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Strategies to generate functionally normal neutrophils to reduce infection and infection-related mortality in cancer chemotherapy☆

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ABSTRACT

Neutrophils form an essential part of innate immunity against infection. Cancer chemotherapy-induced neutropenia (CCIN) is a condition in which the number of neutrophils in a patient's bloodstream is decreased, leading to increased susceptibility to infection. Granulocyte colony-stimulating factor (GCSF) has been the only approved treatment for CCIN over two decades. To date, CCIN-related infection and mortality remain a significant concern, as neutrophils generated in response to administered GCSF are functionally immature and cannot effectively fight infection. This review summarizes the molecular regulatory mechanisms of neutrophil granulocytic differentiation and innate immunity development, dissects the biology of GCSF in myeloid expansion, highlights the shortcomings of GCSF in CCIN treatment, updates the recent advance of a selective retinoid agonist that promotes neutrophil granulocytic differentiation, and evaluates the benefits of developing GCSF biosimilars to increase access to GCSF biologics versus seeking a new mode to fundamentally advance GCSF therapy for treatment of CCIN.

Contents

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; BM, bone marrow; CAK, cyclin-dependent kinase-activating kinase; CCIN, cancer chemotherapy-induced neutropenia; CDK, cyclin-dependent kinase; C/EBP, CCAAT-enhancer-binding protein; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; CRABP, cellular RA-binding protein; FDA, the US Food and Drug Administration; FN, febrile neutropenia; GCSF, granulocyte colony-stimulating factor; GCSFR, GCSF receptor; GMP, granulocyte-monocyte progenitor; HSC, hematopoietic stem cells; IST, immunosuppressive therapy; MAT1, ménage à trois 1; MDS, myelodysplastic syndrome; MEP, megakaryocyte erythroid progenitor; MPP, multipotent progenitor; NK, natural killer cells; PB, peripheral blood; pRb, retinoblastoma protein; RA, all-trans retinoic acid; RARs, retinoic acid receptors; RAREs, retinoic acid response elements; RARαS77, serine-77 of RARα; RARαS77A, phosphorylation-defective RARαS77 mutant; RAS, RA syndrome; RCTs, randomized controlled trials; RNAPII-CTD, RNA polymerase II C-terminal domain; RXRs, retinoid X receptors; SAA, severe aplastic anemia; STAT3, signal transducer and activator of transcription 3; TFIIH, transcription factor IIH.

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1. Introduction

1.1. Neutrophils and their granule-dependent innate immunity

Neutrophil granulocytes, generally referred to as neutrophils, constitute up to 70% of total circulating leukocytes (Stone, Prussin, & Metcalfe, 2002; Döhrmann, Cole, & Nizet, 2016). They may be subdivided into banded and segmented neutrophils that form part of the polymorphonuclear cell family (Nathan, 2006; Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000), together with basophils (~1% of leukocytes) and eosinophils (~3% of leukocytes) (Stone et al., 2002; Falcone, Haas, & Gibbs, 2000; Fulkerson & Rothenberg, 2013). Neutrophils, the most abundant type of leukocyte, function as the major phagocytes and the final effector cells (Kennedy & DeLeo, 2009; Pham, 2006) to form an essential part of human innate immune system against invading microorganisms (Naumenko, Turk, Jenne, & Kim, 2018; Selders, Fetz, Radic, & Bowlin, 2017; Teng, Ji, Ji, & Li, 2017). The development of neutrophil innate immunity is dependent on neutrophil granulocytic differentiation (Cowland & Borregaard, 2016; Lawrence, Corriden, & Nizet, 2018), during which the production of stage-specific neutrophil granules (Borregaard, Sorensen, & Theilgaard-Monch, 2007; Faurschou & Borregaard, 2003) are essential for the ability of neutrophils to fulfill their functions through coordinated direct and alternative antimicrobial mechanisms (Nathan, 2006; Teng et al., 2017).

1.2. Partially differentiated neutrophils with impaired granule formation characterize the limitation of GCSF therapy

CCIN, typically febrile neutropenia (FN), is associated with infection, infectious mortality, chemotherapy dose reduction, therapy delay, and increased medical cost (Dinan, Hirsch, & Lyman, 2015; Michels et al., 2012; Nesher & Rolston, 2014). Prophylactic use of GCSF has been recommended for treatment of CCIN by the American Society of Clinical Oncology because GCSF reduces neutropenia (Smith et al., 2006, 2015). This endorsement is supported by clinical studies showing that GCSF administration quickly restores numbers of neutrophils, leading to reduction of the duration or degree of neutropenia (Crawford et al., 1991; Dale et al., 2018; García-Carbonero et al., 2001; Lyman, Kuderer, & Djulbegovic, 2002; Pfeil et al., 2015; Wang, Baser, Kutikova, Page, & Barron, 2015) (Table 1). In addition, two systematic reviews and meta-analyses of randomized controlled trials (RCTs) have reported that prophylactic GCSF also reduces early deaths (Kuderer, Dale, Crawford, & Lyman, 2007) or all-cause mortality (Lyman et al., 2013) (Table 2). In contrast, by examining the most important beneficial clinical outcome of CCIN treatment, such as infection, infection-related mortality, patient survival, tumor control, time to progression and response rate, many other clinical studies have shown that GCSF therapy does not significantly improve those critical therapeutic parameters, as reported by different RCTs (Hartmann et al., 1997; Heath et al., 2003; Staar et al., 2001; Trillet-Lenoir et al., 1993; Zinzani et al., 1997) or systematic reviews and meta-analyses (Berghmans et al., 2002; Bohlius, Herbst, Reiser, Schwarzer, & Engert, 2008; Gurion et al., 2012; Mhaskar et al., 2014; Renner et al., 2012; Sung, Nathan, Alibhai, Tomlinson, & Beyene, 2007; Wittman, Horan, & Lyman, 2006) in cancer patients receiving chemotherapy (Table 3) or undergoing stem cell transplantation (Elayan, Bachier, Battiwalla, & Siler, 2018; Khoury et al., 2006; Singh et al., 2018; Sung et al., 2007). Thus, a stark conflict appears in the literature. However, there is general agreement that, despite the advancement of supportive care by GCSF prophylaxis, CCIN remains one of the most feared complications of cancer chemotherapy, and is still a major contributing factor for infection, mortality, increased healthcare cost, and results in delays and dose reductions that compromise the efficacy of chemotherapy (Lalami & Klastersky, 2017; Lyman & Rolston, 2010; Marti, Cullen, & Roila, 2009; Nesher & Rolston, 2014). By assessing those reported fundamental limitations of GCSF, the data reveal that GCSF-expanded neutrophilic precursors (Begley, Lopez, Vadas, & Metcalf, 1985; Begley, Nicola, & Metcalf, 1988; Dührsen et al., 1988; Lord et al., 1989; Lord et al., 1991; Molineux, Pojda, Hampson, Lord, & Dexter, 1990) differentiate into morphologically segmented neutrophils with limited granule formation (Dick, Prince, & Sabroe, 2008; Ding et al., 2013). These partially differentiated neutrophils lack neutrophil granules and thus fail to effectively fight infection, e.g., as shown *in vitro* (Dick et al., 2008; Donini et al., 2007; Leavey et al., 1998), *in vivo* (Ding et al., 2013; Li et al., 2016), or in clinical outcomes of CCIN patients (Gurion et al., 2012; Hartmann et al., 1997; Heath et al., 2003; Renner et al., 2012) (see Section 6 for discussion of clinical literature). Therefore, it would be beneficial to complement the action of GCSF on myeloid expansion with a therapeutic agent that can effectively promote terminal granulocytic differentiation. Such a novel combination could advance the current clinical practice of CCIN treatment by producing functionally normal neutrophils that are capable of fighting infection.

1.3. GCSF biosimilars increase access to GCSF for CCIN treatment

GCSF mainly sustains the quantitative production of neutrophilic precursors (Lieschke et al., 1994; Liu, Wu, Wesselschmidt, Kornaga, & Link, 1996) by inducing large amounts of myeloblasts, promyelocytes, and myelocytes (Begley et al., 1985, 1988; Dührsen et al., 1988; Lord et al., 1989, 1991; Molineux et al., 1990). It also mobilizes hematopoietic progenitors from bone marrow (BM) into the peripheral circulation (Begley et al., 1997; Bendall & Bradstock, 2014; Sheridan et al., 1992). It was among the first cytokines to be identified and rapidly translated into clinical practice (Metcalf, 2010). The GCSF biologic drugs, filgrastim and pegfilgrastim, were approved in 1991 and 2002, respectively, for prevention of CCIN by the US Food and Drug Administration (FDA) (Derbyshire, 2017). With the patent terms expiring for filgrastim

Table 1
GCSF reduces the duration and/or severity of CCIN.

Reference	Type of clinical studies	Cancer types	Main findings
Crawford et al., 1991	Multicenter, randomized, double-blind, placebo-controlled trial; 211 patients	Small-cell lung cancer	Reductions in the incidence of fever, culture-confirmed infections, as well as the duration and severity of grade IV CCIN
García-Carbonero et al., 2001	Multicenter RCT with GCSF plus antibiotics vs. antibiotics alone; 210 patients	Solid tumor	Reductions in the duration of CCIN, antibiotic use, and hospitalization
Lyman et al., 2002	Systematic review and meta-analysis of 8 RCTs; 1144 patients	Solid tumor or malignant lymphoma	Reductions in the risk of FN and documented infection
Pfeil et al., 2015	Systematic review and meta-analyses of 5 RCTs (n = 3908), 11 non-RCTs (n = 4551); and 17 observational studies (n=50,891)	Solid tumor or lymphoma or AML[a]	Reductions in the incidence of CCIN and FN
Wang et al., 2015	Systematic review and meta-analysis of 30 RTCs; 6036 participants	Solid tumor or non-Hodgkin lymphoma	Reduction in FN risk
Dale et al., 2018	Systematic review and meta-analysis of 18 RCTs, 2 nonrandomized trials, and 5 observational studies; 9018 patients	Solid tumor or non-Hodgkin's lymphoma or ALL[a]	Reductions in FN and grade 3 or 4 CCIN incidence

[a] AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia.

Table 2
GCSF reduces early stage death or overall death of CCIN patients.

Reference	Type of clinical studies	Cancer types	Main findings
Kuderer et al., 2007	Systematic review and meta-analysis of 17 RCTs; 3,493 patients	Solid tumor or malignant lymphoma	Reduction of FN risk and early deaths (including infection-related deaths)
Lyman et al., 2013	Systematic review and meta-analysis of 59 RCTs; 24,793 patients	Solid tumor or lymphoma	Reduction of all-cause mortality, by which 4,251 deaths occurred in 11,337 patients receiving GCSF (37.5%) compared with 5188 deaths of 13,456 patients without GCSF (38.6%)

(2013 in the US and 2006 in Europe) and pegfilgrastim (2015 in the US and 2017 in Europe) (Derbyshire, 2017), opportunities for introducing GCSF biosimilars have emerged (Matikas, Georgoulias, & Kotsakis, 2016). The FDA approved filgrastim biosimilar, Zarxio, in 2015 and pegfilgrastim biosimilar, Fulphila, in 2018. Currently, many biopharmaceutical companies have submitted applications for approval of their GCSF biosimilars, or are in the process of developing filgrastim biosimilars (http://www.gabionline.net/Biosimilars/General/Biosimilars-of-filgrastim) or pegfilgrastim biosimilars (http://www.gabionline.net/Biosimilars/General/Biosimilars-of-pegfilgrastim). GCSF biosimilars and GCSF biologics share the same mechanism of action, and GCSF biosimilars are non-inferior to GCSF biologics in terms of safety and effectiveness. Hence, while GCSF biosimilars promote competition in the marketplace, potentially bringing costs down (Editor, 2017; Matikas et al., 2016), they still suffer from the same limitations of GCSF biologics, namely they fail to produce functionally normal neutrophils to effectively fight infection.

1.4. An alternative mode of CCIN therapy potentially overcoming the limitations of GCSF

An effective treatment for CCIN requires quickly producing large amounts of functionally normal neutrophils. Since GCSF-expanded neutrophilic precursors (Begley et al., 1985, 1988; Dührsen et al., 1988; Lord et al., 1989, 1991; Molineux et al., 1990) differentiate into partially mature neutrophils that are incapable of effectively fighting infection (Dick et al., 2008; Ding et al., 2013; Donini et al., 2007; Leavey et al., 1998; Li et al., 2016), an alternative mode is needed to synergize GCSF-mediated myeloid expansion with terminal neutrophil granulocytic differentiation. This synergy should result in sufficient numbers of functional neutrophils to effectively fight infection in CCIN patients. It is well known that all-trans retinoic acid (RA), a natural derivative of vitamin A, regulates transcription to control granulocytic differentiation (Collins, 2008; Dimberg & Oberg, 2003; Hu & Zuckerman, 2014; Kastner & Chan, 2001; Lawson & Berliner, 1999; Melnick & Licht, 1999; Tenen, Hromas, Licht, & Zhang, 1997). RA acts by binding to the nuclear receptor transcription factor retinoic acid receptors (RARs), including α, β, and γ subunits. Liganded RARs interact with retinoic acid response elements (RAREs) in the promoter regions of target genes (de The, Vivanco-Ruiz, Tiollais, Stunnenberg, & Dejean, 1990; Dilworth & Chambon, 2001) as a heterodimer with retinoid X receptors (RXRs), i.e., RXRα, β, or γ, respectively, to induce transcription of RA-target genes (Chambon, 1996; Gudas, 1994; Kagechika, 2002; Umemiya et al., 1997). Among them, RARα is preferentially expressed in myeloid cells and identified with myeloid development (Collins, Robertson, & Mueller, 1990; de The, Marchio, Tiollais, & Dejean, 1989; Gallagher

Table 3
CCIN-related infection and mortality remain a significant concern in GCSF therapy for CCIN treatment.

Reference	Type of clinical studies	Cancer types	Main findings
Trillet-Lenoir et al., 1993	RCT; 130 patients	Small cell lung cancer	1) No significant difference in terms of response or survival. 2) Reductions in hospitalization and antibiotic use.
Zinzani et al., 1997	RCT; 158 patients	High-grade non-Hodgkin's lymphoma	1) No significant modifications to dose intensity, complete response rate, and relapse-free survival. 2) Reduction of infection and CCIN rate.
Hartmann et al., 1997	Randomized, double-blind, placebo-controlled trial; 138 afebrile CCIN patients	Solid tumor or lymphoma	1) No difference in the rate of hospitalization, number of days in the hospital, use of parenteral antibiotics, or culture-positive infections. 2) Reduction in the duration of afebrile CCIN.
Staar et al., 2001	Multicenter randomized trial with radio-chemotherapy vs. radiotherapy; 240 patients.	Head-and-neck cancer	Prophylactic GCSF was a poor prognostic factor, and resulted in an unexpected reduced local control of tumor.
Heath et al., 2003	RCT, 287 patients	Children with high-risk ALL	1) No significant difference in FN, positive blood cultures, serious infections, overall survival, or 6-year event-free survival. 2) Reduction in the duration of CCIN.
Berghmans et al., 2002	Systematic review and meta-analysis of FN in 11 RCTs with 1,218 FN episodes occurred in 1,141 patients.	Solid tumor, lymphoma, ALL, or hematological malignancy	1) No effect on FN-related mortality. 2) Small reduction in hospitalization and the duration of CCIN.
Wittman et al., 2006	Systematic review and meta-analysis of 16 RCTs; 804 patients.	Pediatric ALL, lymphoma, or solid tumor	1) No effect on documented infections. 2) Reductions in FN, durations of severe CCIN, and antibiotic use.
Sung et al., 2007	Systematic review and meta-analysis of 148 RCTs; 16,839 participants or cycles.	Leukemia, solid tumor, lymphoma, or undergoing SCT[a]	1) Little or no effect on short-term all-cause mortality or infection-related mortality. 2) Reductions in the duration of FN, rates of infection, and hospitalization.
Bohlius et al., 2008	Systematic review and meta-analysis of 13 RCTs; 2607 patients.	lymphoma	1) No effect on infection-related mortality, overall survival, tumor response, freedom from treatment failure, or antibiotic use. 2) Reductions in the risk of CCIN, FN, and infection.
Renner et al., 2012	Systematic review and meta-analysis of 8 RCTs; 2156 participants.	Breast cancer	1) No effect on infection-related mortality, infections, severe CCIN, or maintaining dose intensity. 2) Reduction of FN. Weak evidence of decreasing early mortality and hospital care.
Gurion et al., 2012	Systematic review and meta-analysis of 19 RCTs; 5256 patients.	AML	No differences in all-cause mortality, overall survival, complete remission rate, relapse rate, disease-free survival, bacteremia, or invasive fungal infection.
Mhaskar et al., 2014	Systematic review and meta-analysis of 14 RCTs with GCSF plus antibiotics vs. antibiotics alone; 1553 participants.	Hematological or solid tumors or mix	1) No effect on overall mortality or infection-related mortality. 2) Reductions in the duration of CCIN and antibiotic use, together with a faster recovery from fever.

[a] SCT: stem cell transplantation.

et al., 1989; Largman, Detmer, Corral, Hack, & Lawrence, 1989). Unliganded RARα delays myeloid differentiation (Chomienne et al., 1991; Kastner et al., 2001; Kastner & Chan, 2001), whereas the myeloid-specific effect of RA-liganded RARα on regulating granulocytic differentiation has been demonstrated in normal and malignant myeloid tissues (Breitman, Selonick, & Collins, 1980; Collins et al., 1990; Douer, Ramezani, Parker, & Levine, 2000; Fazi et al., 2005; Gratas, Menot, Dresch, & Chomienne, 1993; Luo et al., 2007; Walkley et al., 2004; Wang et al., 2002; Wang et al., 2006). Notably, RA-modified RARα action can induce terminal granulocytic differentiation of acute promyelocytic leukemia (APL) cells harboring PML-RARα fusion products (Melnick & Licht, 1999; Pitha-Rowe, Petty, Kitareewan, & Dmitrovsky, 2003; Wang et al., 2006). This discovery has led to the first and thus far the most successful clinical application of differentiation therapy for APL patients (Ablain & de The, 2014; Grimwade, Mistry, Solomon, & Guidez, 2010; Tenen, 2003; Warrell Jr. et al., 1991). In recent years, some synthetic RA agonists with better efficacy than RA in selectively modulating RAR activity have been discovered (Marchwicka, Cunningham, Marcinkowska, & Brown, 2016), e.g., RA agonist *VTP-195183* mainly enhances GCSF-mediated mobilization of hematopoietic stem cells (HSC) and progenitors (Beard et al., 2002; Brown, Marchwicka, Cunningham, Toellner, & Marcinkowska, 2017; Chee, Hendy, Purton, & McArthur, 2013; Herbert et al., 2007) (https://clinicaltrials.gov/ct2/show/NCT02749708?term=VTP+195183&rank=2), whereas RA agonist Am80 (Kagechika, Kawachi, Hashimoto, Himi, & Shudo, 1988; Miwako & Kagechika, 2007) effectively promotes terminal granulocytic differentiation *in vitro* (Hashimoto et al., 1994; Hashimoto et al., 1995; Jimi et al., 2007; Kagechika, 2002; Umemiya et al., 1997), *in vivo* (Ding et al., 2013; Li et al., 2016), and in APL patients (Kitamura et al., 1997; Shinjo et al., 2000; Takeshita et al., 1996; Takeuchi et al., 1998; Tobita et al., 1997). Here, we review the literature with focus on: 1) terminal neutrophil granulocytic differentiation and the underlying regulatory mechanisms of action modulated by key transcription factors; 2) RA: RARα-coordinated transcriptional regulation and cell cycle control of terminal neutrophil granulocytic differentiation versus biological action of GCSF in mediating expansion of neutrophilic precursors; 3) the limitations of GCSF therapy for CCIN; and 4) the effects of Am80 on promoting terminal granulocytic differentiation to produce functional neutrophils effective in fighting infection. Hence, this review aims to provide insight that may be useful for the development of novel synergistic treatment of CCIN by combining the selective RA agonist Am80 with GCSF.

2. Hematopoiesis and granulopoiesis

2.1. Granulocytic progenitors derived from HSC

Hematopoiesis-inducing granulocytic progenitors occur primarily within the BM where blood cells are formed (Akashi, Traver, Miyamoto, & Weissman, 2000; Jagannathan-Bogdan & Zon, 2013; Rieger & Schroeder, 2012). Stage-specific hematopoiesis, characterized by the expression of surface markers (Giebel & Punzel, 2008), first gives rise to multipotent progenitor (MPP) (Oguro, Ding, & Morrison, 2013). MPP further develops to common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). Whereas CLP progenitors will finally become T cells, B cells, or natural killer (NK) cells, CMP differentiates into either a megakaryocyte erythroid progenitor (MEP) or a granulocyte-monocyte progenitor (GMP) (Challen, Boles, Chambers, & Goodell, 2010; Nandakumar, Ulirsch, & Sankaran, 2016). Starting from the stage of GMP, the granulocyte population is formed together with other myeloid populations of monocytes, thrombocytes, and erythrocytes (Fig. 1) under distinct regulatory mechanisms (Dykstra et al., 2007; Muller-Sieburg, Cho, Karlsson, Huang, & Sieburg, 2004; Schroeder, 2010).

2.2. Early myeloid proliferation and late terminal granulocytic differentiation in granulopoiesis

Granulopoiesis, the process of producing sequential stage-specific granules, starts from the myeloblast stage of committed neutrophilic precursors (Bainton, Ullyot, & Farquhar, 1971; Cowland & Borregaard, 2016; Lübbert, Herrmann, & Koeffler, 1991). It gives rise to several morphologically distinct stages that are readily identified by their characteristic nuclear shape and granule contents. At least four subtypes of granules, including primary (azurophil) granules, secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles, have been characterized (Borregaard, Theilgaard-Mönch, Sørensen, & Cowland, 2001; Lawrence et al., 2018; Pham, 2006). These granules are essential for neutrophils to fulfill their role as key effector cells of innate immunity against infection (Borregaard et al., 2007; Faurschou & Borregaard, 2003). The sequential formation of different granules has been characterized as two stages of granulocytic differentiation toward maturation of neutrophils, in which early myeloid expansion is associated with formation of primary granules, whereas the productions of secondary, tertiary, and secretory granules occur at the late terminal granulocytic differentiation stage (Cowland & Borregaard, 2016; Lawrence et al., 2018; Pham, 2006). Myeloblasts derived from GMP are the first neutrophilic precursors committed to granulopoiesis. Granule formation within the developing neutrophil begins between the myeloblast and promyelocyte stages, which acquires primary granule and forms a round nucleus (Bainton et al., 1971; Borregaard et al., 2007; Borregaard & Cowland, 1997; Faurschou & Borregaard, 2003). During this early development, both myeloblasts and promyelocytes are still actively proliferating cells (Begley et al., 1985; Boll & Fuchs, 1970; Donohue, Reiff, Hanson, Betson, & Finch, 1958; Lawrence et al., 2018). The next maturation sequence starts from myelocytes that feature the appearance of secondary granules and a round nucleus. Although myelocytes still retain some proliferative capacity, their cell division terminates at transition to the stage of metamyelocytes with a kidney-shaped nucleus (Cowland & Borregaard, 2016; Klausen, Bjerregaard, Borregaard, & Cowland, 2004; Mora-Jensen et al., 2011). After this irreversible cell cycle exit from proliferation, metamyelocytes differentiate into banded neutrophils where tertiary granules are formed, showing a horse-shoe shaped nucleus. Granulopoiesis is completed with the development of secretory vesicles, in which neutrophils acquire a characteristic segmented nucleus (Fig. 2) (Borregaard et al., 2007; Cowland & Borregaard, 2016; Lawrence et al., 2018; Pham, 2006).

3. Transcriptional regulation of neutrophil development

3.1. Coordinated transcriptional network controls granulocytic differentiation

Transcription factors are essential regulators that modulate granulocytic differentiation and govern the transition between proliferation and differentiation, through which the first stage of myeloid

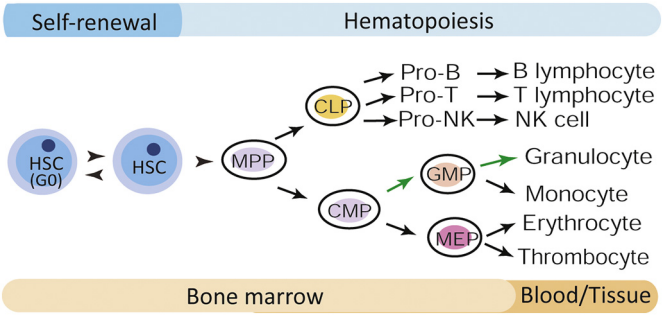

Fig. 1. Conventional model of hematopoiesis. The granulopoietic pathway is marked in green.

proliferation and the second stage of terminal neutrophil granulocytic differentiation are tightly controlled (Bretones, Delgado, & León, 2015; Cowland & Borregaard, 2016; Friedman, 2002; Tenen, 2003; Tenen et al., 1997). The CCAAT-enhancer-binding protein (C/EBP) is a family of transcription factors composed of six members named from C/EBPα to C/EBPζ. They play critical roles in both myeloid expansion and granulocytic differentiation. Among them, C/EBPα and C/EBPε are the two key regulators governing granulopoiesis at the early and late developmental stages, respectively (Borregaard et al., 2001; Cheng et al., 1996; Dahl et al., 2003; Lekstrom-Himes, 2001; Radomska et al., 1998). Equally, transcription factors RARα, c-Myc, and PU.1 play essential roles in a coordinated manner to modulate myeloid expansion, transition to irreversible cell cycle arrest, and granule formation (Bretones et al., 2015; Dimberg & Oberg, 2003; Friedman, 2002; Uribesalgo, Benitah, & Di Croce, 2012), as described below.

3.2. Synergized transcriptional regulation mediated by C/EBPα, RARα, PU.1, and c-Myc in early stage of granulopoiesis

C/EBPα is the predominant C/EBP isoform expressed in immature myeloid cells during the developmental stages of myeloblast and promyelocyte (Bjerregaard, Jurlander, Klausen, Borregaard, & Cowland, 2003). Absence of C/EBPα ceases neutrophil development (Zhang et al., 1997), and C/EBPα is involved in regulating expression of primary *myeloperoxidase* and *neutrophil elastase* granules (Oelgeschläger, Nuchprayoon, Lüscher, & Friedman, 1996; Wang, Wang, Ward, Touw, & Friedman, 2001). Expression of C/EBPα also results in production of neutrophilic cells that express mRNAs encoding specific *lactoferrin* and *collagenase* granules (Radomska et al., 1998). Of note in early stage of granulocytic differentiation, both C/EBPα and RARα can induce transcription of *PU.1* (Kummalue & Friedman, 2003; Mueller et al., 2006; Saeed, Logie, Stunnenberg, & Martens, 2011; Wang, Wang, et al., 2010; Yeamans et al., 2007), a hematopoietic transcription factor that modulates the formation of myeloperoxidase (Ford et al., 1996), neutrophil elastase (Oelgeschläger et al., 1996), lysozyme (Ahne & Strätling, 1994), and proteinase-3 primary granules (Sturrock, Franklin, & Hoidal, 1996).

c-Myc regulates myeloid expansion by promoting cell growth in actively proliferating myeloblasts and promyelocytes during early granulopoiesis (Collins, 1987; Dalla-Favera, Wong-Staal, & Gallo, 1982; Gonda & Metcalf, 1984; Obaya, Kotenko, Cole, & Sedivy, 2002; Obaya, Mateyak, & Sedivy, 1999). c-Myc induces most of the critical positive cell cycle regulatory genes to promote cell proliferation, including E2F transcription factors, cyclin-dependent kinases (CDKs), and cyclins (Bretones et al., 2015; Claassen & Hann, 2000; Hu & Zuckerman, 2014; McArthur et al., 2002; Obaya et al., 1999; Peukert et al., 1997; Vlach, Hennecke, Alevizopoulos, Conti, & Amati, 1996; Wu et al., 2003). In E2F−/− cells, c-Myc fails to induce S-phase (Leone et al., 2001), and transcription of E2F genes by c-Myc allows E2F to activate transcription of target genes promoting cell cycle progression (Adams, Sears, Nuckolls, Leone, & Nevins, 2000; Sears, Ohtani, & Nevins, 1997). In proliferating myeloid cells, Myc-Max heterodimers induce E-box-containing genes and repress RARα-target genes, leading to a block of differentiation while cells are proliferating (Uribesalgo et al., 2011, 2012). To promote proliferation, c-Myc also inhibits the transcription of CDK inhibitor $p21^{Cip/Kip}$ (Claassen & Hann, 2000) by interacting with the initiator-binding transcription factor Miz-1 (Peukert et al., 1997; Wu et al., 2003), and down-regulates CDK inhibitor $p27^{Cip/Kip}$ by inducing SKP2, a protein involved in degradation of $p27^{Cip/Kip}$ (Bretones et al., 2011, 2015).

RARα, upon completion of early myeloid expansion and primary granule formation, plays essential role in promoting the transition from myeloid proliferation to late terminal granulocytic differentiation by down-regulating c-Myc expression (Bretones et al., 2015; Dimberg et al., 2002; Dimberg, Karlberg, Nilsson, & Oberg, 2003; Dimberg & Oberg, 2003; Hu & Zuckerman, 2014; Lawrence et al., 2018). At the myelocyte stage, RA-induced suppression of c-Myc expression is the first critical event required for immature myeloid cells to commit to terminal differentiation (Dimberg et al., 2002; Dimberg et al., 2003; Dimberg & Oberg, 2003). Down-regulation of *c-Myc* by RA occurs via its effect on transcript elongation (Bentley & Groudine, 1986). Both RA treatment and blocking *c-Myc* mRNA significantly inhibit c-Myc expression and induce granulocytic differentiation (Bentley & Groudine, 1986; Holt, Redner, & Nienhuis, 1988). Importantly, phosphorylated c-Myc by Pak2 kinase following RA stimulation forms a complex with RARα on RAREs of RA-responsive genes to induce transcription of RARα-target genes (Uribesalgo et al., 2011), leading to irreversible cell cycle arrest and terminal granulocytic differentiation (Dimberg et al., 2002; Uribesalgo et al., 2012). Coordinately, C/EBPα also down-regulates *c-Myc* expression to promote terminal granulocytic differentiation (Johansen et al., 2001).

3.3. Coordinated transcriptional regulation by C/EBPε, RARα, and PU.1 is pivotal in terminal neutrophil differentiation

C/EBPε, predominantly expressed in late granulopoiesis beyond the promyelocyte stage (Bjerregaard et al., 2003; Morosetti et al., 1997), is required for terminal neutrophil differentiation (Borregaard, 2010; Lekstrom-Himes, 2001; Nakajima et al., 2006) by modulating production of secondary and tertiary granules (Gombart et al., 2001; Gombart et al., 2003; Khanna-Gupta, Zibello, Sun, Gaines, & Berliner, 2003; Khanna-Gupta, Zibello, Sun, Lekstrom-Himes, & Berliner, 2001; Nakajima et al., 2006). Moreover, overexpression of C/EBPε in human myeloid cells leads to down-regulation of c-Myc, whereas the interaction of C/EBPε with E2F1 results in repression of E2F1-mediated transcriptional activity (Gery, Gombart, Fung, & Koeffler, 2004). Also, induction of C/EBPε in myeloid cells leads to up-regulation of $p27^{Cip/Kip}$, induction of CD11b, and down-regulation of CDK4/6 and cyclin D2/E/A, which are critical events that arrest cell cycle while promoting maturation of neutrophils (Nakajima et al., 2006).

Importantly, RA-mediated RARα activities are essential to inhibit cell cycle progression while promoting terminal neutrophil

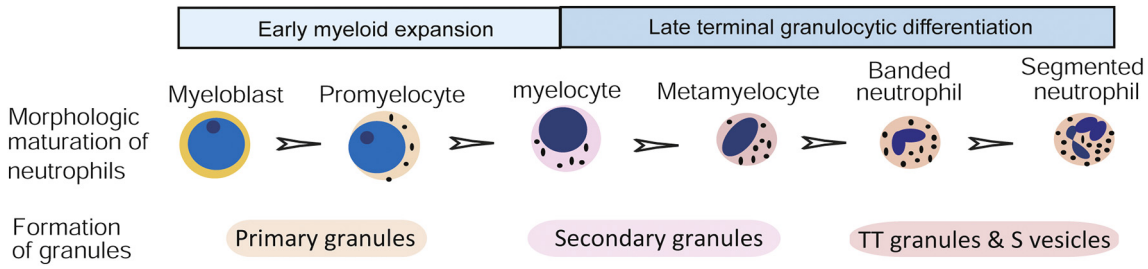

Fig. 2. Schematic illustration of stage-specific granule formation during granulopoiesis. Changes in nucleus distinguish morphologic maturation stages of neutrophils. Banded neutrophils are an intermediate step prior to the complete maturation of segmented neutrophils. Sequential formation of the three different neutrophil granules and secretory vesicles begins with the differentiation of myeloblasts into promyelocytes, and occurs throughout the subsequent stages of neutrophil granulocytic differentiation. TT granules, tertiary granules; S vesicles, secretory vesicles.

differentiation (Collins, 2002; Collins et al., 1990; Dimberg et al., 2002; Dimberg & Oberg, 2003; Gratas et al., 1993; Hu & Zuckerman, 2014; Luo et al., 2007; Purton, Bernstein, & Collins, 1999; Walkley et al., 2004). RARα directly activates transcription of *C/EBPε* (Chih, Chumakov, Park, Silla, & Koeffler, 1997; Park et al., 1999), CDK inhibitor $p21^{Cip/Kip}$ (Jiang et al., 1994; Liu, Iavarone, & Freedman, 1996), tumor suppressor $RAR\beta_2$ (de The et al., 1990; Sucov, Murakami, & Evans, 1990), and the complement receptor-3 (CR3) component of the leukocyte integrin β_2 subunit *CD18* (Bush, St Coeur, Resendes, & Rosmarin, 2003). These RARα targets are responsible for cell cycle arrest and terminal granulocytic differentiation in normal and malignant myeloid cells (Alvarez et al., 2007; Collins, 2002; Collins, 2008; Coqueret, 2003; Dimberg & Oberg, 2003; Hu & Zuckerman, 2014; Mayadas & Cullere, 2005; Soprano, Qin, & Soprano, 2004). RARα also activates PU.1 promoter and restores PU.1 expression to induce neutrophil differentiation in both human myeloid cell lines and primary cells (Mueller et al., 2006; Saeed et al., 2011; Wang, Wang, et al., 2010). To inhibit cell cycle progression while sustaining terminal granulocytic differentiation, RA activates $p21^{Cip/Kip}$ at the transcriptional level whereas up-regulates $p27^{Cip/Kip}$ by sustaining the stability of $p27^{Cip/Kip}$ through ubiquitin-mediated degradation of the F-box protein SKP2 (Dimberg et al., 2002; Dimberg & Oberg, 2003; Dow, Hendley, Pirkmaier, Musgrove, & Germain, 2001; Shimizu, Awai, & Takeda, 2000). Dominant-negative RARα blocks neutrophil differentiation at the promyelocyte stage (Collins et al., 1990; Tsai et al., 1992; Tsai & Collins, 1993). Mice lacking both RARα and RARγ have immature myeloid cells with blocked neutrophil differentiation at the myelocyte stage (Labrecque et al., 1998), which is similar to that observed in C/EBPε (-/-) mice with myelodysplasia (Yamanaka et al., 1997). Such RA-modulated irreversible cell cycle arrest and terminal granulocytic differentiation reflect a tight transcriptional regulation (Dimberg & Oberg, 2003; Hu & Zuckerman, 2014). An initial wave of RARα action resulting in down-regulation of *c-Myc* and *E2F* is immediately followed by up-regulation of $p21^{Cip/Kip}$ and $RAR\beta_2$, together with decreasing *cyclin E* and *cyclin D1/D3* at G_1 exit while suppressing *cyclin A/B* at later stages (Dimberg et al., 2002; Jiang et al., 1994; Liu, Iavarone, & Freedman, 1996; Walkley et al., 2004; Wang et al., 2002, 2006). This leads to decreased cyclin/CDK activities in the phosphorylation of retinoblastoma protein (pRb) and RARα that ultimately mediates cell cycle arrest (Juan, Li, & Darzynkiewicz, 1998; Luo et al., 2007; Mihara et al., 1989; Wang et al., 2006; Wang, Alimova, et al., 2010). A second wave of RARα action is involved in activating transcription of *C/EBPε* (Chih et al., 1997; Park et al., 1999) and *PU.1* (Mueller et al., 2006; Saeed et al., 2011; Wang, Wang, et al., 2010) to promote terminal granulocytic differentiation.

PU.1 plays important roles in mediating production of secondary and tertiary granules as well as secretory vesicles in the late stage of terminal granulocytic differentiation. PU.1 can directly activate transcription of *C/EBPε* (Yoshida et al., 2007). Increased expression of PU.1 occurs throughout the late stage of granulocytic differentiation (Bjerregaard et al., 2003; Cheng et al., 1996), and deletion of PU.1 severely reduces neutrophil production (McKercher et al., 1996; Scott, Simon, Anastasi, & Singh, 1994). PU.1-deficient neutrophils do not express secondary (collagenase, lysozyme, and lactoferrin) and tertiary (gelatinase) granules, resulting in a limited ability to combat infection (Anderson, Smith, Pio, Torbett, & Maki, 1998; Iwama et al., 1998). Binding of PU.1 to the promoters of CR3 signaling molecules CD11b and CD18 are required to modulate the activity of CD11b/CD18 secretory vesicles (Böttinger, Shelley, Farokhzad, & Arnaout, 1994; Chen, Pahl, Scheibe, Zhang, & Tenen, 1993; Rosmarin, Caprio, Levy, & Simkevich, 1995). Moreover, it has been reported that increased expression of PU.1 is involved in transcriptional repression of the c-Myc promoter to inhibit *c-Myc* expression (Kihara-Negishi et al., 2001).

In summary, many studies have demonstrated that C/EBPα, RARα, C/EBPε, PU.1, and c-Myc constitute a key transcriptional regulatory network to mediate granulopoiesis. C/EBPα, C/EBPε, and PU.1 mainly regulate granule production, whereas RARα activates transcription of *C/EBPε* and *PU.1* to modulate stage-specific granule formation, as well as coordinates with c-Myc and C/EBPα to balance proliferation and differentiation (Fig. 3).

4. The role of GCSF in mediating neutrophil granulocytic differentiation

4.1. C/EBPα and C/EBPε signaling in mediating granulopoiesis is independent of GCSF

Studies have shown that GCSF-promoted granulopoiesis occurs via activation of signal transducer and activator of transcription 3 (STAT3) (Shimozaki, Nakajima, Hirano, & Nagata, 1997). GCSF-activated STAT3 binds to C/EBPα, leading to enhancement of transcriptional activity of C/EBPα during granulopoiesis (Numata et al., 2005). Moreover, C/EBPα regulates the GCSF receptor (GCSFR) promoter (Smith, Hohaus, Gonzalez, Dziennis, & Tenen, 1996). Mice with targeted disruption of C/EBPα lose expression of GCSFR and have a selective block in neutrophil differentiation (Zhang et al., 1997). Because C/EBPα can act independent of GCSF signaling to induce granulocytic differentiation (Wang & Friedman, 2002; Wang, Scott, Sawyers, & Friedman, 1999), C/EBPα-modulated expression of GCSFR may in general balance proliferation and differentiation in response to extracellular GCSF signaling *in vitro* and *in vivo* (Wang et al., 1999; Wang et al., 2001; Zhang et al., 1997). GCSF also up-regulates C/EBPε, whereas the region surrounding Tyr-703 of GCSFR is required to generate this induction signal (Nakajima & Ihle, 2001). However, although expression of C/EBPε facilitates GCSF-mediated effects on modulating limited granulocytic differentiation, C/EBPε alone is sufficient to induce morphologically and functionally mature granulocytes (Nakajima & Ihle, 2001).

4.2. GCSF differentiates neutrophilic precursors into partially mature neutrophils with limited granule formation

GCSF is a regulatory cytokine glycoprotein that mediates granulocytic development and stem cell mobilization (Bendall & Bradstock, 2014; Metcalf, 2010). Soon after purified recombinant human GCSF became available, GCSF was found to be capable of mediating limited leukemic cell differentiation by inducing expression of granulocyte membrane antigen (Begley, Metcalf, & Nicola, 1987). GCSF stimulates the production of committed neutrophil progenitors *in vitro* (Burgess & Metcalf, 1980). In human and mice, GCSF profoundly induces large amounts of committed neutrophilic precursors at the stages of myeloblast, promyelocyte, and myelocyte with elevated rates of proliferation (Begley et al., 1985, 1988; Lord et al., 1989, 1991). GCSF-induced stem cell mobilization is characterized by a huge increase of hematopoietic progenitor cells in peripheral blood (PB) (Begley et al., 1997; Dührsen et al., 1988; Molineux et al., 1990; Sheridan et al., 1992). Also, GCSFR- or GCSF-null mice still retain neutrophils but in significantly reduced numbers (Lieschke et al., 1994; Liu, Wu, et al., 1996). Since GCSF lacks the ability to independently modulate terminal neutrophil differentiation, GCSF-induced neutrophilic precursors are differentiated into partially mature neutrophils with limited granule formation and impaired microbicidal function (Dick et al., 2008; Ding et al., 2013; Donini et al., 2007; Leavey et al., 1998; Li et al., 2016).

5. Mechanisms of RA:RARα action in regulating terminal granulocytic differentiation

5.1. RARα links transcriptional regulation and cell cycle control in granulopoiesis

RA stimulation is transduced by RARα to induce terminal granulocytic differentiation after the promyelocyte stage (Collins et al., 1990; Gratas et al., 1993; Labrecque et al., 1998; Purton et al., 1999; Tsai et al., 1992; Tsai & Collins, 1993; Wang et al., 2002; Wang et al., 2006) at both

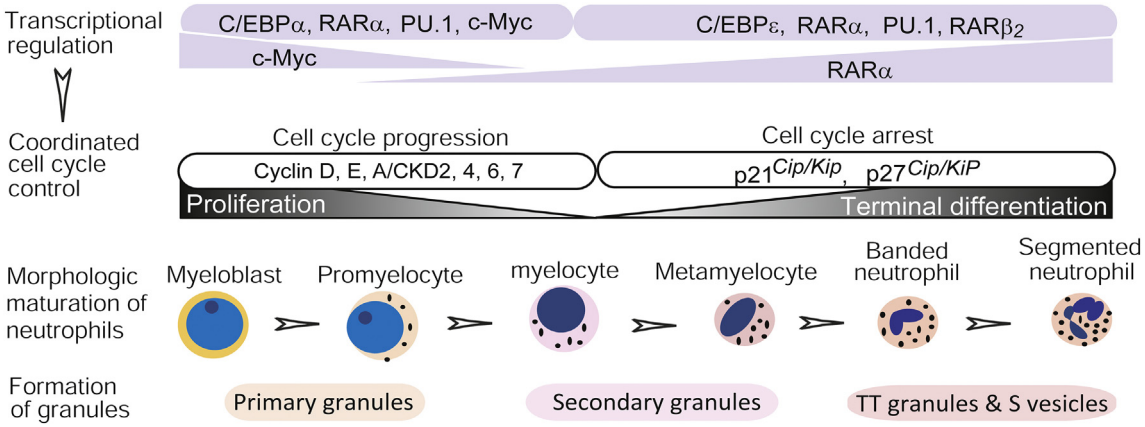

Fig. 3. Transcriptional regulation of myeloid expansion and neutrophil granulocytic differentiation. Schematic diagram of the key transcription factors regulating early myeloid expansion and late terminal granulocytic differentiation. RA-activated RARα induces an irreversible cell cycle arrest and sustains terminal granulocytic differentiation by suppressing c-Myc expression, inducing c-Myc phosphorylation, and modulating c-Myc interaction with the RAREs of target genes. TT granules, tertiary granules; S vesicles, secretory vesicles.

transcription and cell cycle levels (Dimberg & Oberg, 2003; Friedman, 2002; Hu & Zuckerman, 2014; Tenen, 2003; Tenen et al., 1997). This cross-linked regulation mainly results from RA-inhibited phosphorylation of serine-77 of RARα (RARαS77) by either cyclin-dependent kinase-activating kinase (CAK) complex or general transcription factor IIH (TFIIH)-containing CAK complex. CAK regulates cell cycle progression (Fuss & Tainer, 2011; Nigg, 1996) by phosphorylation-activation of different CDKs (Diehl & Sherr, 1997; Fesquet et al., 1993; Kaldis, Russo, Chou, Pavletich, & Solomon, 1998; Kato, Matsuoka, Polyak, Massague, & Sherr, 1994; Wu et al., 2001; Yu et al., 2007), or serves as a kinase subunit of the TFIIH complex (Drapkin, Le Roy, Cho, Akoulitchev, & Reinberg, 1996; Fisher, 2005; Fuss & Tainer, 2011; Shiekhattar et al., 1995) to mediate transcription through phosphorylation of the RNA polymerase II C-terminal domain (RNAPII-CTD) (Busso et al., 2000; Drapkin et al., 1996; Fuss & Tainer, 2011; Helenius et al., 2011; Shiekhattar et al., 1995). Notably, both free CAK and TFIIH-containing CAK phosphorylate RARα (Bour et al., 2005; Rochette-Egly, Adam, Rossignol, Egly, & Chambon, 1997; Wang et al., 2002) to modulate RARα-dependent transcription and cell cycle (Crowe & Kim, 2002; Wang et al., 2002, 2006; Wang, Alimova, et al., 2010).

5.2. RA-suppressed CAK phosphorylation of RARα induces cell cycle arrest and promotes granulocytic differentiation

Decreased CAK phosphorylation of RARα results from RA-mediated MAT1 (ménage à trois 1) ubiquitin-proteolysis (He, Peng, Collins, Triche, & Wu, 2004; Wang et al., 2002), and MAT1 is an assembly factor and targeting subunit of CAK (Fisher, Jin, Chamberlin, & Morgan, 1995; Tassan et al., 1995; Wu et al., 2001). The first evidence that RARα hypophosphorylation induces cell cycle arrest is obtained by expression of the phosphorylation-defective RARαS77 mutant (RARαS77A) (Rochette-Egly et al., 1997) in a human squamous cell carcinoma cell line, by which RARαS77A mimics the effect of RA on inhibiting proliferation (Crowe & Kim, 2002). Later *in vitro* and *in vivo* studies have demonstrated that decreased CAK phosphorylation of RARα can be induced by either RA-mediated MAT1 degradation (He et al., 2004; Wang et al., 2002), RNA-antisense abrogation of *MAT1* expression (Wu et al., 1999, 2001; Zhang et al., 2004), mimicking MAT1 fragmentation (Lou et al., 2013; Luo et al., 2007), or RARαS77A-mimicked CAK hypophosphorylation of RARα (Luo et al., 2007; Wang, Alimova, et al., 2010). The resultant RARα hypophosphorylation induces neutrophil granulocytic differentiation in either normal primary human hematopoietic precursors or myeloid leukemia cells, which is associated with CAK hypophosphorylation of pRb and RNAPII-CTD (Lou et al., 2013; Luo et al., 2007; Wu et al., 2001), up-regulated expression of RA-target genes, and cell cycle G_1 arrest (Chaudhry, Yang, Wagner, Jong, & Wu, 2012; Lou et al., 2013; Luo et al., 2007; Wang et al., 2002; Wang et al., 2006; Wang, Alimova, et al., 2010). Hence, RA-mediated MAT1 degradation leads to CAK hypophosphorylation of RARα, pRb, and RNAPII-CTD, resulting in RARα-mediated transcription of target genes to coordinate cell cycle G_1 arrest and terminal granulocytic differentiation (Fig. 4).

5.3. RA-coordinated action of RARα, c-Myc, and CAK synergizes myeloid expansion and terminal granulocytic differentiation

RA-decreased CAK phosphorylation of RARα induces transcription of RA-target genes to suppress myeloid proliferation and induce granulocytic differentiation (Lou et al., 2013; Luo et al., 2007; Wang et al., 2002; Wang et al., 2006; Wang, Alimova, et al., 2010), in which hypophosphorylated RARα reduces its binding on the RAREs of target genes, leading to dissociation from transcription repressors and recruitment of co-activators (Bastien & Rochette-Egly, 2004; Wang, Alimova, et al., 2010). RA also induces changes in c-Myc phosphorylation to alter c-Myc interaction with RARα, resulting in a switch of c-Myc function from sustaining myeloid expansion to promoting terminal neutrophil differentiation (Uribesalgo et al., 2012). In the absence of RA, c-Myc phosphorylation by Pak2 kinase is inhibited in leukemia cells (Nisimoto & Ogawa, 2002; Uribesalgo et al., 2011). Unphosphorylated c-Myc forms a complex with Max and targets to RAREs by its direct interaction with RARα, resulting in the suppression of RA-responsive genes required for differentiation. Upon RA stimuli, c-Myc is down-regulated and phosphorylated by Pak2 kinase. Phosphorylated c-Myc thereby dissociates from Max but retains its binding to RARα, leading to recruitment of co-activators that activate transcription of the same genes regulating granulocytic differentiation (Uribesalgo et al., 2011; Uribesalgo et al., 2012). Moreover, CAK subunits cyclin H, CDK7, and MAT1 are increased by c-Myc via a posttranscriptional mechanism, and c-Myc promotes proliferation through CDK7 by activating both CAK and TFIIH (Cowling & Cole, 2007; Mateyak, Obaya, & Sedivy, 1999). Equally, c-Myc (-/-) cells show reduced levels of CDK7 and elevated levels of CDK inhibitor $p27^{Cip/Kip}$ (Mateyak et al., 1999; Obaya et al., 2002). While RA suppresses *c-Myc* expression, RA induces degradation of F-box protein Skp2 to promote accumulation of $p27^{Cip/Kip}$ and inactivates cyclin E/CDK2 to block G_1/S transition (Dow et al., 2001; Nishioka et al., 2009).

6. Unmet needs of GCSF prophylaxis in current CCIN therapy

6.1. GCSF-induced partially mature neutrophils mitigate neutropenia but fail to reduce neutropenia-related infection and mortality

GCSF prophylaxis mitigates neutropenia (Smith et al., 2006; Smith et al., 2015) by inducing large amounts of neutrophils to reduce the

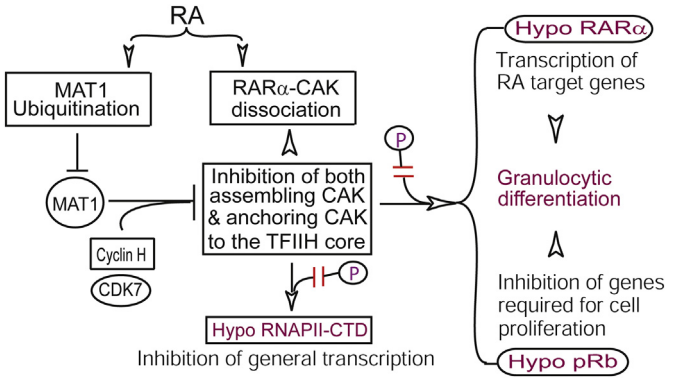

Fig. 4. Hypophosphorylated RARα modulates granulocytic differentiation. RA-induced MAT1 degradation suppresses CAK assembly, interrupts RARα-CAK interaction, and inhibits CAK's attachment to the TFIIH-core. The resultant CAK hypophosphorylation of RARα, pRb, and RNAPII-CTD cross-links transcription and cell cycle to regulate granulocytic differentiation. Hypo: hypophosphorylated.

duration or severity of CCIN (Crawford et al., 1991; Dale et al., 2018; García-Carbonero et al., 2001; Lyman et al., 2002; Pfeil et al., 2015; Wang et al., 2015) (Table 1). Unfortunately, GCSF-induced neutrophils are partially differentiated with limited granule formation, and thus have impaired innate immunity that fails to effectively reduce infection *in vitro* or *in vivo* (Dick et al., 2008; Ding et al., 2013; Donini et al., 2007; Leavey et al., 1998; Li et al., 2016). As such, the resultant reduction of CCIN by GCSF falls short of reducing infection and infection-related mortality, the fundamental limitations of GCSF prophylaxis that have been demonstrated by different clinical studies (Table 3). A RCT of 138 afebrile CCIN patients with severe neutropenia concludes that, although use of GCSF can reduce the duration of neutropenia, there is no effect on the rate of hospitalization, number of days in the hospital, duration of treatment with potential antibiotics, or number of culture-positive infections (Hartmann et al., 1997). A systematic review and meta-analysis of 11 RCTs with 1141 patients shows no advantage in FN-related mortality in GCSF recipients (Berghmans et al., 2002). In a RCT of 287 children with high risk acute lymphoblastic leukemia (ALL) who receive intensive induction therapy, no significant differences are observed between patients receiving and not receiving GCSF at the incidence of FN, positive blood cultures, severe infections, duration of hospitalization, induction therapy completion times, event-free survival and overall survival, despite significantly shortened neutropenia duration in GCSF recipients (Heath et al., 2003). Systematic review and meta-analysis of 13 RCTs, with a total enrollment of 2,607 patients receiving chemotherapy for malignant lymphoma, shows that GCSF does not confer significant advantages in complete tumor response, freedom from treatment failure, use of antibiotics, infection-related mortality, or overall survival (Bohlius et al., 2008). Similarly, there is a large systematic review and meta-analysis of 148 RCTs with a total of 16,839 participants/cycles receiving cancer chemotherapy or conditioning for stem cell transplantation before neutropenia developed. The results show that although the reductions in FN duration and documented infection are observed, GCSF has no effect on infection-related mortality or all-cause mortality (Sung et al., 2007). Systematic review and meta-analysis of 8 RCTs, comprising 2156 participants with different stages of breast cancer undergoing chemotherapy, shows no significant differences between GCSF and control groups in reduction of severe neutropenia, infection, infection-related mortality, or maintenance of scheduled chemotherapy dose (Renner et al., 2012). Consistently, a systematic review and meta-analysis of 19 RCTs, with a total of 5256 acute myeloid leukemia (AML) patients post-chemotherapy, reveals that GCSF has no effect on complete remission, relapse, disease-free survival, overall survival, rate of bacteremias, and invasive fungal infections (Gurion et al., 2012). Moreover, a systematic review and meta-analysis of 14 RCTs has summarized the evidence for the use of GCSF plus antibiotics or antibiotics alone in the treatment of CCIN among 1553 cancer patients. Statistical analyses demonstrate that both overall mortality and infection-related mortality are not significantly improved with GCSF plus antibiotics, although addition of GCSF induces faster neutrophil recovery and reduces hospital stay (Mhaskar et al., 2014).

6.2. GCSF-mediated myeloid overexpansion may lead to leukemogenesis or metastasis

GCSF can profoundly induce large amount of committed neutrophilic precursors (Begley et al., 1985, 1988, 1997; Dührsen et al., 1988; Lord et al., 1989, 1991; Molineux et al., 1990; Sheridan et al., 1992). Use of GCSF to mobilize HSC and progenitors in unrelated volunteers has shown no significant differences in the incidence of malignancy relative to persons not exposed to GCSF (Hölig et al., 2009; Pulsipher et al., 2009). However, in other clinical applications, several lines of study have shown that GCSF-mediated myeloid overexpansion might be associated with leukemogenesis (Beekman & Touw, 2010; de Figueiredo, de Abreue Lima, & Rego, 2004). In a population of women aged 65 or older with stages I to III breast cancer, adjuvant chemotherapy plus GCSF is associated with a doubling in the risk of subsequent AML or myelodysplastic syndrome (MDS) (Hershman et al., 2007). Increasing evidence shows that use of GCSF for neutrophil recovery in breast cancer patients following chemotherapy can promote metastasis (Mouchemore, Anderson, & Hamilton, 2018). The administration of GCSF to children with ALL may increase the risk for developing a therapy-related secondary myeloid malignancy (Relling et al., 2003). Moreover, use of GCSF together with immunosuppressive therapy (IST) for severe aplastic anemia (SAA) patients increases the incidence of AML/MDS. As such, adding GCSF to IST is not standard treatment for SAA (Socie et al., 2007). Also, severe congenital neutropenia patients who receive lifelong treatment with GCSF may have an elevated risk of developing AML/MDS, which has become a major clinical concern particularly in patients harboring mutations in the gene encoding the GCSFR (Beekman & Touw, 2010; Freedman et al., 2000; Rosenberg et al., 2006).

7. Am80 and its clinical use as an effective stimulant of terminal granulocytic differentiation

7.1. Am80 and its mechanism of action in mediating terminal neutrophil differentiation

RA activates RARα, RARβ and RARγ (Chambon, 1996; Glass, DiRenzo, Kurokawa, & Han, 1991; Hashimoto, Kagechika, & Shudo, 1990; Hashimoto & Shudo, 1991; Umemiya et al., 1997), whereas Am80 (Tamibarotene) is a synthetic RA agonist (Fig. 5) with selective affinity for RARα and RARβ (Hashimoto et al., 1990; Kagechika, 2002; Kagechika et al., 1988; Umemiya et al., 1997). The difference of binding affinities between RA and Am80 (Table 4) (Umemiya et al., 1997) essentially constitutes the novel functions of Am80. It has been demonstrated that RARα is a regulatory factor for Am80-induced growth inhibition (Jimi et al., 2007). Am80 has >4-fold binding affinity to RARα than to RARβ while no binding to RARγ (Fukasawa, Iijima, Kagechika, Hashimoto, & Shudo, 1993; Umemiya et al., 1997). RARγ enhances HSC self-renewal (Purton et al., 2006), and RARβ, known as a tumor suppressor, inhibits proliferation (Alvarez et al., 2007; Collins, 2002; Soprano et al., 2004). Hence, Am80 retains RARβ-mediated growth inhibition while eliminating RARγ's action of promoting HSC self-renewal, amplifying the effect of RARα on promoting terminal neutrophil granulocytic differentiation. Unlike RA, Am80 has a lower binding affinity for cellular RA-binding protein (CRABP) and thus generally does not induce drug metabolism (Takagi et al., 1988). Critically, Am80 alters RA-mediated transcription to more effectively induce key differentiation regulators, e.g., *C/EBPε*, *RARβ*$_2$, *CD66c*, and *CD18*. Moreover, Am80 concurrently activates transcription of *C/EBPε* and *RARβ*$_2$ to promote

terminal neutrophil granulocytic differentiation while inhibiting proliferation of committed neutrophilic precursors (Li et al., 2016). Compared to RA, Am80 is about 10 times more potent in leukemia cells and 50 times more potent in normal primary hematopoietic precursors in promoting terminal granulocytic differentiation (Ding et al., 2013; Hashimoto et al., 1995; Kagechika et al., 1988). Compared to GCSF, Am80 not only significantly promotes production of stage-specific granules and secretory vesicles, but also induces significantly higher levels of co-expression of CD66 antigens with CD11b or CD18 to cross-link granulocyte activation and neutrophil immunity response (Ding et al., 2013; Li et al., 2016).

7.2. Am80 has a more favorable safety/efficacy profile compared to RA

Am80, an oral medication, has been approved for treatment of relapsed or refractory APL since 2005 in Japan (Miwako & Kagechika, 2007). Compared to RA, Am80 is more potent at inducing terminal granulocytic differentiation in APL patients (Kitamura et al., 1997; Shinjo et al., 2000; Takeshita et al., 1996; Takeuchi et al., 1998; Tobita et al., 1997). RA resistance develops rapidly in APL patients after a few treatments, which is at least in part due to increased plasma clearance of RA (Marill, Idres, Capron, Nguyen, & Chabot, 2003). Compared to RA, the plasma clearance of Am80 is low after continuous oral administration (Tobita et al., 1997). This retained plasma level by Am80 is likely due to its lower binding affinity for CRABP (Takagi et al., 1988). The toxicity profile of Am80, including adverse effects as well as RA syndrome (RAS) observed in treatment of APL with RA (Breccia et al., 2008), are known to be similar but milder than that of RA (Kitamura et al., 1997; Takeshita et al., 1996; Takeuchi et al., 1998; Tobita et al., 1997). These have been representatively described by a clinical study from Ohno's group (Table 5) (Tobita et al., 1997). The skin irritation resulting from RA binding to RARγ (Chen et al., 1995; Look et al., 1995; Standeven, Teng, & Chandraratna, 1997) has been significantly eliminated by Am80 in APL patients (Kitamura et al., 1997; Shinjo et al., 2000; Takeshita et al., 1996; Takeuchi et al., 1998; Tobita et al., 1997) due to its lack of binding to RARγ (Kagechika, 2002; Kagechika et al., 1988; Miwako & Kagechika, 2007; Umemiya et al., 1997). To date, no severe toxicity related to Am80 has been reported. Moreover, following the successful clinical use of Am80 for APL patients in Japan, many clinical trials in different countries and regions have explored the therapeutic effect of Am80 on treatment of APL and other diseases (Table 6).

8. Am80 differentiates GCSF-induced neutrophilic precursors into functional neutrophils

8.1. Am80 significantly promotes granulocytic differentiation of existing neutrophilic precursors

In cultured normal primary human hematopoietic $CD34^+$ precursors, Am80 generates 46% morphologically segmented neutrophils compared to 18% by GCSF (Ding et al., 2013). More importantly, ultrastructural assessments of those morphologically segmented neutrophils reveal that GCSF-induced neutrophils exhibit limited nuclear segmentation, more intracytoplasmic vacuoles, and much less granule development (Dick et al., 2008; Ding et al., 2013). Further, by comparing bactericidal activity and granule production in equal numbers of Am80- and GCSF-induced morphologically segmented neutrophils, the results show that neutrophils induced by Am80 have significantly higher levels of bacterial killing, granule formation, and granule secretion than those induced by GCSF (Ding et al., 2013). These data demonstrate that Am80-induced neutrophil maturation from existing neutrophil precursors occurs at both morphological and functional levels, whereas GCSF-induced neutrophils show partial morphological maturation and are deficient in both granule formation and innate immunity development (Ding et al., 2013), similar to data reported by other groups (Dick et al., 2008; Donini et al., 2007; Leavey et al., 1998). These observations are further demonstrated by using a mouse model designed to specifically mimic the early neutrophil-decrease stage of CCIN. The results demonstrate that Am80 induces significantly higher numbers of neutrophils in PB with few remaining in BM. In contrast, GCSF induces significantly lower numbers of neutrophils in PB but retains much higher numbers in BM. Also, PB neutrophils induced by Am80 kill significantly more bacteria than those induced by GCSF (Li et al., 2016). Hence, in the early stage of CCIN when granulopoiesis is inhibited in the CCIN mice, Am80-enhanced bacterial killing is the result of Am80-induced differentiation of existing granulocytic precursors into functional neutrophils. These data demonstrate that the effectiveness of Am80 in producing functional neutrophils is significantly superior to GCSF.

Table 4
Differentiated binding affinity of Am80 for RARs and RXRs.

	RARS/RXRs					
	K_i values, $\times 10^{-9}$ M					
Agonist	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
RA	3.3	4.1	0.04	330	54	120
Am80	6.5	30	NB	NB	NB	NB

NB: no binding, as shown that 1000-fold excess of a test compound did not affect the binding of the receptors.

8.2. Am80 can differentiate large amounts of GCSF-induced neutrophilic precursors into functional neutrophils to reduce infection and infection-related mortality

GCSF-treated CCIN mice produce large amounts of morphologically segmented neutrophils that cannot effectively reduce infection of CCIN mice. However, by combining Am80 and GCSF treatment, Am80 differentiates GCSF-induced partially mature neutrophils into functional neutrophils, leading to an effective reduction of infection in CCIN mice (Li et al., 2016). Further, extensive survival tests, covering a full cycle of mouse CCIN with perpetual systemic intravenous bacterial infection, prove that Am80 synergizes with GCSF to induce sufficient numbers of functional neutrophils to significantly reduce infectious death without myeloid overexpansion (Li et al., 2016). These *in vivo* data demonstrate that without myeloid overexpansion caused by GCSF alone, the combination of Am80-GCSF can effectively induce

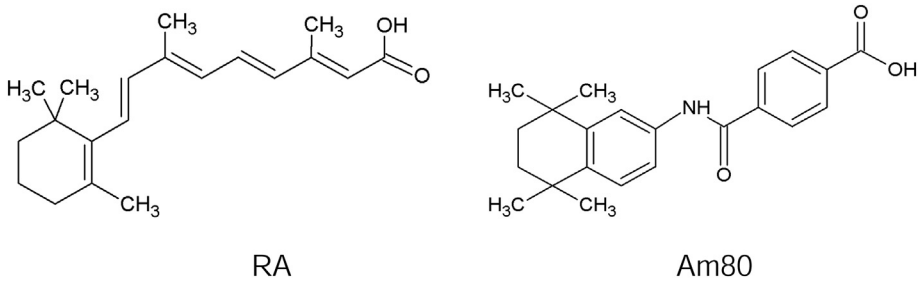

Fig. 5. Molecular structures of RA (All-trans) and Am80 (Tamibarotene).

Table 5
Adverse events in Am80 treatment of APL patients.

Event	Incidence (N, %)			
	Mild	Moderate	Severe	Total (N = 24)
Cheilitis	8 (33%)	0	0	8 (33%)
Xerosis	8 (33%)	1 (4%)	0	9 (38%)
GI trouble	2 (8%)	0	0	2 (8%)
Bone pain	0	5 (21%)	0	5 (21%)
Headache	3 (13%)	3 (13%)	0	6 (25%)
Dermatitis	0	5 (21%)	0	5 (21%)
RA syndrome	0	0	1 (4%)	1 (4%)
Liver damage	3 (13%)	0	0	3 (13%)
Hyperleukocytosis[a]	3 (13%)	0	1 (4%)	4 (17%)
Hypercholesterolemia[b]	10 (42%)	4 (17%)	1 (4%)	15 (63%)
Hypertrigliceridemia[b]	2 (8%)	5 (21%)	9 (38%)	16 (67%)

[a] Mild = <20 × 10^9/L; severe = ≥50 × 10^9/L.
[b] Mild = <3.0 g/L; moderate = 3.0–4.99 g/L; severe = ≥5.0 g/L.

adequate numbers of functional neutrophils (Fig. 6) to reduce CCIN-related infection and mortality (Li et al., 2016).

8.3. Am80-GCSF alters transcriptional regulation to coordinate myeloid expansion with terminal granulocytic differentiation

In normal primary human hematopoietic precursors, the Am80-GCSF combination synergizes myeloid expansion with effective granulocytic differentiation to generate significantly larger amounts of functional neutrophils than Am80 alone. Conversely, in primary human AML specimens, Am80-GCSF inhibits malignant growth while producing functional neutrophils against infection significantly superior to GCSF alone (Li et al., 2016). How can Am80-GCSF have such differential effects on normal versus malignant cells? Compared to RA, Am80 concurrently activates transcription of *C/EBPε* and *RARβ*$_2$ (Li et al., 2016). It is known that RARβ$_2$ is a master tumor suppressor (Alvarez et al., 2007; Soprano et al., 2004) and that C/EBPε is a key regulator of terminal granulocytic differentiation (Lekstrom-Himes, 2001; Park et al., 1999). Both *RARβ*$_2$ and *C/EBPε* are the direct transcriptional targets of RARα (de The et al., 1990; Melnick & Licht, 1999; Park et al., 1999). Of note, Am80-GCSF synergy alters transcription patterns mediated by Am80 or GCSF in both normal and malignant cells to induce significantly higher co-expression of *RARβ*$_2$ and *C/EBPε*. Both GCSF and Am80-GCSF induce significantly higher expression of *RARβ*$_2$ and *C/EBPε* than Am80 alone in the late differentiation induction stage in normal primary cells. However, only Am80-GCSF can consistently induce significantly higher expression of both *RARβ*$_2$ and *C/EBPε* compared to GCSF alone throughout the full differentiation induction period in primary human AML specimens (Li et al., 2016). This suggests that only Am80-GCSF can differentially modulate expression of *RARβ*$_2$ and *C/EBPε* in normal

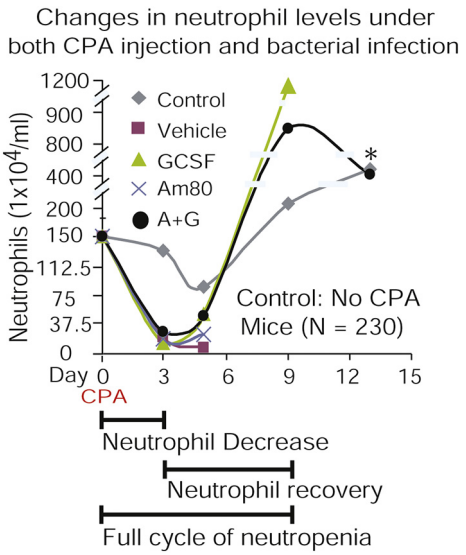

Fig. 6. Am80-GCSF induces sufficient numbers of functional neutrophils without causing myeloid overexpansion in CCIN mice. At the peak of neutrophil recovery on day 9, neutrophils induced by Am80-GCSF were higher than those in control group while lower than those induced by GCSF. Neutrophils induced by Am80-GCSF were reduced to the level similar to those in control group 3 days post-CCIN. CPA injection was on day 0. Based on the designs for different mouse models (Li et al., 2016), regimen treatment was performed at either 4 h, 24 h, or 48 h after CPA injection, whereas tail injection of Gram-positive S. aureus bacteria was performed on day 2, day 3, or day 4. *: no mice survived in GCSF group after day 9; CPA: cyclophosphamide.

versus malignant cells to balance granulocytic differentiation with limited myeloid expansion (Fig. 7).

Additionally, RARα is a substrate for CAK complex (Rochette-Egly et al., 1997) that cross-regulates transcription and cell cycle (Busso et al., 2000; Fuss & Tainer, 2011; Helenius et al., 2011; Nigg, 1996). RA-suppressed CAK phosphorylation of RARα induces granulocytic differentiation in both normal and malignant human hematopoietic precursors (Chaudhry et al., 2012; Lou et al., 2013; Luo et al., 2007; Wang et al., 2002; Wang et al., 2006; Wang, Alimova, et al., 2010). Similarly, RA induces c-Myc phosphorylation to alter interaction of c-Myc with RARα, resulting in the cessation of proliferation sustained by c-Myc to ensure RARα regulation of terminal granulocytic differentiation (Nisimoto & Ogawa, 2002; Uribesalgo et al., 2011; Uribesalgo et al., 2012). Am80 is much more potent than RA in modulating RARα-dependent granulocytic differentiation *in vitro* (Ding et al., 2013; Hashimoto et al., 1995; Kagechika et al., 1988), *in vivo* (Ding et al., 2013; Hashimoto et al., 1995; Kagechika et al., 1988) and in APL patients (Kitamura et al., 1997; Takeshita et al., 1996; Takeuchi et al., 1998; Tobita et al., 1997). Hence,

Table 6
Clinical trials with Am80 (Tamibarotene) treatment of different diseases.

Conditions	Phase	Status	Locations	Reference
Acute myeloid leukemia Myelodysplastic syndrome	II	Recruiting	United States, France	a, b, d, e
Acute promyelocytic leukemia	II	Completed	United States	a, b, d
Alzheimer's disease	II	Recruiting	Japan	a, b, c
Lupus nephritis	II	Unknown Status	Japan	a, b
Relapse and refractory pediatric solid tumor	I	Recruiting	Japan	c
Relapse and refractory neuroblastoma	II	Recruiting	Japan	c
Chronic graft-versus-host disease	II	Follow-up complete	Japan	c
Crohn's disease	II	Completed	Japan	a, b
HTLV-I-associated myelopathy	II, III	Unknown Status	Japan	a, b
Non-small cell lung cancer	II	Terminated	United States, Bulgaria, India, Mexico, Russian Federation, Ukraine	a, b, e

[a] https://clinicaltrials.gov/ct2/results?cond=&term=Am80%2FTamibarotene&cntry=&state=&city=&dist=
[b] http://www.drugbank.ca/drugs/DB04942
[c] https://rctportal.niph.go.jp/en/result
[d] https://www.ema.europa.eu/en/search/search?search_api_views_fulltext=Tamibarotene
[e] https://www.clinicaltrialsregister.eu/ctr-search/search?query=Tamibarotene

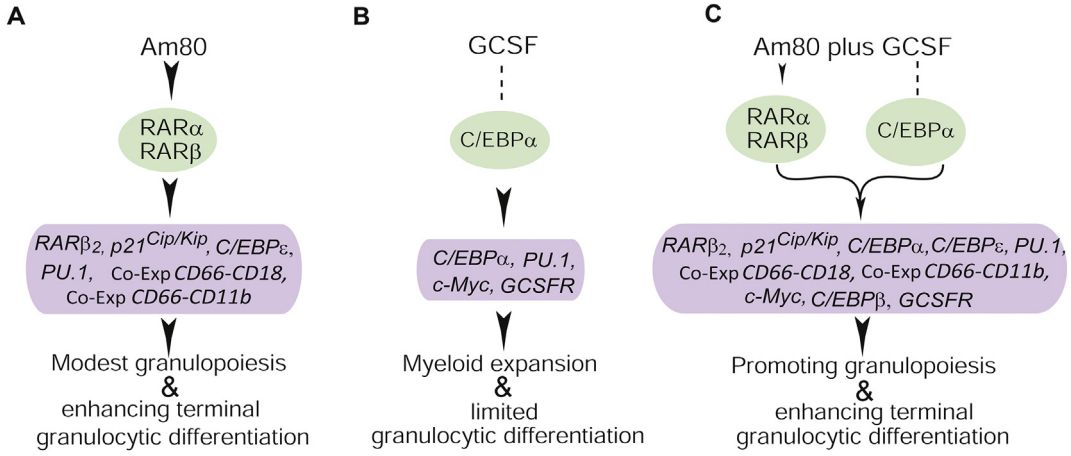

Fig. 7. Altered transcriptional regulation by Am80-GCSF combination synergizes myeloid expansion with terminal neutrophil granulocytic differentiation. (A) Am80 activates transcription of RARα and RARβ to induce RA-target genes, resulting in modest myeloid expansion and effective neutrophil differentiation. (B) GCSF induces gene expression through C/EBPα-dependent transcriptional regulation. (C) Am80 synergizes with GCSF to induce transcription of target genes that effectively coordinate myeloid expansion and terminal granulocytic differentiation. Co-exp: Co-expression; Dotted line: indirect effect on gene transcription.

Am80-GCSF combination may cross-link regulation of transcription and cell cycle by differentially modulating the phosphorylation status of RARα and c-Myc, leading to induction of a coordinated adjustment of growth and differentiation rate to generate sufficient numbers of functional neutrophils without myeloid overexpansion.

9. Concluding remarks

Although GCSF quickly induces large amounts of committed neutrophilic precursors, GCSF can only differentiate them into partially mature neutrophils with limited granule formation that cannot effectively fight infection. This results in the aforementioned unmet need of addressing CCIN in the age of GCSF biologics and biosimilars. Am80, an RA agonist that selectively activates RARα and RARβ$_2$, is much more potent than RA in promoting terminal neutrophil granulocytic differentiation. As an orally administered medicine with better safety/efficacy profile compared to RA, Am80 has been approved for treatment of APL since 2005 in Japan. In different CCIN mouse models, Am80 can differentiate large amounts of GCSF-expanded neutrophilic precursors into functional neutrophils to effectively reduce CCIN-related infection and mortality while avoiding myeloid overexpansion. Hence, the discovery of Am80-GCSF synergy, capable of inducing sufficient numbers of functional neutrophils to effectively fight infection, suggests a novel therapeutic approach that can compensate for the fundamental limitation of GCSF in CCIN treatment. Moreover, GCSF prophylaxis for AML patient post-chemotherapy encounters more limitations than for other cancer patients with CCIN. Since Am80-GCSF combination can induce functional neutrophils while inhibiting malignant growth, Am80-GCSF synergy may also provide an opportunity for treatment of AML patient post-chemotherapy, a population that needs supportive care desperately.

Declaration of Competing Interest

LW is a named inventor on patent applications and issued patents related to use of Am80 as well as Am80-GCSF combination for treatment of neutropenia, both of which are filed by and assigned to Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, CA, USA.

All other authors declare that they have no conflict of interest.

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