

# Involvement of IL-10 and granulocyte colony-stimulating factor in the fate of monocytes controlled by galectin-1

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**Abstract.** The process of differentiation from monocytes to dendritic cells is critical in immune modulation. Monocyte apoptosis is a key regulator in balancing the immune response. Galectin-1 has been reported to induce tolerogenic dendritic cells by the autocrine interleukin (IL)-10 in monocytes. However, IL-10 has been found to induce apoptosis in IL-4/granulocyte macrophage colony-stimulating factor (CSF) stimulating and non-stimulating monocytes, whereas galectin-1 has not. After analyzing the factors secreted by galectin-1-activated CD14 monocytes isolated from the peripheral blood, the present study revealed that galectin-1 upregulates IL-10 and granulocyte (G)-CSF expression. Furthermore, G-CSF inhibited IL-10-induced apoptosis, implying that galectin-1 may enhance the immune-modulating functions of G-CSF by inducing tolerogenic dendritic cells and maintaining their survival. Therefore, G-CSF may be further applied in immune therapy, particularly in the IL-10-presenting microenvironment.

## Introduction

Dendritic cells (DCs) are specific antigen-presenting cells critical for the induction of adaptive immunity and tolerance by interacting with T cells (1). DC differentiation from monocytes is a key step in infections and numerous other conditions. DC turnover is similarly important for maintaining the steady state of the immune system. Circulating monocytes usually undergo spontaneous apoptosis within days (2); however,

the life span of monocytes is extended to weeks following differentiation into DCs, induced by treatment with interleukin (IL)-4/granulocyte macrophage colony-stimulating factor (GM-CSF).

Previous studies have observed that T-helper (Th)1 cytokines, including IL-2 and IL-12, inhibit myeloid cell apoptosis, whereas Th2 cytokines, such as IL-4 and IL-10, enhance apoptosis in these cells (3,4). IL-10-induced myeloid cell apoptosis is mediated through the caspase-dependent signaling pathway, which is blocked by caspase-3 inhibitors and pan-caspase inhibitors (2). Galectin-1 (Gal-1) exhibits the ability to induce IL-10 expression in T cells (5,6) and in DCs (7,8), but does not induce apoptosis in monocytes (9,10).

Granulocyte colony-stimulating factor (G-CSF, also termed CSF3) was identified in an attempt to define the normal regulators present in cell supernatants that induced terminal differentiation of the WEHI-3B D<sup>+</sup> murine myeloid leukemia cell line (11). Recently, Romero-Weaver *et al* reported the ability of G-CSF to promote the proliferation of bone marrow stem cells and inhibit granulocyte apoptosis (12). G-CSF also improved the recovery from spinal cord injury in mice (13) and improved memory and neuro-behavior in an amyloid- $\beta$ -induced experimental model of Alzheimer's disease (14). However, the direct effects of G-CSF on differentiating monocytes have not been discussed. In present study, the role of G-CSF in galectin-1-treated monocytes was examined, particularly its role in preventing cell apoptosis.

## Materials and methods

**Materials.** Gal-1 and G-CSF were purchased from ProsPec-Tany TechnoGene, Ltd. (Ness-Ziona, Israel). Human recombinant IL-10 was purchased from R&D Systems (Minneapolis, MN, USA). Human recombinant GM-CSF and IL-4 were purchased from Millipore Corp. (Billerica, MA, USA).

**Isolation and culture of human monocytes.** Human CD14<sup>+</sup> monocytes were isolated from the peripheral blood mononuclear cells (PBMCs) of healthy donors without any known cancers or immunological disease. Briefly, PBMCs were collected from interphase subsequent to Ficoll paque plus separation (GE

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**Key words:** galectin-1, monocyte, IL-10, granulocyte colony-stimulating factor, apoptosis

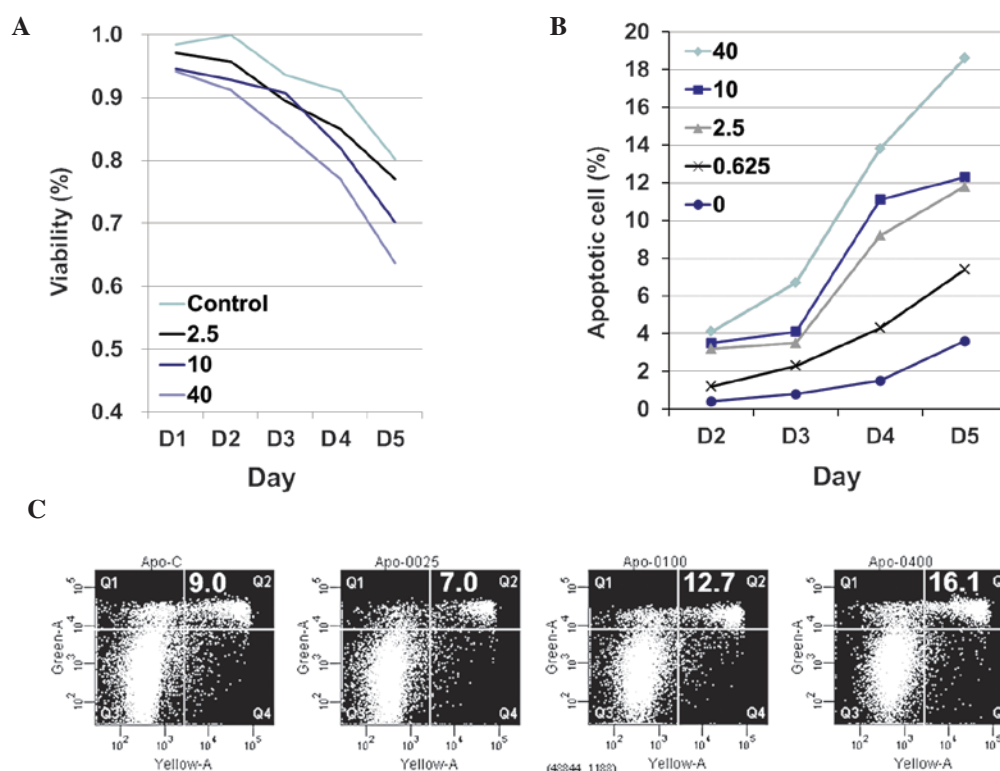


Figure 1. IL-10 induces apoptosis in IL-4/GM-CSF-stimulated monocytes. (A) Different concentrations of IL-10 (2.5, 10 and 40 ng/ml) were added to the culture media and cell viability was determined by trypan blue exclusion assay at various time-points. (B and C) Stimulated monocytes from different treatments were placed into a 96-well plate, stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide and analyzed by flow cytometry. The results of single experiments are shown and all the results are representative of at least three independent experiments. GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

Healthcare Bio-Sciences, Little Chalfont, UK) and washed twice in phosphate-buffered saline (PBS). CD14<sup>+</sup> monocytes were isolated using the MACS<sup>®</sup> system (MACS MicroBeads; Miltenyi Biotec Ltd, Bergisch Gladbach, Germany) following the manufacturer's instructions and cultured in RPMI-1640 containing 10% fetal bovine serum (Invitrogen Life Sciences, Carlsbad, CA, USA) for five days in the presence of 20 ng/ml IL-4/GM-CSF with or without 1  $\mu$ g/ml Gal-1, 10 ng/ml G-CSF and IL-10 as indicated. Monocyte viability was determined by trypan blue exclusion staining.

The Institutional Review Board of Kaohsiung Medical University Hospital (Kaohsiung, Taiwan) approved the study. All patients provided informed consent in accordance with the Declaration of Helsinki.

**Flow cytometry and detection of Annexin V staining and CD14 expression.** Two-color flow cytometry was performed by FACSarray<sup>™</sup> (BD Biosciences, Franklin Lakes, NJ, USA) using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit I (BD Biosciences) according to the manufacturer's instructions. Briefly, the treated cells were centrifuged at 200  $\times$  g for 5 min and washed twice with cold PBS. The cells were resuspended in 100  $\mu$ l 1X binding buffer, and 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide (PI) were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Subsequently, the cells were centrifuged at 200  $\times$  g for 5 min, washed twice with 1X binding buffer and resuspended in 100  $\mu$ l 1X binding

buffer. The samples were analyzed using a FACSarray<sup>™</sup> flow cytometer.

**Measurement of secreted factors.** The cultured supernatants from monocytes were collected following centrifugation. The samples were analyzed for IL-10 and G-CSF by multiple cytokine analyses using the cytometric bead array (CBA; BD Biosciences). The CBA technique is based on two bead populations with distinct fluorescence intensities that are coated with capture antibodies specific for each cytokine. The fluorescent dye had a maximal emission wavelength of  $\sim$ 650 nm (FL-3), which was detectable by flow cytometry. The cytokine capture beads were mixed with the phycoerythrin-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following the acquisition of sample data on the FACSarray<sup>™</sup> flow cytometer, the sample results were analyzed using FACSArray<sup>™</sup> software version 3.0 (BD Biosciences). A standard calibration curve was established for each cytokine; the maximum and minimum limits of detection for each cytokine were 1.0 and 5,000 pg/ml, respectively.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. Statistical comparisons of the results were performed by analysis of variance and two-sided Student's t-test using Excel 2010 (Microsoft Corp., Redmond, WA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference between the means of the two groups.

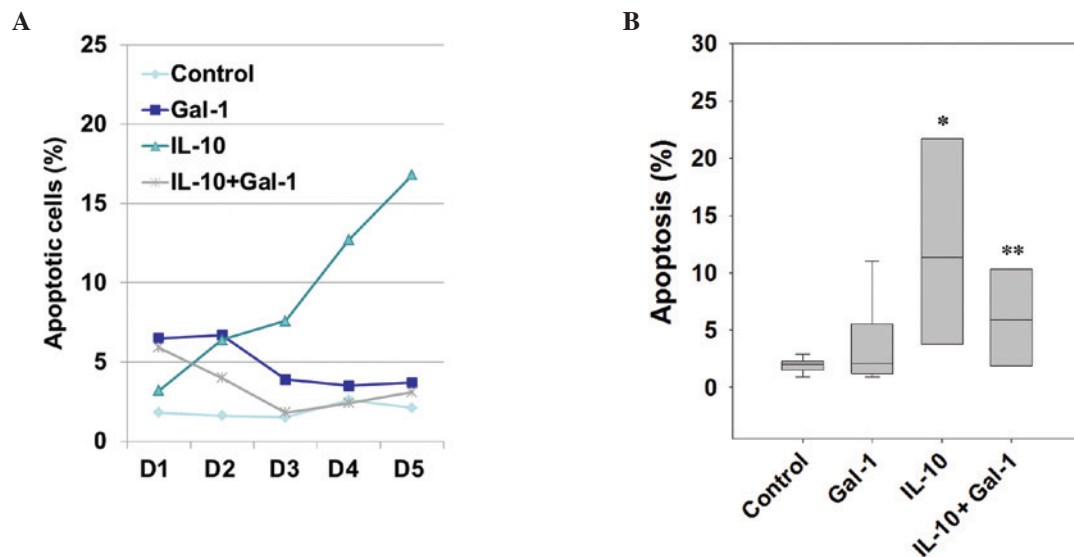


Figure 2. Gal-1 inhibits IL-10-induced apoptosis in stimulated monocytes. (A) The percentage of apoptotic cells was determined by Annexin V-propidium iodide staining at each time-point. (B) The percentage of monocyte apoptosis following different treatments on day five was determined in five healthy donors. Each value signifies the mean  $\pm$  standard deviation of five independent experiments. \* $P < 0.05$  vs. control; \*\* $P < 0.05$  vs. IL-10 group, by Kruskal-Wallis non-parametric analysis of variance. Gal-1, galectin-1; IL, interleukin.

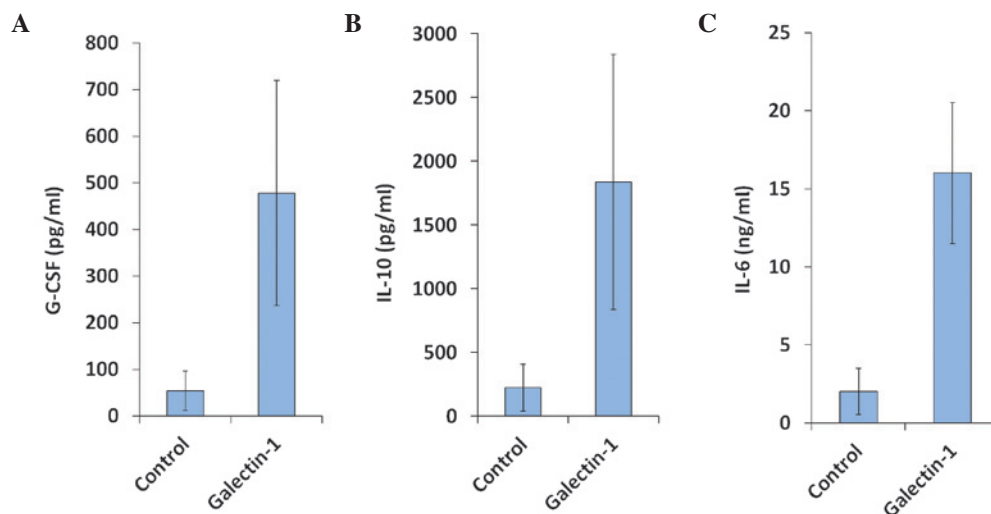


Figure 3. Galectin-1 induces IL-10 and G-CSF in stimulated monocytes. (A-C) Cytokine concentrations as determined by cytometry bead array. All results are representative of at least three independent experiments. G-CSF, granulocyte colony-stimulating factor; IL, interleukin.

## Results

**IL-10 induces apoptosis in monocytes.** Monocytes isolated from PBMCs of healthy donors usually died after several days due to a constitutively active cell death program (15). This spontaneous cell death was reduced by 20% following stimulation with IL-4 and GM-CSF for five days (Fig. 1A). The viability of the stimulated monocytes, determined by trypan blue exclusion assay, was reduced when IL-10 was added and the proportion of trypan blue-stained cells increased following treatment with higher IL-10 concentrations (Fig. 1A). Similarly, Annexin V-PI staining revealed that the proportion of apoptotic cells was elevated with increasing IL-10 concentration and increased culture duration (Fig. 1B and C). The apoptosis induced by recombinant human IL-10 was significantly increased at concentrations  $>2.5$  ng/ml.

**Gal-1 protects monocytes from IL-10-induced apoptosis.** The percentage of apoptotic cells was determined by Annexin V-propidium iodide staining of the IL-4/GM-CSF-stimulating monocyte culture media with and without 1  $\mu$ g/ml Gal-1 and/or 10 ng/ml IL-10. Stimulated monocyte apoptosis in the IL-10-only group continuously increased over five days. The Gal-1-only group exhibited no increase in apoptosis after three days (Fig. 2A). Furthermore, IL-10+Gal-1-stimulated monocyte apoptosis was not increased after three days (Fig. 2A). The same phenomenon was observed in monocytes isolated from five donors, although the percentage of apoptotic cells varied (Fig. 2B).

**Gal-1 induces IL-10 and G-CSF in stimulated monocytes.** The supernatants of the Gal-1 only group were collected after five days of incubation and analyzed by the CBA system. The

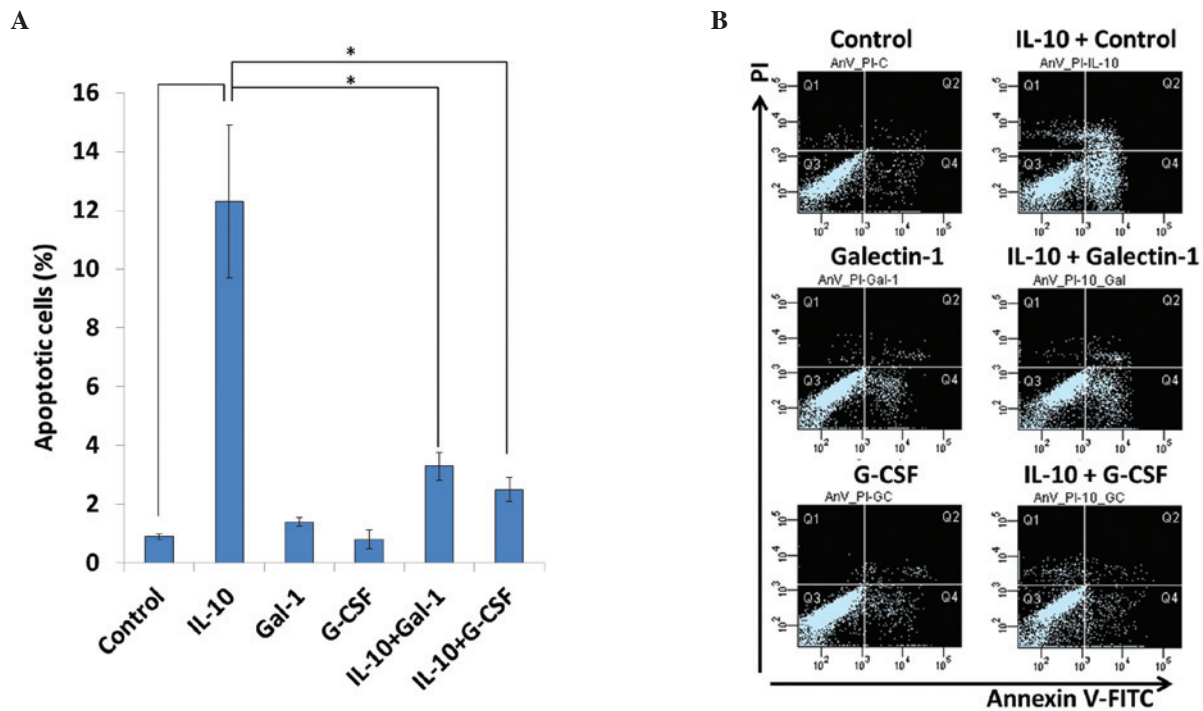


Figure 4. G-CSF inhibits IL-10-induced apoptosis in stimulated monocytes. (A-B) The percentage of apoptotic cells on day five was determined by Annexin V-PI staining and repeated in three healthy donors. \* $P < 0.05$  vs. IL-10-only group, as determined by one-way analysis of variance. G-CSF, granulocyte colony-stimulating factor; Gal-1, galectin-1; IL, interleukin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

concentrations of >10 cytokines (i.e. IL-1, -4, -6, -8, -10, -11, -12, -17 and -21, interferons (IFNs), the tumor necrosis factors (TNFs), basic fibroblast growth factor, vascular endothelial growth factor and G-CSF) were determined, with GM-CSF serving as an internal control. Gal-1 enhanced the expression levels of IL-6, IL-10 and G-CSF, but not those of the other cytokines (Fig. 3A-C).

**G-CSF inhibits IL-10-induced apoptosis in monocytes.** When IL-10 (10 ng/ml) was added to the IL-4/GM-CSF-stimulated monocyte culture media with and without Gal-1 (1  $\mu$ g/ml) and G-CSF (10 ng/ml), analysis of stimulated monocyte apoptosis revealed that recombinant human G-CSF or Gal-1 significantly inhibited IL-10-induced apoptosis ( $P < 0.05$  as compared with IL-10-only treated cells; Fig. 4A and B).

## Discussion

The fate of monocytes is regulated by different signaling pathways, including those of NF- $\kappa$ B, Fas-Fas ligand (FasL) and the B-cell lymphoma 2 (Bcl-2) family. A previous study reported that spontaneous monocyte apoptosis was inhibited by treatment with inflammatory mediators, including TNF, lipopolysaccharide (LPS), CD40 ligand (CD154), growth factors and cytokines, including GM-CSF and IFN- $\gamma$  (16). Alone, IL-4 does not inhibit spontaneous apoptosis, and may inhibit the anti-apoptotic effects of IL-1 and LPS (3,17). However, co-treatment with GM-CSF and IL-4, according to the monocyte-derived DC protocol, inhibits the spontaneous apoptosis of monocytes (17). This implies that the signaling pathway involved in the anti-apoptotic effect mediated by GM-CSF may be different from the signaling pathway induced by IL-1 and LPS.

Receptors of pro-inflammatory mediators, including TNF receptor, IL-1R, Toll-like receptor 4 and CD14, activate the NF- $\kappa$ B signaling pathway and upregulate anti-apoptotic genes (18). Conversely, the GM-CSF receptor activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT)5 signaling pathway and upregulates Bcl-2 in neural progenitor cells and mouse hematopoietic precursors (19,20).

Studies regarding IL-4 and IL-6 in monocytes support the hypothesis that IL-4 inhibits IL-6 production by reducing nuclear NF- $\kappa$ B levels (21,22). However, the interaction between the IL-4 signaling pathway and STAT5 in monocytes has not been reported. Notably, in the present study, apoptosis enhanced by another Th2 cytokine, IL-10, was not inhibited by the presence of GM-CSF, suggesting a difference between IL-10-induced apoptosis and apoptosis enhanced by IL-4. Hashimoto *et al* (23) obtained similar results and further demonstrated that IL-10 inhibited the phosphorylation of STAT5 induced by GM-CSF. In another study, Schmidt *et al* (24) found that CD95 ligand-neutralizing antibody significantly inhibited IL-10-induced apoptosis. In conclusion, IL-10 may induce apoptosis by inhibiting STAT5 and by activating the Fas/FasL signaling pathway.

Galectins are a family of 15  $\beta$ -galactoside-binding proteins. Gal-1 is a 14.5 kDa protein and was the first galectin family member to be described. Dimeric Gal-1 binds to glycoproteins and activates signaling pathways, including those of CD4, CD7, CD43 and CD45 (25-28). Numerous studies have demonstrated that Gal-1 induces apoptosis in T cells (25,28-32) and macrophages (33), which may be involved in the regulation of immune responses. The signaling pathway involved in Gal-1-mediated T-cell



death requires clarification, as data remain inconclusive due to variations in Gal-1 interacting proteins and concentrations (34).

A study revealed that Gal-1 regulates the T-cell immune response through upregulating IL-10 expression; Gal-1 did not induce apoptosis in myeloid lineage and Th cells, but did increase the regulatory T-cell population (35). In another model, recombinant Gal-1 enhanced IL-10 expression levels up to seven-fold, but the apoptosis induced by high dosages of IL-10 was not observed, implying that other signaling pathways activated by Gal-1 inhibit the pro-apoptotic effects of IL-10 (36). In the present study, Gal-1 enhanced IL-6 and G-CSF expression levels up to twelve- and nine-fold, respectively, but not the expression levels of pro-inflammatory cytokines (i.e. TNF, IFN and IL-12; data not shown). Mangan and Wahl (37) reported that IL-6 exerted no effect on non-stimulating apoptosis; this was also observed in later studies (5,6). The present study demonstrated that IL-6 did not inhibit IL-10-induced apoptosis in IL-4/GM-CSF-stimulated monocytes. However, another hematopoietic growth factor induced by Gal-1, G-CSF, was found to reduce IL-10-induced apoptosis.

G-CSF is the predominant regulator of neutrophil production under basal conditions of hematopoiesis. G-CSF maintains neutrophil survival (38,39) and regulates the survival and mobilization of cardiomyocytes and neurons (40-42). The G-CSF receptor belongs to the cytokine receptor type I superfamily, which engages the canonical JAK/STAT, Ras/Raf/mitogen-activated protein kinase and protein kinase B signaling pathways, all of which are crucial for the anti-apoptotic function of G-CSF (43,44).

The present study demonstrated that G-CSF not only exerted an anti-apoptotic effect on monocytes, but also inhibited IL-10-induced apoptosis without affecting the tolerogenic function of IL-10 (data not shown). Examining the network of cytokines that regulate the fate of monocytes, this implies that Gal-1 reinforces its immune modulating effects by simultaneously upregulating IL-10 and G-CSF. Therefore, G-CSF may be further applied in immune therapy, particularly in the IL-10-presenting microenvironment.

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