti-gp130 antibody.

B: DNA was labelled with PI and cells were analyzed on a FACScan apparatus. The percentage of the S phase of the cell cycle is indicated and is determined using the ModFit LT software.

Results are from one experiment representative of three.

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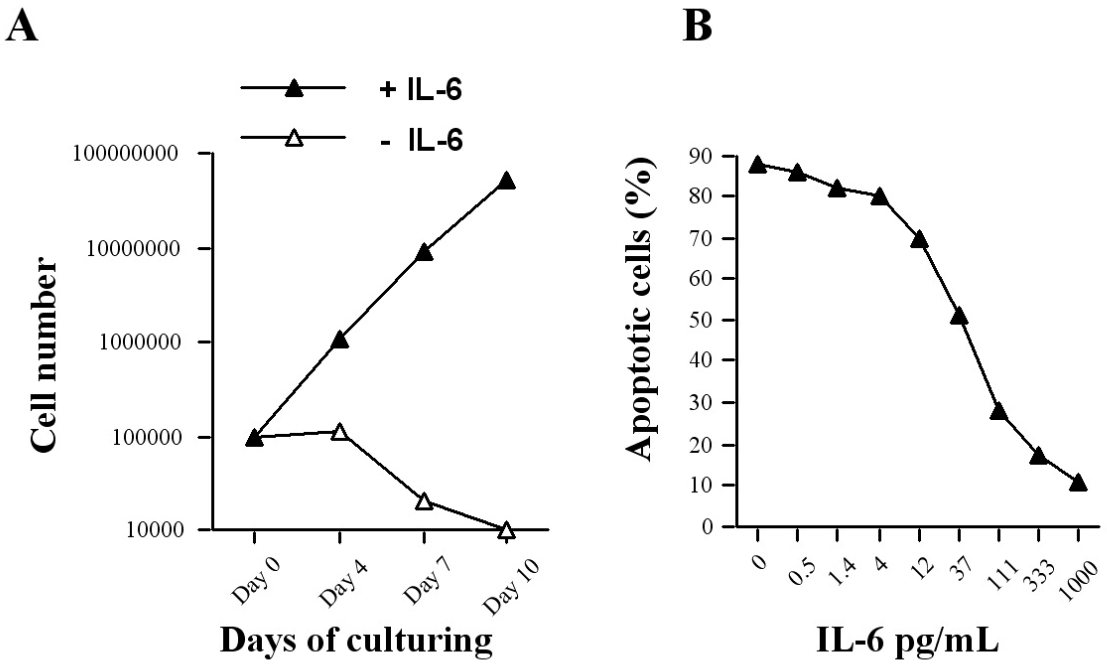

Figure 1

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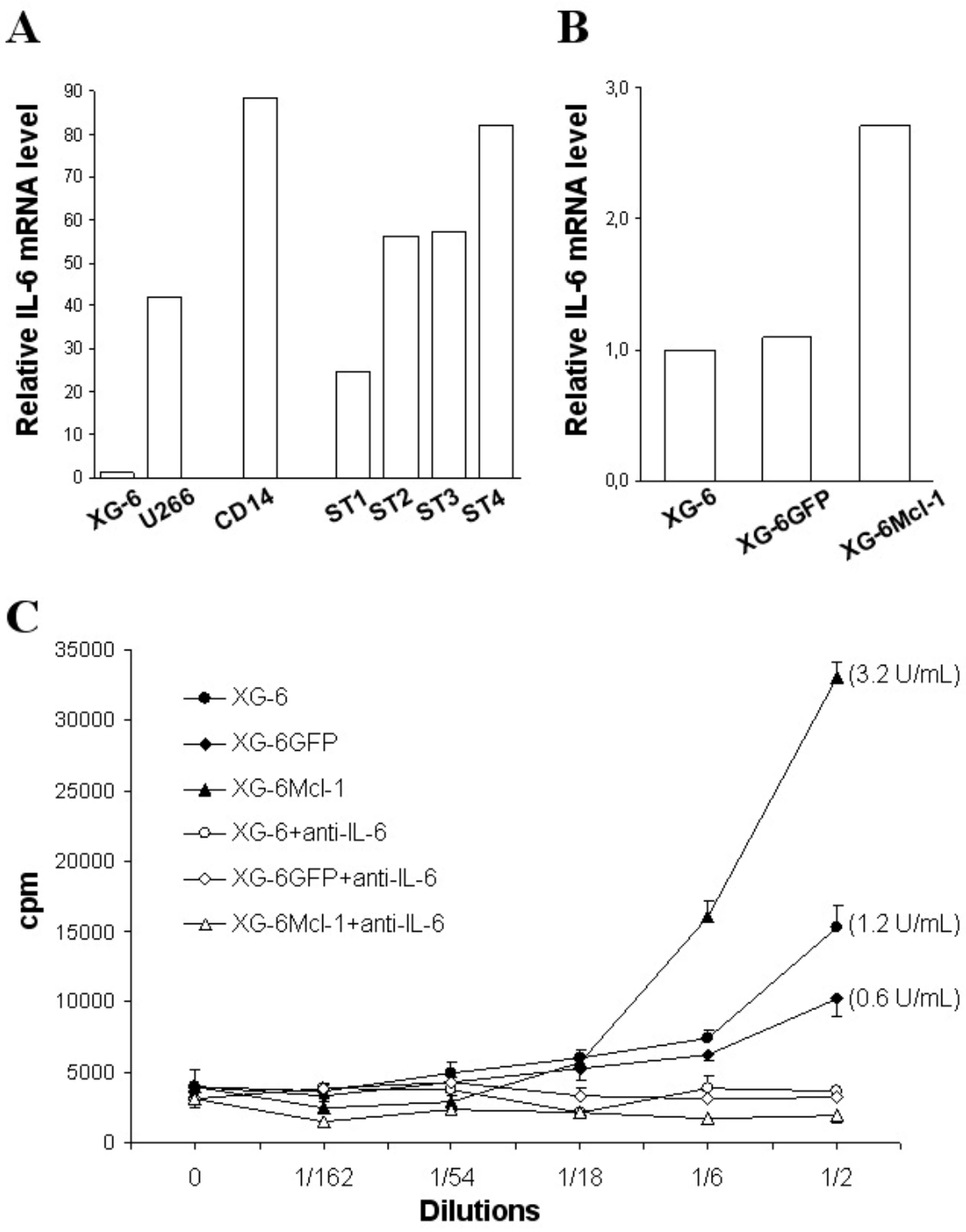

Figure 2

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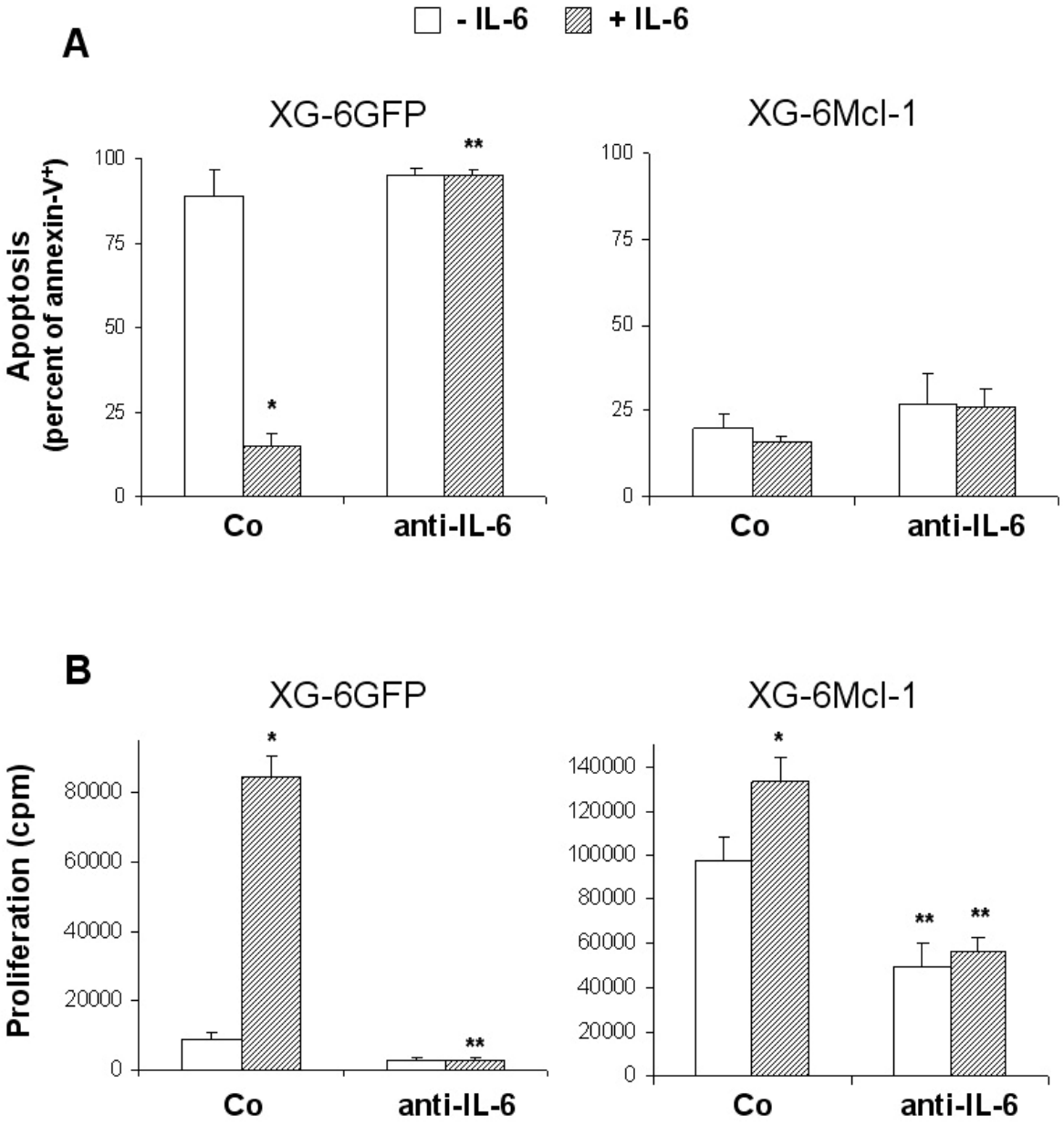

Figure 3

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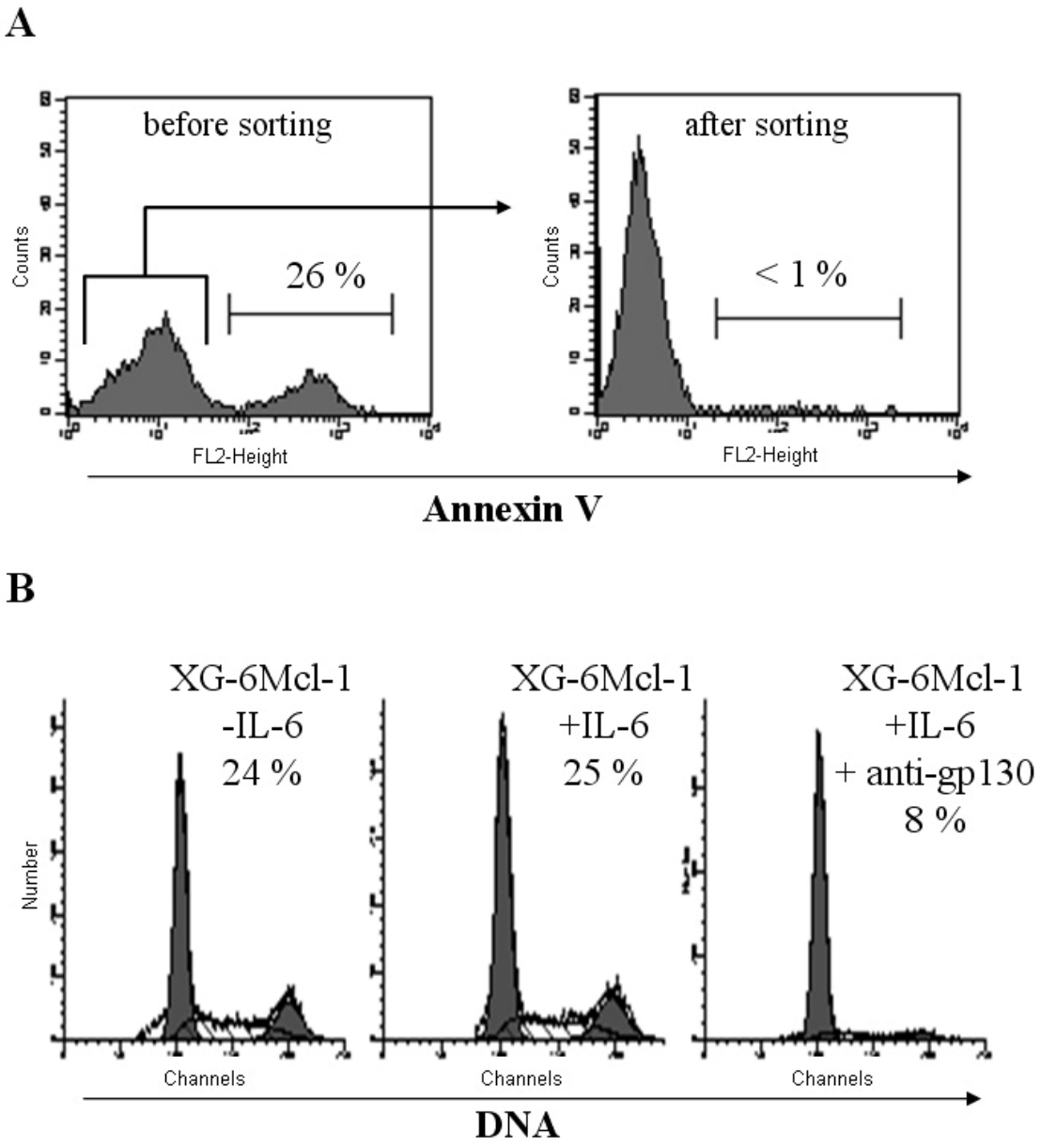

Figure 4