

# The Role of CD40/CD40 Ligand Interactions in Bone Marrow Granulopoiesis

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The CD40 ligand (CD40L) and CD40 are two molecules belonging to the TNF/TNF receptor superfamily, and their role in adaptive immune system has widely been explored. However, the wide range of expression of these molecules on hematopoietic as well as nonhematopoietic cells has revealed multiple functions of the CD40/CD40L interactions on different cell types and processes such as granulopoiesis. CD40 triggering on stromal cells has been documented to enhance the expression of granulopoiesis growth factors such as granulocyte-colony-stimulating factor (G-CSF) and granulocyte/monocyte-colony-stimulating factor (GM-CSF), and upon disruption of the CD40/CD40L-signaling pathway, as in the case of X-linked hyperimmunoglobulin M (IgM) syndrome (XHIGM), it can lead to neutropenia. In chronic idiopathic neutropenia (CIN) of adults, however, under the influence of an inflammatory microenvironment, CD40L plays a role in granulocytic progenitor cell depletion, providing thus a pathogenetic cause of CIN.

**KEYWORDS:** CD40L, CD40, granulopoiesis, G-CSF, GM-CSF, Flt3-L, neutropenia, apoptosis, tumor necrosis factor family, and granulocytic progenitor cells

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## 1. INTRODUCTION

CD40 ligand (CD40L) is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) family, and its gene is located on the q arm of chromosome X [1]. Following activation, it can be upregulated predominantly on CD4<sup>+</sup> T-cells and platelets, but also on CD8<sup>+</sup> T-cells, natural killer (NK) cells, B-cells, dendritic cells (DCs), monocytes/macrophages, basophils, eosinophils, and endothelial cells [1, 2]. Its receptor, CD40, is a type I transmembrane protein, belonging to the TNF receptor superfamily and is encoded by the gene located on the q arm of chromosome 20 [3]. CD40 is constitutively expressed on normal and leukemic B cells, as well as on monocytes, macrophages, endothelial cells, epithelial cells, smooth muscle cells (SMCs), DCs, fibroblasts, and adipocytes [1, 4].

The cytoplasmic domain of CD40 lacks intrinsic signaling activity, and upon ligation, it recruits adaptor proteins, namely, the tumour receptor-associated factors (TRAFs) to initiate different downstream signaling pathways resulting in a wide spectrum of cell-type specific actions [5]. The role of CD40-CD40L interactions on adaptive immunity has been extensively studied [6, 7]. Upon ligation, CD40 stimulates B-cell proliferation and differentiation into plasma cells, germinal center formation, immunoglobulin switching, and somatic hypermutations [8, 9], while it is also involved in the development of thymocytes and differentiation of naïve CD4<sup>+</sup> T cells [10–13]. Furthermore, CD40-40L interactions play an important role in innate immunity [6, 14], apoptosis [15, 16], inflammation, and a number of autoimmune diseases [17–20].

The role of CD40-CD40L dyad, however, has not been elucidated in normal granulopoiesis as well as in neutropenia states. In this paper we will highlight the role of CD40L in the development of granulocytic cells.

## 2. GRANULOPOIESIS

Granulopoiesis is a complex process in which a large number of granulocytes is formed from a small number of hemopoietic stem cells (HSCs) that can replicate and differentiate into multilineage-(common myeloid progenitors; CMPs), double-lineage-(granulocyte/macrophage progenitors; GMPs), and unilineage-committed progenitor cells which further multiply and differentiate into functional mature neutrophils [21, 22]. In early granulopoiesis, granulocyte/monocyte-colony-forming units (CFU-GM), which express the CD34<sup>+</sup> surface antigen, give rise to granulocyte-colony-forming units (CFU-G), while in the later stages of neutrophil maturation, myeloblasts, being the first morphologically distinguishable cells of granulocytic lineage expressing both CD34 and CD33 antigens, can divide and differentiate into promyelocytes (CD34<sup>-</sup>/CD33<sup>+</sup>), myelocytes (CD34<sup>-</sup>/CD33<sup>+</sup>), which further differentiate into metamyelocytes, band cells (CD33<sup>-</sup>/CD15<sup>+</sup>), and finally neutrophils [23–26].

The whole process is strictly controlled by transcription factors, such as CCAAT/enhancer-binding protein alpha (c/EBP $\alpha$ ), PU.1, c-Myb, lymphoid enhancer-binding factor-1 (LEF-1), c/EBP $\epsilon$  [27–30], and growth factors and cytokines like stem-cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3-L), interleukin(IL)-3, granulocyte/monocyte-colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) [31–36]. *In vivo*, the growth factors that promote granulopoiesis are produced mainly in the bone marrow (BM) stromal microenvironment as indicated from *in vitro* experiments with long-term BM cultures (LTBMCs), where a stromal layer of adherent cells consisting mostly of fibroblasts, macrophages, endothelial cells, and adipocytes can produce constitutively or after stimulation the above mentioned growth factors [37–40].

Among the major regulators of granulopoiesis are the GM-CSF and G-CSF that can act through their receptors on a range of hematopoietic as well as nonhematopoietic cells [41–43]. G-CSF is produced by monocytes and macrophages, fibroblasts, and endothelial cells following stimulation with lipopolysaccharide (LPS), IL-1, IL-3, GM-CSF, TNF $\alpha$ , and interferon (IFN)- $\gamma$  [44–48]. It controls the survival, proliferation, and differentiation of cells along the granulocytic pathway and is necessary for their terminal differentiation to mature neutrophils [49–51]. GM-CSF is produced by macrophages, T lymphocytes,

fibroblasts, endothelial cells, and stromal cells, which in most cases require stimulation with cytokines, antigens, or inflammatory agents [39, 52–55].

GM-CSF promotes the proliferation and maturation of neutrophils, and macrophages from BM progenitors can interact with other factors, which may elevate or decrease cell growth in the presence of different amounts of the cytokine [35, 36, 56, 57].

### 3. THE GRANULOPOIESIS-PROMOTING EFFECT OF CD40L

As mentioned above, BM stromal cells express constitutively or under stimulation CD40 on their surface, and upon engagement with CD40L and/or other stimuli, they can upregulate the expression of GM-CSF and G-CSF, two of the key regulators of granulopoiesis. It has been demonstrated that CD40 triggering on endothelial cells as well as on SMCs enhances the production of GM-CSF [58, 59]. Endothelial cells and macrophages have also been reported to upregulate IL-1 $\beta$  and IFN $\gamma$  expression upon CD40 ligation [6, 60–62], which, in turn, act synergistically with CD40L on fibroblasts to induce GM-CSF expression [63]. Consistent with these hypothesis was the observed upregulation of GM-CSF on thymic epithelial cells following activation with IL1, IFN $\gamma$ , and CD40L [64]. Furthermore, in a recent work from our group, it was demonstrated that CD40 engagement on BM stromal cells from healthy donors resulted in increased levels of both G- and GM-CSF in the supernatants of LTBMcs [65]. In addition, when BM mononuclear cells (BMMCs) obtained from healthy subjects were cocultured with the adherent-cell layer of normal LTBMcs in the presence of CD40L and assessed for clonogenic progenitor cells, the number of granulocytic-colony-forming units (CFU-G) was increased comparing with the untreated cultures [65]. Our data corroborate the granulopoiesis-inducing effect of CD40/CD40L through G- and GM-CSF induction BM stroma.

Flt3-L, which is another hematopoiesis/granulopoiesis-promoting factor that acts on primitive and myeloid-committed hematopoietic cells [66, 67], was shown to be upregulated upon CD40 stimulation on fibroblasts, endothelial cells, and stromal cells from LTBMcs. Furthermore, in CD40L-induced cocultures of endothelial cells (ECs) with CD34<sup>+</sup> cells, the production of Flt3-L increased the number of clonogenic cells [68]. In the same work, CD40 ligation on all stromal cell types resulted in thrombopoietin (TPO) expression, a regulator of early hematopoiesis [69].

### 4. CD40L AND NEUTROPENIA

The CD40-CD40L couple is implicated in different types of neutropenia with distinct pathogenetic cause. The X-linked hyperimmunoglobulin M (IgM) syndrome (XHIGM) is a rare immunodeficiency disease characterized by normal or elevated serum IgM, reduced levels of IgG, IgA, and IgE, and defective T-cell function. The most common clinical signs are infections, arthritis, and mucosal ulcers. In the majority of patients, the syndrome is due to mutations of the gene encoding for CD40L on chromosome X, and almost 70% of these patients have neutropenia, 45% of whom having chronic neutropenia, without the presence of antineutrophil antibodies [70–72] (Table 1). The etiology of neutropenia in XHIGM is not well known, but it has been hypothesized that abnormal CD40L interactions with stromal cells resulting in ineffective synthesis of granulocyte-inducing growth-factors may have a role. In favor of this hypothesis is the finding that treatment of patients with recombinant G-CSF results in increased or normal levels of neutrophil counts [73, 74].

Chronic idiopathic neutropenia (CIN) of adults is an acquired form of neutropenia representing the mild form of the spectrum of BM failure syndromes that are characterized by T-cell and cytokine-mediated suppression of hematopoiesis. The pathogenetic cause of neutropenia is in large due to impaired BM granulopoiesis, and it has been documented defective CFU-G growth potential of BMMCs as well as a lower frequency of CD34<sup>+</sup>/CD33<sup>+</sup> cells which was correlated with Fas overexpression and accelerated Fas-mediated apoptosis within this strictly defined cell compartment [76]. In addition, an inflammatory BM microenvironment with elevated levels of TNF $\alpha$ , IL-1 $\beta$ , TGF $\beta$ 1, IL-6 as well as IFN- $\gamma$  and Fas-ligand-producing activated T-lymphocytes has been documented previously in CIN patients [75, 77, 78] (Table 1).

**TABLE 1:** Pathogenetic and pathophysiologic features of X-linked Hyper IgM Syndrome and Chronic Idiopathic Neutropenia.

	X-linked hyper-IgM syndrome	Chronic idiopathic neutropenia
Clinical features	Infections, arthritis, mucosal ulcers, and neutropenia [70–72]	Neutropenia, and osteopenia/osteoporosis, usually uncomplicated course [75]
Immunologic Features/cytokines	Elevated IgM, reduced IgG, IgA, and IgE levels [70–72]	Elevated TNF $\alpha$ , IFN $\gamma$ , TGF $\beta$ , IL-1 $\beta$ , IL-6, and FasL [75–77]
Pathophysiologic features	Mutations in the CD40L gene, abnormal T-cell mediated signals, failure of B-cell differentiation, and defective regulation of immunoglobulin isotype switching [70–72]	Impaired BM granulopoiesis and T-cell and cytokine-mediated suppression of hematopoiesis [75–78]

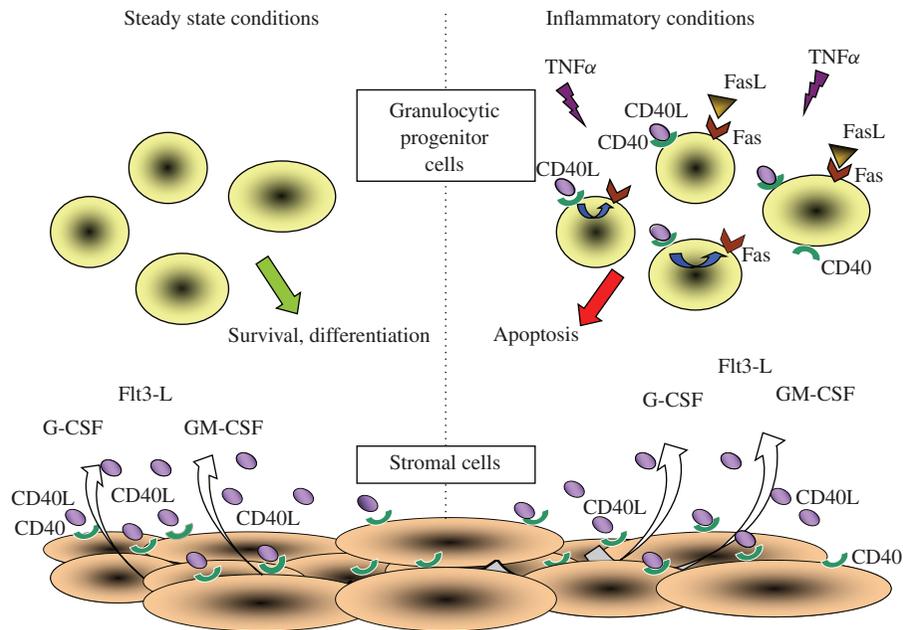
Abbreviations: IgM, immunoglobulin-M; IgG, immunoglobulin-G; IgA, immunoglobulin-A; IgE, immunoglobulin-E; TNF $\alpha$ , tumour necrosis factor- $\alpha$ ; TGF $\beta$ , transforming growth factor- $\beta$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6, interleukin-6; BM, bone marrow.

In a recent study of our group, it was demonstrated that CD40 was minimally expressed on normal BM granulocytic progenitor and precursor cells, namely, the CD34<sup>+</sup>, CD34<sup>-</sup>/CD33<sup>+</sup>, and CD34<sup>-</sup>/CD33<sup>-</sup>/CD15<sup>+</sup> cell subpopulations; however, CD40 was upregulated upon TNF $\alpha$  stimulus in *in vitro* cultures, and upon ligation with CD40L, it resulted in the increased amount of apoptotic cells in the CD40<sup>+</sup> cell compartment compared to untreated cells or TNF $\alpha$ -treated cells [65]. Furthermore, CD40 triggering resulted in Fas expression on these cell populations, which upon stimulation with rhFasL in the presence of both TNF $\alpha$  and CD40L, it resulted in a further increase of apoptotic cells in an additive way. However, when Fas receptor was blocked, CD40L could still initiate apoptosis on granulocytic subpopulations, leading to the assumption that CD40-CD40L interactions can act either directly or indirectly via the Fas-FasL system to induce apoptotic effects on granulocytic progenitor and precursor cells [65]. By evaluating the clonogenic potential of normal and CIN BMNCs under the influence of rhCD40L, it was shown that the number of CFU-G of both groups was decreased although not in a statistically significant way in healthy controls. The prominent decrease of CFU-Gs in CIN patients could be due to increased endogenous TNF $\alpha$  levels in these patients [75] with a subsequent overexpression of CD40 on progenitor and precursor cell surface, rendering those cells more susceptible to the apoptotic effect of CD40 as shown above [65].

Finally, when clonogenic assays were performed with the nonadherent cells of LTBMNCs following coculture with the adherent cells of stromal layer of LTBMNCs in the presence of CD40L alone, the number of CFU-Gs was increased in the case of normal supernatant cells incubated on normal stromal layer as opposed to the decrease in the CFU-Gs in the case of CIN supernatant cells incubated on CIN stromal layer [65]. However, when the nonadherent cell of LTBMNCs of CIN patients were incubated with normal stromal layer in the presence of CD40L alone, the decrease in CFU-Gs previously seen was less (unpublished data). These observations lead to the assumption that CD40L which can be expressed by activated T-lymphocytes present in the BM of CIN patients is a potent effector of granulocytic progenitor cell depletion, resulting in neutropenia, even counterbalancing the beneficial effect of elevated G-CSF found in those patients [75].

## 5. CONCLUSION

The fact that CD40L induces the expression of granulopoiesis-promoting factors such as G-CSF, GM-CSF, and Flt3-L from normal stromal cells leads to the hypothesis that the cytokine could act as a granulopoiesis stimulating molecule under steady state conditions. However, under the influence of an inflammatory microenvironment as in the case of CIN, where there are elevated levels of proinflammatory cytokines like



**FIGURE 1:** Schematic diagram of the CD40/CD40L interactions in the bone marrow. Under steady state conditions, CD40 is minimally expressed on the BM granulocytic progenitor cells, but it is constitutively expressed on BM stromal cells, and upon ligation with CD40 ligand (CD40L), it induces the production of FMS-like tyrosine kinase 3 ligand (Flt3-L), granulocyte-colony-stimulating factor (G-CSF), and granulocyte/monocyte-colony-stimulating factor (GM-CSF). Under inflammatory conditions, involving increased tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), Fas ligand (FasL), and CD40L production, as found in the BM microenvironment of chronic idiopathic neutropenia (CIN) patients, CD40 expression is upregulated in all stages of the granulocytic differentiation, and upon activation with CD40L, it induces the apoptotic cell death both directly and indirectly through Fas upmodulation, counterbalancing the beneficial effect of G-CSF and GM-CSF produced by BM stromal cells.

TNF $\alpha$  and IFN- $\gamma$ , which can induce further the expression of CD40 on granulocytic progenitor and precursor cells, CD40L can act as a granulopoiesis-inhibitory molecule due to increased apoptosis of these cell populations. Accordingly, the CD40-CD40L interactions display a dual effect on granulopoiesis (Figure 1).

## AUTHOR'S CONTRIBUTION

I. Mavroudi wrote the paper and H. A. Papadaki critically reviewed and revised the paper.

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