PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Retinoid agonist Am80-enhanced neutrophil bactericidal activity arising from granulopoiesis in vitro and in a neutropenic mouse model

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Despite advances in the therapeutic use of recombinant granulocyte colony-stimulating factor (G-CSF) to promote granulopoiesis of human hematopoietic stem cells (HSCs), neutropenia remains one of the most serious complications of cancer chemotherapy. We discovered that retinoid agonist Am80 (tambirabotene) is more potent than G-CSF in coordinating neutrophil differentiation and immunity development. Am80-induced neutrophils (AINs) either in vitro or in neutropenic mouse model displayed strong bactericidal activities, similar to those of human peripheral blood neutrophils (PBNs) or mouse peripheral blood neutrophils (MPBNs) but markedly greater than did G-CSF–induced neutrophils (GINs). In contrast to GINs but similar to PBNs, the enhanced bacterial killing by AINs accompanied both better granule maturation and greater coexpression of CD66 antigen with the integrin β2 subunit CD18. Consistently, anti-CD18 antibody neutralized Am80-induced bactericidal activities of AINs. These studies demonstrate that Am80 is more effective than G-CSF in promoting neutrophil differentiation and bactericidal activities, probably through coordinating the functional interaction of CD66 with CD18 to enhance the development of neutrophil immunity during granulopoiesis. Our findings herein suggest a molecular rationale for developing new therapy against neutropenia using Am80 as a cost-effective treatment option. (Blood. 2013;121(6):996-1007)

Introduction

Neutrophils, the most common granulocytes, constitute up to 70% of circulating leukocytes that primarily defend pathogen infections. Cancer chemotherapy-induced neutropenia is a hematologic disorder marked by large decrease in the number of neutrophils in the bloodstream. It has been more than 2 decades since G-CSF was first used to treat acquired and congenital neutropenia by promoting granulopoiesis of hematopoietic stem cells (HSCs). Despite the considerable clinic benefits of this agent when used as primary prophylaxis,2 neutropenia induced by chemotherapy in cancer patients still remains a devastating issue with substantial morbidity, mortality, and cost, which places a significant burden on the individual patient and the healthcare system.1,3-6 An earlier pioneering study with G-CSF administered to normal individuals reveals that this agent adversely affects neutrophil chemotaxis and bactericidal activity against Staphylococcus aureus (S. aureus), due in part to the reduced assembly of neutrophil F-actin and altered cytosolic calcium mobilization.7 Recent studies also show that in contrast to peripheral blood neutrophils (PBNs), the impaired bacterial killing in neutrophils induced by G-CSF from CD34+ cells is associated with lack of mature granules, because of abnormal granulopoiesis early in the differentiation process.8 Thus, success in devising more cost-effective therapy for neutropenia may depend on determining how granulopoiesis is coordinated with the development of neutrophil-based immunity.

The retinoid agonist Am809,10 is designed to ameliorate the side effects of all-trans retinoic acid (RA) through its selective binding to retinoic acid receptor α (RARα),9,11 a transcription factor activated by RA12 to regulate granulocytic differentiation of both leukemic myeloblasts and HSCs.13-17 RA, a naturally occurring form of vitamin A, plays key roles in the development of the body plan and induces the differentiation of many types of normal and malignant cells.18-20 To date, RA treatment of acute promyelocytic leukemia (APL) represents the best example of successful differentiation-induction therapy in clinical oncology21; however, the side effects associated with RA therapy are generally serious and RA resistance is a common event.22-24 Several studies have demonstrated that RARα regulates Am80-induced granulocytic differentiation.25-27 Moreover, Am80 is approximately 10-fold more efficient, with lower toxicity, than either RA or other retinoids used as differentiation therapy in APL patients.10,28 Currently, Am80 has been approved for the treatment of APL in Japan10 and tested clinically.
for several other cancers/diseases in the United States and Europe (http://www.cytrx.com/tamibarotene; http://clinicaltrials.gov). The advances in the use of Am80 to induce granulocytic differentiation led us to test this agent as a means to enhance neutrophil bactericidal activity arising from granulopoiesis during immune development. We report here that Am80 possesses significantly greater activity than G-CSF as an inducer of neutrophil differentiation and immune development, probably through its promotion of HSC-derived granulopoiesis by mediating the differential effects of CD66 on CD18 activation.

### Methods

#### Human PBNs, cells, and cell culture

Human peripheral blood (PB) was taken from healthy volunteers in accord with a protocol approved by the Children’s Hospital Los Angeles/University of Southern California Keck School of Medicine (CHLA/USC) Committee on Clinical Investigations. Informed consent was obtained in accordance with the Declaration of Helsinki. Details for cells, cell culture, and purification of PBNs are available in supplemental Methods (available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

For transmission electron microscopy and magnetic sorting of CD15+ neutrophils, see supplemental Methods.

#### Phagocytic and bacterial killing assays

Each 1×10^5 to 2×10^6 of freshly purified PBNs, mouse peripheral blood neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (AINs) were suspended with 500 neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINs), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB neutrophils (C-MPBNs), G-CSF–induced neutrophils (GINS), and Am80–induced neutrophils (MPBNs), cyclophosphamide (CPA)–neutropenic mice PB

#### Flow cytometric analysis

Flow cytometric analysis with immunofluorescence staining is detailed in supplemental Methods.

#### Neutropenic mouse model

Mouse work was performed according to guidelines under protocols approved by the Children’s Hospital Los Angeles Institutional Animal Care and Use Committee. Twenty female C57BL6/J mice (The Jackson Laboratory), aged 6 to 8 weeks, were randomly divided into 1 control and 3 experimental groups. To create neutropenic mouse model, as described,31,32 experimental mice received a single 200 mg/kg intraperitoneal dose of cyclophosphamide (CPA; Baxter Healthcare) at day 0, whereas control mice with PBS. To induce neutrophil recovery, experimental mice at day 3 were treated with either 250 μg/kg G-CSF by subcutaneous injection32 or 5 mg/kg Am80 or PBS intraperitoneally for consecutive 2 or 6 days. Each 50 μL of PB was collected from the tail vein every other day for monitoring the baseline of leukocytes (WBCs) and neutrophils with Vetscan HM5 (Abaxis). Mice were killed by CO2 asphyxiation at days 5 or 9, and PB samples were collected by cardiac puncture immediately after sacrificing of mice.

#### Statistical analysis

See supplemental Methods for statistical analysis.

### Results

#### Am80 promotes neutrophil differentiation more effectively than does G-CSF

Am80, CH55, and ITYA are a group of retinoid agonists that were synthesized by introducing heteroatoms into RA-like structures (supplemental Figure 1). We first compared the efficiencies of these compounds with G-CSF and RA in mediating granulopoiesis from CD34+ cells, using our established methodology.17,29 We demonstrated that G-CSF was less effective than RA at inducing morphologic differentiation of CD34+ cells to granulocytes, accompanied by a higher induction of monocytes (supplemental Figure 2A–B). However, G-CSF induced greater cell proliferation and a lower rate of cell death compared with RA (supplemental Figure 2C). Of the other retinoid agonists, Am80 (10nM) and CH55 (5nM) promoted >75% granulocytic differentiation by day 13, in contrast to ITYA (5nM) that produced >60% monocytes (supplemental Figure 2D–E). Although both Am80 and CH55 inhibited cell proliferation (supplemental Figure 2F), the cell death rate associated with Am80 treatment was lower than seen with either CH55 or RA (supplemental Figure 2C,F). Together, these data show that G-CSF is significantly less efficacious as an inducer of granulocytic differentiation than are RA, CH55, and Am80, whereas ITYA mainly induces monocyctic differentiation. Importantly, although Am80, RA, and CH55 promote granulocytic differentiation with similar effectiveness, Am80 induces a lower rate of cell death.

Because the lower level of CD34+ cell differentiation to granulocytes induced by G-CSF on day 12 was associated with a higher level of monocyctic induction (supplemental Figure 2A–B), we considered that G-CSF might induce granulocytic differentiation more effectively in a short period of time. Thus, we treated CD34+ cells with G-CSF for 6, 9, and 12 days. By analysis of morphologic differentiation of those cells, we found more effective granulocytic differentiation with the lowest rate of monocyctic induction on day 6 compared with either day 9 or day 12 (Figure 1A). Using this optimal 6-day induction condition, we again compared G-CSF and Am80 for their ability to induce granulocytic
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in inducing neutrophil morphologic differentiation, with a similar consistently induced monocytes (approximately 10%; Figure 1A,D). In addition, G-CSF myeloblasts as well as promyelocytes and myelocytes than banded sufficiently induced by Am80. By contrast, G-CSF induced more analysis showed that sequential development of neutrophils was treated with G-CSF or Am80 for 6 days. Granulocytic morphologic period for G-CSF is also suitable for Am80 (2.5nM), promoting us 1C). Hence, these results indicate that the 6-day optimal induction Am80 stimulated proliferation while preventing cell death (Figure 1A). (B) Similar to G-CSF, 2.5nM of Am80 gible monocytic induction compared with G-CSF (Figure 1B). In contrast to 5nM of Am80 but similar to G-CSF, however, 2.5nM of Am80 stimulated proliferation while preventing cell death (Figure 1C). Hence, these results indicate that the 6-day optimal induction period for G-CSF is also suitable for Am80 (2.5nM), promoting us to apply such drug exposure time and drug dose for the remainder of the study.

We next analyzed neutrophil differentiation of CD34+ cells on the promyelocyte, myelocyte, metamyelocyte, and band neutrophil stages to segmented mature neutrophil stage. CD34+ cells were treated with G-CSF or Am80 for 6 days. Granulocytic morphologic analysis showed that sequential development of neutrophils was sufficiently induced by Am80. By contrast, G-CSF induced more myeloblasts as well as promyelocytes and myelocytes than banded and segmented neutrophils (Figure 1D). In addition, G-CSF consistently induced monocytes (approximately 10%; Figure 1A,D). These results demonstrate that Am80 is more effective than G-CSF in inducing neutrophil morphologic differentiation, with a similar rate of cellular toxicity.

**AINs are more effective than GINs in producing and secreting granules.**

During neutrophil differentiation, heterogeneous populations of granule proteins are produced sequentially and stored in cytoplasm for first-line defense against different pathogens.33,34 We thus investigated whether Am80-enhanced neutrophil maturation is associated with increased granule production. Neutrophils induced for 6 days from CD34+ cells by G-CSF or Am80 were analyzed by transmission electron microscopy. The ultrastructural images showed that, at the segmented neutrophil level, GINs possessed variable number of vesicles often containing less dense and amorphous material, together with few primary and secondary-like granules (Figure 2A), similar to the observations reported before. By contrast, vesicles found in AINs were frequently filled with dense material or with both amorphous and dense material (Figure 2B). Compared with GINs, AINs contained increased numbers of primary and secondary-like granules, though their numbers were considerably less than those in PBNs (Figure 2A-C). Thus, our data indicate marked differences in vesicle formation and granule production between GINs and AINs.

We next verified whether Am80-induced granulocytic differentiation was indeed associated with sufficient granule production, and tested the degranulation ability of AINs upon bacterial stimuli. CD34+ cells were treated with G-CSF or Am80 for 6 days. The resultant GINs and AINs were then incubated with or without *E. coli* or formyl-met-leu-phe (fMLP), followed by analyzing granule production and secretion. By assessing the activity of primary granule neutrophil elastase (NE) release, we found that both AINs and GINs showed much lower activity of NE release than did PBNs, upon challenge with fMLP (Figure 2D). On the other hand, WB analysis using both cell lysates and supernatants showed that compared with GINs, AINs displayed a greater production and secretion of the cleaved 38-kDa product of primary myeloperoxidase (MPO) granule.35 Moreover, we found that lactoferrin, a secondary granule that has potent broad-spectrum anti-microbial activity,36,37 was stored in AINs and secreted into the medium in sufficient quantities upon bacterial stimuli. By contrast, although the level of lactoferrin was increased with bacterial stimuli in GINs, the efficiency of lactoferrin secretion by GINs was much lower than seen with AINs (Figure 2F). Similarly, a high level of secondary granule LL-37 was observed in AINs, and upon bacterial stimuli, LL-37 granules were effectively released into the medium. GINs, on the other hand, showed both lack of LL-37 production and degranulation (Figure 2G). Thus, MPO, lactoferrin, and LL-37 are effectively produced and/or secreted by AINs but not GINs.

**Figure 1.** Am80 promotes neutrophil differentiation more effectively than G-CSF while stimulating proliferation and preventing cell death similar to G-CSF. (A) Better granulocytic induction associated with lower monocytic induction in CD34+ cells treated with G-CSF for 6 versus 9 or 12 days. (B-C) Reduced concentration of Am80 (2.5nM) leads to more effective induction of granulocytic differentiation with negligible monocytic induction compared with G-CSF (B). Similar to G-CSF, 2.5nM of Am80 displayed capacity of stimulating proliferation while preventing cell death (C). (D) Overall comparison of Am80 and G-CSF under conditions found to be optimal for granulocytic differentiation of CD34+ cells because 10nM of Am80 induced more cell death (supplemental Figure 2F), a reduced Am80 concentration (2.5nM or 5nM) was substituted in the tests. We found that, similar to the effect of 5nM of Am80, 2.5nM of this agent markedly induced granulocytic differentiation with negligible monocytic induction compared with G-CSF (Figure 1B). In contrast to 5nM of Am80 but similar to G-CSF, however, 2.5nM of Am80 stimulated proliferation while preventing cell death (Figure 1C). Hence, these results indicate that the 6-day optimal induction period for G-CSF is also suitable for Am80 (2.5nM), promoting us to apply such drug exposure time and drug dose for the remainder of the study.

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Interestingly, even in the absence of E. coli, we found that GINs secreted MMP-9 (tertiary granules) into the medium, and that this secretion was inhibited by bacterial stimuli (Figure 2H). On the other hand, although E. coli increased the level of MMP-9 in AINs, bacterial stimuli failed to induce AINs to secrete MMP-9 (Figure 2H). These observations indicate possible defects in MMP-9 induction or degranulation (or both) in ex vivo–differentiated GINs and AINs.

AINs possess significantly higher phagocytotic and bactericidal activities than do GINs

The above studies show that Am80 is more effective than G-CSF in promoting both granulocytic morphologic differentiation and granule production/secretion (Figures 1-2). We therefore asked whether this higher degree of neutrophil differentiation induced by Am80 translates into greater neutrophil immunity against bacterial infection. Because Am80 induces more band-form and segmented neutrophils than does G-CSF (Figure 1), we first tested whether sorted band and segmented neutrophils from GINs and AINs possessed similar bactericidal activities. Because neutrophils are CD15+ cells, we purified GINs and AINs using anti-CD15 antibody conjugated to magnetic MicroBeads. After purification, the proportion of band/segmented neutrophils in unsorted GINs increased from 21% to 63% in sorted GINs, similar to findings in sorted AINs (Figure 3Aii versus Aiv). Moreover, the fraction of residual monocytes in sorted GINs was only approximately 1% (Figure 3C). In addition, the size of these ex vivo–differentiated neutrophils is larger than that of freshly isolated PBNs from healthy donor (Figure 3B), as previously observed.8 These unsorted and sorted GINs and AINs, together with PBNs consisting of > 95% segmented neutrophils (Figure 3B), were then tested for their capacity to phagocytize E. coli. Of note, the CFU counts indicated that only a few viable bacteria were recovered from intracellular compartments of PBNs and unsorted AINs compared with unsorted GINs (Figure 3E).
Moreover, the sorted GINs were also significantly less able than sorted AINs to clear the ingested bacteria (Figure 3F). Such enhanced bacterial clearance in AINs was further confirmed by in situ labeling of *E. coli*, using anti-OmpA antibody that specifically recognizes the outer membrane of *E. coli* (Figure 3G). These results indicate that the levels of neutrophil differentiation arising from granulopoiesis induced by Am80 are essential to effective neutrophil immunity against bacterial infection. This observation is supported by the data that GINs with segmented neutrophil morphology still display a lower level of granule-like molecules and contain a greater number of less dense, amorphous vesicles (Figure 2A-C).

To thoroughly compare bactericidal activities between AINs and GINs, we treated CD34<sup>+</sup>/H11001 cells with G-CSF or Am80 for 6 days, followed by analyses of phagocytosis and bacterial killing. Using the methodology previously described, we incubated GINs, AINs, or PBNs with *E. coli* at an MOI of 5 for 15 or 60 minutes, or use of *E. coli* in the absence of neutrophils to monitor bacterial growth. The results showed (Figure 4A) that the extracellular bacteria were significantly decreased in both PBN and AIN samples. PBNs rapidly killed bacteria within 15 minutes, while by 60 minutes there were only a few viable intracellular bacteria in either PBN or AIN samples. By contrast, GINs showed substantially impaired clearance of intracellular bacteria by 60 minutes of infection and was deficient in both phagocytosis and bacterial killing. To confirm that AINs possess greater bactericidal activity than do GINs, we examined bacterial killing in situ, using confocal microscopy of both viable bacteria labeled with SYTO9 fluorescent dye (green) and killed bacteria labeled with propidium iodide (PI) fluorescent dye (red). The results demonstrated that significantly more surviving *E. coli* (green) were retained in GIN samples, where we found much less dead bacteria (red) in contrast to observations in AIN or PBN samples (Figure 4B). Quantifying both extracellular bacteria after infection as well as in situ dead bacteria confirmed that both AINs and PBNs possessed greater bactericidal activities than did GINs (Figure 4C). Because *S. aureus* remains a significant
cause of morbidity and mortality in neutropenic patients whereas superoxide-producing activity of mature neutrophils is involved in oxygen-dependent killing of this bacterium, we examined the oxidative activity of GINs, AINs, and PBNs. White arrow indicates surviving (green) or dead bacteria (red), whereas black arrow indicates example of neutrophil nucleus stained in red simultaneously by PI fluorescent dye. Representative confocal fluorescence images of bacteria were reproduced at 60× magnification. (C) Quantification of both extracellular bacteria after infection and in situ killed bacteria in panel B (*GINs versus AINs or PBNs, P < .04 at least). (D-E) Similar to panels A and B, phagocytic and bactericidal activities against S aureus infection were determined in GINs, AINs, and PBNs (*GINs versus AINs, P < .04 at least). (F-H) Ultrastructural images of bacterial infection of GINs (F), AINs (G), and PBNs (H) with electron microscope. Lb indicates living bacteria; Db, suggestive of dead bacteria; Pg, primary granule; and Sg, secondary granule.

Figure 4. AINs possess significantly higher phagocytic and bactericidal activities than do GINs. (A) Phagocytic and bactericidal activities against E coli infection were determined by the number of extracellular bacteria, phagocytized bacteria, recovered intracellular bacteria, and killed bacteria. There was a 1.26–0.01-fold increase in bacterial numbers over the 45 minutes of the experiment (*GINs versus AINs, P < .03 at least). (B) Confocal fluorescence microscopy of surviving and dead bacteria in GINs, AINs and PBNs. White arrow indicates surviving (green) or dead bacteria (red), whereas black arrow indicates example of neutrophil nucleus stained in red simultaneously by PI fluorescent dye. Representative confocal fluorescence images of bacteria were reproduced at 60× magnification. (C) Quantification of both extracellular bacteria after infection and in situ killed bacteria in panel B (*GINs versus AINs or PBNs, P < .04 at least). (D-E) Similar to panels A and B, phagocytic and bactericidal activities against S aureus infection were determined in GINs, AINs, and PBNs (*GINs versus AINs, P < .04 at least). (F-H) Ultrastructural images of bacterial infection of GINs (F), AINs (G), and PBNs (H) with electron microscope. Lb indicates living bacteria; Db, suggestive of dead bacteria; Pg, primary granule; and Sg, secondary granule.
with increased numbers of primary and secondary-like granules (Figure 2B). In GINs, AINs, and PBNs, there were also a few vesicles with degraded materials suggestive of dead bacteria (Figure 4F-H). Considered together, these data suggest that Am80-induced granulocytic differentiation is associated with enhanced neutrophil innate immunity against bacterial infection.

Granulocytes induced by Am80 show higher coexpression of CD66 and CD18 surface markers than do those induced by G-CSF

Our observation, AINs possessed profound bactericidal activity similar to that in PBNs but greater than GINs (Figure 4), raises an intriguing question: What signaling regulation against bacterial infection has developed during neutrophil differentiation? Previous studies suggest that both CD66 antigens and complement receptor-3 (CR3), a heterodimeric integrin αMβ2 that consists of CD11b and CD18, regulate neutrophil innate immunity against diverse pathogens.43,44 Hence, we investigated the expression of CD66, CD11b, and CD18 surface markers in these cells. Flow cytometric analysis showed that granulocytes induced by G-CSF or Am80 expressed similar levels of CD11b, CD18, or CD66 surface markers with CD11b surface marker expression being significantly lower in GINs and AINs than in PBNs (supplemental Figure 4A-D). Moreover, GINs and AINs coexpressed CD11b-CD18 surface markers at similar levels that were lower than found in PBNs (supplemental Figure 4E). These data indicate that both GINs and AINs are characterized by defective expression of CD11b or coexpression of CD11b-CD18 surface markers.

We next compared the coexpression of CD66 with CD11b or CD18 surface markers in GINs versus AINs versus PBNs using flow cytometry analysis. The results revealed an additional subpopulation coexpressing either CD66-CD18 (supplemental Figure 5A top panel) or CD66-CD11b (supplemental Figure 5B top panel) surface markers in G-CSF–induced granulocytes. Such subpopulations were not observed in either Am80-induced granulocytes or PBNs. Moreover, there was higher coexpression of CD66-CD11b or CD66-CD11b surface markers in Am80-induced granulocytes than in those induced by G-CSF (supplemental Figure 5A-B). These data indicate that during differentiation, AINs gain some properties similar to those of PBNs, as shown by a single population expressing CD66-CD18 or CD66-CD11b surface markers.

Because CD66a and CD66b are involved in mediating neutrophil aggregation and CR3 activation,45,46,47 we next investigated coexpression of CD66a-CD18, CD66a-CD11b, CD66b-CD18, and CD66b-CD11b surface markers in granulocytes induced from CD34+ cells by G-CSF or Am80. Flow cytometric analyses showed that coexpression of CD66a-CD18 (Figure 5A-C) and CD66a-CD11b (Figure 5B,D) surface markers in GINs was significantly lower than in AINs or PBNs samples (Figure 5A-D). Furthermore, we found that similar to PBNs, significantly higher coexpression of CD66b-CD18 (Figure 5E,G) and CD66b-CD11b (Figure 5F,H) surface markers were induced in Am80-induced granulocytes compared with those induced by G-CSF (Figure 5E-H). These findings suggest that Am80 is more effective than G-CSF to enhance the development of a CD66-CR3 signaling network during differentiation.

Anti-CD18 antibody neutralizes Am80-enhanced neutrophil bactericidal activity

Because Am80-enhanced neutrophil bactericidal activity (Figure 4) was associated with higher coexpression of CD66-CD18 surface markers (supplemental Figure 5; Figure 5), this suggests that during granulopoiesis, Am80 simultaneously promotes neutrophil differentiation and development of the CD66-CD18 signaling network against bacterial infection. To test this idea using bacterial killing assay, we disrupted CD66-CD18 signaling by neutralization of CD18 function with anti-CD18 antibody. Freshly isolated PBNs, AINs, GINs were first incubated with or without anti-CD18 antibody for 30 minutes. The cells were then exposed to log-phase E.coli for 60 minutes. E.coli in the neutrophil-free condition were used to monitor bacterial growth. The changes in phagocytotic and bactericidal activities in the presence and absence of anti-CD18 antibody were determined by calculating extracellular, phagocytized, intracellular, and killed bacteria. The results (Figure 6A) showed that similar to PBNs, AINs displayed significantly higher phagocytotic and bactericidal activities than did GINs, and that addition of anti-CD18 antibody significantly neutralized such activities, as reflected by recovered numbers of extracellular bacteria, suppressed phagocytotic capacity, and reduced bacterial killing. Further to assess the effect of CD66-CD18 signaling on regulating neutrophil immunity we performed in situ bacterial infection and killing in the presence of normal IgG (control) or anti-CD18 antibody. Confocal microscope imaging showed that both AINs and PBNs indeed had higher in situ bactericidal activities in killing of E.coli than did GINs, whereas addition of anti-CD18 antibody significantly reversed such bacterial killing in either AIN or PBN samples (Figure 6B-C). Furthermore, such Am80-promoted neutrophil differentiation and development of the CD66-CD18 signaling network were evaluated by testing the effect of AINs against S.aureus infection. We found that similar to the results in Figure 6A-C, the markedly higher bactericidal activity of AINs against S.aureus infection was significantly neutralized in the presence of anti-CD18 antibodies (Figure 6D-E). Hence, higher coexpression of CD66-CD18 surface markers induced by Am80 (supplemental Figure 5; Figure 5) correlates with Am80-enhanced neutrophil immunity arising from granulopoiesis, supporting the notion that in AINs, CD18 cross-links the differential effect of CD66 on neutrophil activation with CR3-dependent neutrophil innate immunity against bacterial infection.

Neutrophils mobilized by Am80 in neutropenic mice display greater bactericidal activity than those by G-CSF

Using a neutropenic mouse model induced by a single dose of CPA, as described,31,32 we tested whether in vivo mobilized neutrophils by Am80 indeed possess the same greater neutrophil immunity against bacterial infection than those by G-CSF, as observed in the ex vivo model (Figures 4, 6). We found that a severe reduction of both WBCs and neutrophils occurred in all experimental mice 3 days after injection of CPA, compared with control mice (Figure 7A). Rapidly thereafter at day 5 with injection of G-CSF or Am80 or vehicle for 2 consecutive days, a remarkably accelerated neutrophil recovery was induced by G-CSF compared with Am80, whereas neutrophil counts in vehicle group also returned nearly to control value (Figure 7A-B). These neutrophils were purified from PB of different mice, as shown by GIN sample (Figure 7C), and used for analyzing of bactericidal activities against S.aureus infection. We found (Figure 7D) that extracellular bacteria were eliminated significantly by either MPBNs, AINs, or GINs than neutrophils isolated from C-MPBNs treated with vehicle, whereas AINs were markedly more effective on eliminating bacteria than both GINs and C-MPBNs. Similar to MPBNs, AINs phagocytized and killed significantly more bacteria than either GINs or C-MPBNs. Because the accelerated neutrophil recovery ceased at day 7 (Figure 7E), we purified neutrophils from PB of different mice at
day 9 (Figure 7F) to compare their bactericidal activities after cessation of accelerated neutrophil recovery. The results showed that both MPBNs and AINs still displayed significantly higher bactericidal activity than did C-MPBNs, whereas there was no difference in either phagocytosis or bacterial killing between GINs and C-MPBNs (Figure 7G). These findings demonstrate that similar to MPBNs, neutrophils mobilized by Am80 in neutropenic mice are significantly more efficacious against \textit{S. aureus} infection than those by G-CSF, even though G-CSF can induce remarkably more neutrophils than do Am80 at earlier stage of neutrophil recovery. Moreover, although C-MPBN counts reach significantly higher level than control values at later stage of neutrophil recovery, the bactericidal activities of C-MPBNs are still significantly lower than MPBNs or AINs.

**Discussion**

\textit{Am80-enhanced bactericidal activity arises from granulopoiesis during neutrophil differentiation}

Granulopoiesis refers to the stage-specific differentiation of HSCs to common myeloid progenitors to granulocyte progenitors and finally to mature granulocytes. Neutrophil innate immunity develops over the entirety of granulocytic differentiation. Recent studies demonstrate that impaired bacterial killing by neutrophils induced from CD34\(^+\) cells with G-CSF is associated with the lack of mature granules.\(^8\) We now show that Am80 is more effective than G-CSF in inducing neutrophil differentiation of CD34\(^+\) cells (Figure 1).
This property can be attributed to the sequential manner in which Am80 induces this process, beginning with the promyelocyte stage and expanding finally to segmented neutrophils (Figure 1D), all associated with the production of granule proteins and their secretions upon bacterial stimulation (Figure 2). By contrast, GINs with segmented neutrophil morphology exhibit much less dense/amorphous vesicles and a lower level of granules than do AINs and PBNs (Figure 2A-C). Such deficiency in GINs is accompanied by defects in degranulation compared with AINs (Figure 2E-G). Moreover, coexpression of CD66-CD18 surface markers in AINs and PBNs is significantly higher than in GINs (supplemental Figure 5; Figure 5). Such higher levels of CD66-CD18 correlate with the significantly greater bactericidal activity of AINs, PBNs, and MPBNs versus GINs (Figures 4, 6, 7), explaining why Am80-enhanced phagocytosis and bacterial killing can be neutralized with anti-CD18 antibody (Figure 6). These findings support the notion that CD66-CD18 signaling is essential in the regulation of neutrophil innate immunity against bacterial infection. Further evidence favoring the cost-effective use of Am80 against neutropenia needs to be generated from determining that in a neutropenic mouse model, Am80 is more effective than G-CSF against neutropenia-related different microbial infections in both prophylaxis and treatment settings.

Retinoid-mediated development of neutrophil innate immunity and CD66-CD18 signaling during granulopoiesis

During the past 2 decades, the results of clinical therapy for neutropenia have shown that despite an increased number of neutrophils and reduced neutropenia duration with administration of G-CSF, there are no benefits consistently on reducing infection, morbidity, and mortality.1,3,5,6 This raises a crucial question: How could G-CSF increase the number of neutrophils and shorten the neutropenia duration but only have a marginal effect on reducing infection, morbidity, and mortality? In 1998, Ambruso’s group initiated a critical in vivo study to...
address this issue by administering G-CSF to normal individuals. They found that neutrophils mobilized by G-CSF displayed significantly lower chemotaxis and bacterial killing, probably because of the reduced assembly of neutrophil F-actin and altered calcium signaling. This study revealed, for the first time, that the real benefit of G-CSF therapy might lie in enhanced number and survival of neutrophils, although these cells harbor defects in bacterial killing. In later studies using an ex vivo granulopoiesis system, Dick et al. show that neutrophils induced by G-CSF are defective in normal granule development, leading to marked impairment in bacterial killing. The studies we report here demonstrate that neutrophils mobilized by Am80 in vitro (Figures 1-2) or in vivo (Figure 7) possess greater innate immunity against bacterial infection than do those induced with G-CSF (Figures 3, 4, 6, 7). Such enhanced innate immunity arises during granulopoiesis in the differentiation process (Figures 1, 2, 5; supplemental Figure 7).

Figure 7. Neutrophils mobilized by Am80 in neutropenic mice display greater bactericidal activity than those by G-CSF. Twenty C57BL6J mice were randomly divided into 4 groups for the experiments. (A) G-CSF induced a remarkably accelerated neutrophil recovery compared with mice treated with Am80 or vehicle at day 5. (B) Morphologic analysis of PB neutrophils at day 5 (*G-CSF versus Am80, P < 1.2 × 10^{-6}; G-CSF versus control, P < 4.0 × 10^{-6}; G-CSF versus vehicle, P < 8.2 × 10^{-6}; Am80 versus vehicle, P < 1.3 × 10^{-6}; Am80 versus control, P < .016. (C) Representative purity of PB neutrophils, as shown by freshly isolated GINs from mice. (D) Phagocytic and bactericidal activities of neutrophils, isolated from PB of different mice, were reflected by the number of extracellular bacteria, phagocytosed bacteria, and killed bacteria. *Extracellular: AINs versus GINs, P < .043; AINs versus C-MPBNs, P < 1.1 × 10^{-4}; MPBNs versus C-MPBNs, P < 3.7 × 10^{-4}; GINs versus C-MPBNs, P < .049. *Phagocytosis: AINs versus GINs, P < .026; AINs versus C-MPBNs, P < .02; MPBNs versus GINs, P < .042; MPBNs versus C-MPBNs, P < .003; GINs versus C-MPBNs, P < .009. *Killing: AINs versus GINs, P < .026; AINs versus C-MPBNs, P < .005; MPBNs versus GINs, P < .015; MPBNs versus C-MPBNs, P < .003; GINs versus C-MPBNs, P < .009. (E) Accelerated recoveries of WBC and neutrophil were ceased at day 7 after 96 hours of stimuli with G-CSF or Am80 or vehicle. (F) Morphologic analysis of PB neutrophils at day 9 (*G-CSF versus control, P < .005; Am80 versus control, P < 3.9 × 10^{-4}; Am80 versus vehicle, P < .03; Vehicle versus control, P < .03). (G) AINs possessed significantly higher phagocytic and bactericidal activities than do GINs 48 hours after cessation of accelerated neutrophil recovery (E). *Extracellular: AINs versus GINs, P < .02; AINs versus C-MPBNs, P < .007; MPBNs versus GINs, P < .04; MPBNs versus C-MPBNs, P < .02. *Phagocytosis: AINs versus C-MPBNs, P < .034; MPBNs versus C-MPBNs, P < .043. *Killing: AINs versus C-MPBNs, P < .034; MPBNs versus C-MPBNs, P < .043.
5), probably via coordination of the granule production, neutrophil maturation, and immunity development through the CD66-CD18 signaling pathway (Figures 5-6, supplemental Figure 5). Hence, our data based on comparing the neutrophil maturation and bactericidal activity mobilized by Am80 and G-CSF support the notion: the defect of neutrophil innate immunity mobilized by G-CSF during granulopoiesis primarily results in the lower efficacy against bacterial infection. However, how retinoid signaling coordinates granulopoiesis with development of neutrophil immunity is currently unknown. Am80 is designed to eliminate the side effects of RA by binding to RARα in a more selective manner9,11 to induce granulocytic differentiation.25,27 Using Am80 to induce granulopoiesis of CD34+ cells, we found that AINs and PBNs coexpress CD66-CD18 surface markers at significantly greater levels than do GINs (Figure 5; supplemental Figure 5). Such up-regulated surface marker expression in AINs is associated with increased granule production, sufficient degranulation of MPO, lactoferrin, and LL-37 on bacterial stimuli, and enhanced bactericidal activities (Figures 2-4). Moreover, the enhanced phagocytic and bactericidal activities of AINs can be neutralized with anti-CD18 antibody (Figure 6). Thus, the enhanced bactericidal activity of AINs appears to operate through Am80-induced RARα signaling to coordinate CD66-dependent neutrophil maturation/activation with CD18-mediated host defense (supplemental Figure 6). Further evaluation of this regulatory signaling using loss-of-function approaches, including shRNA blockade of CD66a, CD66b, and CD18 expression during granulopoiesis, should systemically determine the mechanisms by which Am80-mediated development of neutrophil immunity against bacterial infection proceeds through the CD66-CD18 signaling pathway. This information, in turn, should provide new insights into how CD66 leads to CD18 activation and how CD66-CD18 signaling coordinates granulocytic maturation/activation with the host defense.

Defects arising from ex vivo granulopoiesis and their implications

CD66 and CR3 signaling receptors play essential roles in cross-linking granulocyte activation with neutrophil immunity against bacterial infection,43,44 whereas granule proteins produced and stored sequentially during granulopoiesis provide an innate defense armory against microbial infections.33,48 Thus, adequate coordination of both innate immune receptor expression and granule production during neutrophil differentiation is critical to securing neutrophil bactericidal activity. Indeed, the relatively lower effectiveness with which AINs coexpress CD11b-CD18 (supplemental Figure 4) and secrete NE or MMP-9 (Figure 2D,H) may reduce the anti-microbial activity of these neutrophils against other microorganisms, a possibility that remains to be tested. Moreover, Sabroe’s group shows that compared with PBNs, G-CSF–induced neutrophils have markedly higher surface expression of Toll-like receptors (TLR),5 similar to our observation that the level of TLR4 surface marker is significantly high in both GINs and AINs (data not shown). Interestingly, although neutrophils induced by G-CSF show a greater increase in IL-8 production than do PBNs in response to the TLR agonists, they fail to migrate toward low concentrations of IL-8, whereas PBNs show chemotaxis in response to this chemokine.8 However, we observed that GINs showed the highest chemotaxis compared with AINs and PBNs in response to FMLP stimuli (data not shown). Despite this difference, a higher surface expression of TLR detected by Sabroe’s group and by us indicates an abnormal development of TLR signaling regulation in those ex vivo–differentiated neutrophils.8 Moreover, we have found that in contrast to PBNs, ex vivo–differentiated neutrophils induced by either G-CSF or Am80 display an altered expression pattern of fragment crystallizable γ receptors FcγR, including FcγRI (CD64) and FcγRIII (CD16; supplemental Figures 7-8), which are involved in maintenance of immune homeostasis and regulation of proinflammatory responses.49 Further studies to elucidate the mechanisms of Am80-enhanced neutrophil immunity as well as identify the defects in those Am80-mobilized neutrophils should establish a framework of molecular insights into Am80 signaling, which may catalyze the design of improved retinoid agonists to more effectively mimic human granulopoiesis and development of neutrophil immunity in vivo simultaneously.

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Authorship

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Retinoid agonist Am80-enhanced neutrophil bactericidal activity arising from granulopoiesis in vitro and in a neutropenic mouse model

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