

Hortensia Soto, Willie Hsu, Rena Chen, Juan E. Moyron-Quiroz, John Ransom, and Jie Zhou
 Department of Molecular and Cellular Immunology, BioLegend, San Diego, CA, USA.

Abstract

Myelopoiesis (production of monocytes and granulocytes) is an important component of the major activity of the bone marrow. Hematopoietic stem cells (HSC) are surrounded by a highly regulated environment. Primitive HSCs are located in the bone marrow endosteum niche, and more mature HSC dividing cells are in the vascular or sinusoidal niche. Different cells form and regulate the endosteal niche, such as osteoblasts, osteoclasts, mesenchymal stromal cells (MSCs), Tregs, and macrophages. In the sinusoidal niche, the HSC interact with sinusoidal endothelial cells and leptin receptor⁺ perivascular stromal cells. Myeloid cells are derived from multipotent hematopoietic stem cells (MPC) that will be differentiated to common myeloid progenitors (CMP), and subsequently to granulocyte macrophage precursors (GMP). The key regulator of granulopoiesis is G-CSF that regulates the production of committed-myeloid progenitors. GM-CSF, IL-3, and IL-6 can stimulate granulopoiesis *in vivo*. IL-17-mediated granulopoiesis via G-CSF induces neutrophilia. IL-17 production is induced by IL-23 in neutrophil regulatory T cells (T_N, $\gamma\delta$ TCR); thus, IL-23 has a role in neutrophil homeostasis. CXCL12/SDF-1 is produced constitutively by bone marrow stromal cells and binds to CXCR4; this ligand-receptor interaction plays a key role in neutrophil release from the bone marrow. G-CSF induces downregulation of CXCL12 and upregulation of CXCL1 and CXCL2, thereby modulating release of bone marrow neutrophils. The bioassays for some of the cytokines involved in neutrophil homeostasis are described in this work. GM-CSF and G-CSF activities were measured by induction of TF-1 and M-NFS-60 cell proliferation, respectively. IL-3 and IL-6 activities were measured by induction of M-NFS-60 and 7TD-1 cell proliferation, respectively. IL-17A and IL-23 activities were measured by their ability to induce IL-6 in dermal fibroblasts and IL-17 in T cells, respectively. CXCL1/CXCL2 and CXCL12 activities were measured by chemotaxis of neutrophils and T cells, respectively. We conclude that we have successfully expressed and purified multiple cytokines involved in granulopoiesis, and showed that they have potent biological activity.

Introduction

- Neutrophils develop in the bone marrow at a rate of $5 \times 10^{10} - 10 \times 10^{10}$ cells/day and are the most abundant circulating leukocytes in humans.
- G-CSF is a key regulator in the development of granulocytes, and lack of G-CSF or its receptor results in 70–80% reduction of circulating granulocytes.
- GM-CSF, IL-3, and IL-6 stimulate granulopoiesis *in vivo*, and G-CSF, GM-CSF, and IL-3 show high levels in serum during infections.
- The number of circulating neutrophils is regulated by granulopoiesis, bone marrow release, and clearance.
- CXCL12, produced by bone marrow stromal cells, and its receptor CXCR4 retain neutrophils in the bone marrow.
- The release of neutrophils from the bone marrow is mediated by a decrease of CXCL12 and increase of CXCL1 and CXCL2 production.
- Clearance of apoptotic neutrophils in the tissues is mediated by macrophages and DC, and as a result their IL-23 production is diminished. Therefore, the IL-17 production from T cells ($\gamma\delta$ and NK T cells) is decreased, and finally the secretion of G-CSF and granulopoiesis is reduced in the bone marrow.

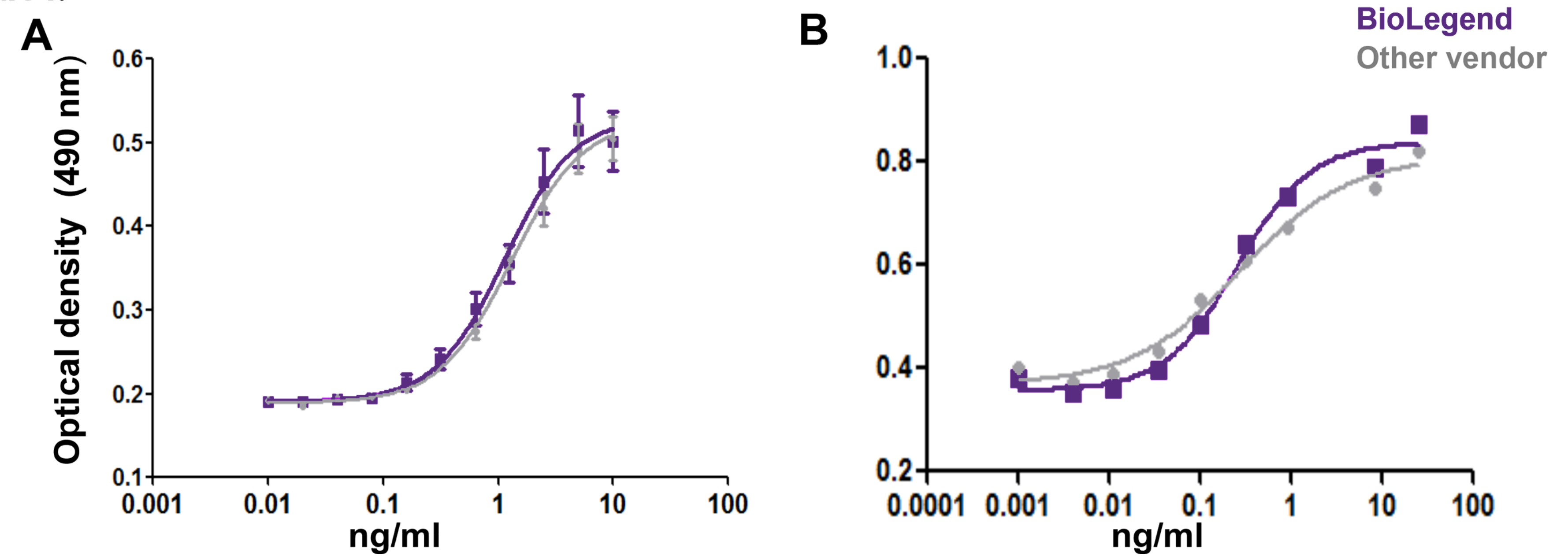
MATERIALS AND METHODS

- **Protein Expression.** The cytokines described in this work were expressed in *E.coli* system, except mouse IL-23 that was expressed in insect cells.
- **Protein purification.** The proteins expressed in *E.coli* were refolded from inclusion bodies. Some of them were purified using IMAC and then followed by Histidine tag removal by sequence specific protease.
- The protein was further purified using ion exchange or reverse phase chromatography.
- **Detection of bioactivity.** The target cells were incubated with different concentrations of cytokine, and the cell proliferation was measured using MTS reagent. In the case of cytokine induction assays, the released cytokine was measured by ELISA in the conditioned medium. Regarding chemotactic experiments, human neutrophils and T cells were purified by conventional methods and used to measure the bioactivity of CXCL2 and CXCL12 respectively.

DISCUSSION

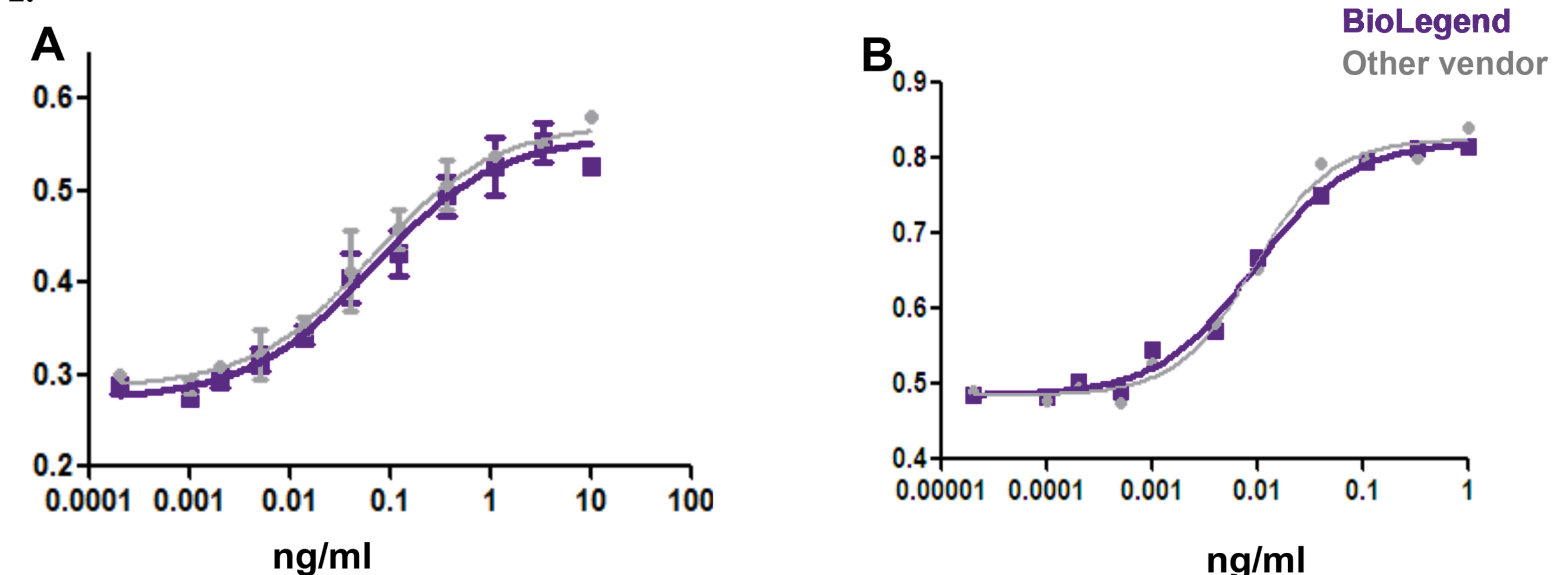
- Immunology research depends on a reliable source of high-quality reagents.
- At BioLegend we have developed an efficient process of cloning, expression, purification, and biotesting for cytokines.
- One of the main objectives at BioLegend is the development of technology for production of high quality secreted proteins in milligram quantities in a dependable and reproducible manner, and made available at low cost.

Figure 1.



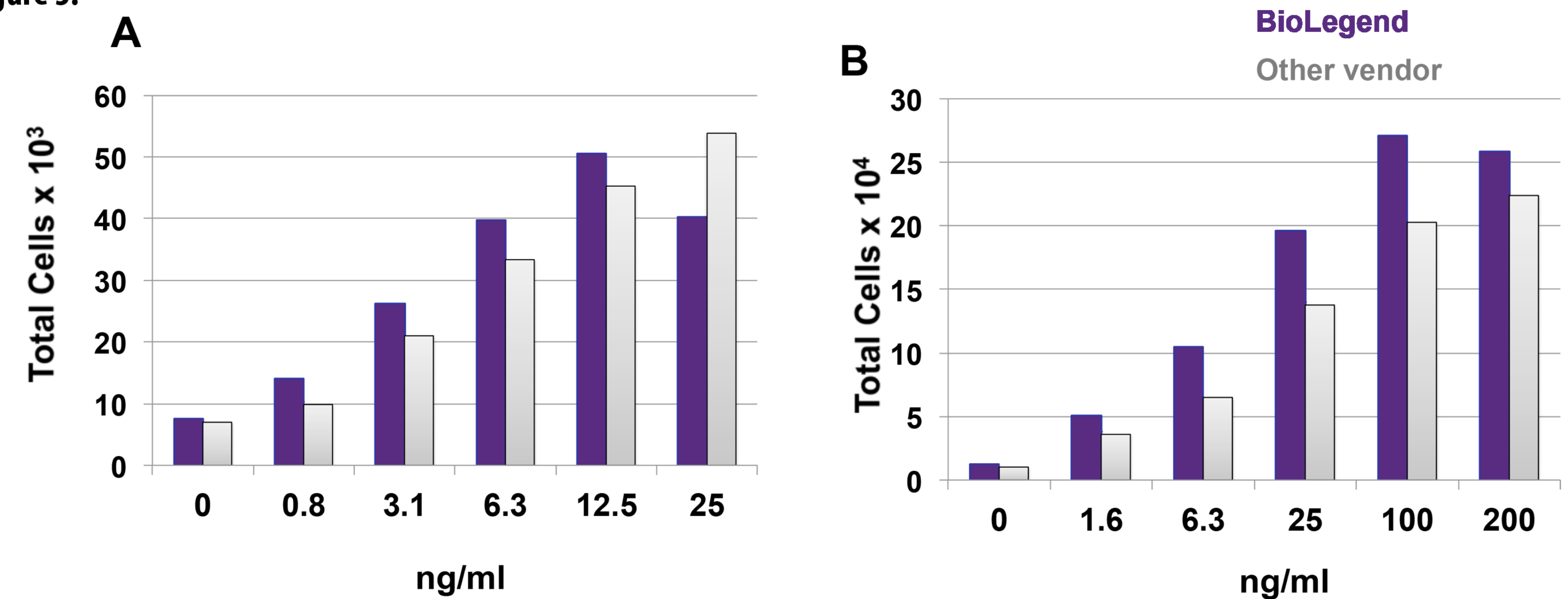
A. Mouse G-CSF induces proliferation in M-NFS-60 cells. Mouse myelogenous leukemia cells were treated with different concentrations of mouse G-CSF. ED50 is 0.5 - 3 ng/ml, corresponding to a specific activity of $0.33 - 2.0 \times 10^6$ units/mg. **B. Human GM-CSF induces TF-1 cell proliferation.** The ED50 is 0.10 - 0.30 ng/ml, corresponding to a specific activity of $0.33 - 1 \times 10^7$ units/mg.

Figure 2.



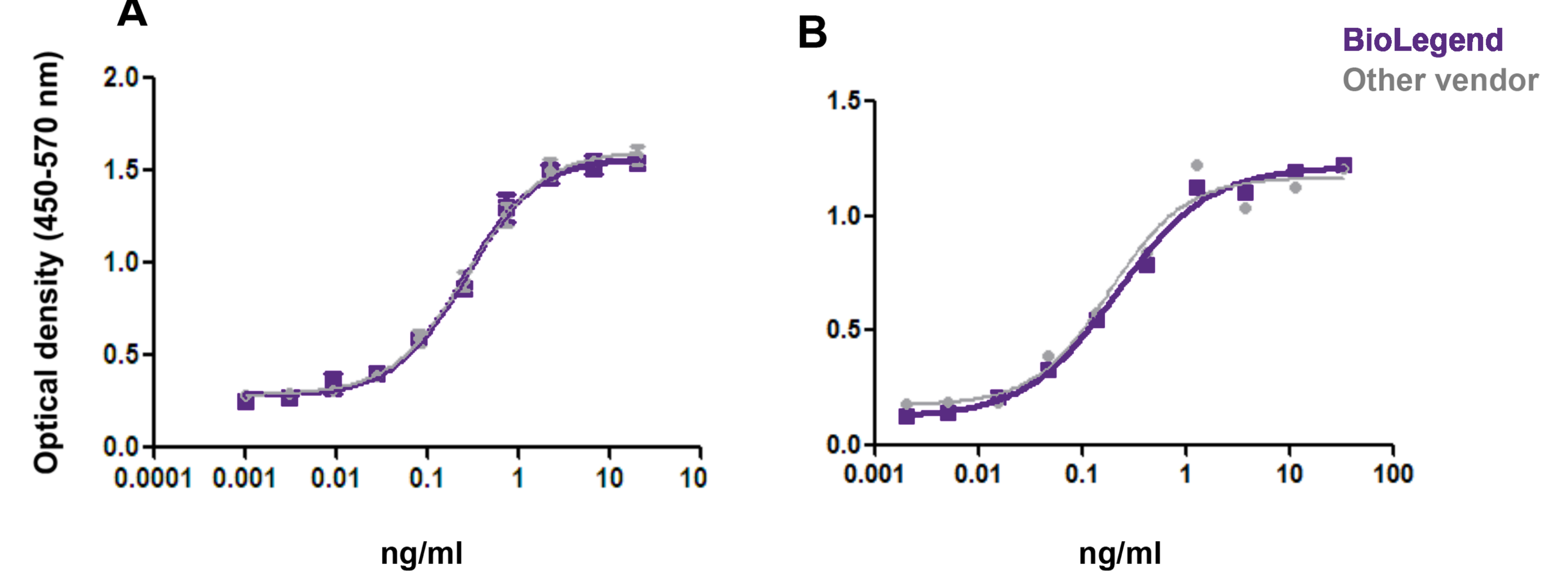
A. Mouse IL-3 induces proliferation in M-NFS-60 cells. ED50 is 0.05 - 0.20 ng/ml, corresponding to a specific activity of $0.5 - 2.0 \times 10^7$ units/mg. **B. Human IL-6 induces proliferation in murine hybridoma 7TD1 cells.** The ED50 is 4 - 20 pg/ml, corresponding to a specific activity of $0.5 - 2.5 \times 10^8$ units/mg.

Figure 3.



A. Mouse CXCL2 chemoattracts human neutrophils. The chemotaxis was performed using transwell inserts, pore size 3 Mm. **B. Human CXCL12 chemoattracts human T cell.** The chemotaxis was performed in transwell inserts, pore size 5 Mm. In both cases the cells were quantified by FACS

Figure 4.



A. Mouse IL-17 induces IL-6 production from mouse skin fibroblasts. The ED50 is 0.25 - 1 ng/ml, corresponding to a specific activity $1.0 - 4 \times 10^6$ units/mg. **B. Mouse IL-23 induces IL-17 production from activated mouse splenocytes.** ED50 is 0.2 - 0.8 ng/ml, corresponding to a specific activity of $1.25 - 5 \times 10^6$ units/mg.