A Truncated Granulocyte Colony-stimulating Factor Receptor (G-CSFR) Inhibits Apoptosis Induced by Neutrophil Elastase G185R Mutant

IMPLICATION FOR UNDERSTANDING CSF3R GENE MUTATIONS IN SEVERE CONGENITAL NEUTROPENIA

Mutations in ELANE encoding neutrophil elastase (NE) have been identified in the majority of patients with severe congenital neutropenia (SCN). The NE mutants have been shown to activate unfolded protein response and induce premature apoptosis in myeloid cells. Patients with SCN are predisposed to acute myeloid leukemia (AML), and progression from SCN to AML is accompanied by mutations in CSF3R encoding the granulocyte colony-stimulating factor receptor (G-CSFR) in ∼80% of patients. The mutations result in the expression of C-terminally truncated G-CSFRs that promote strong cell proliferation and survival. It is unknown why the CSF3R mutations, which are rare in de novo AML, are so prevalent in SCN/AML. We show here that a G-CSFR mutant, d715, derived from an SCN patient inhibited G-CSF-induced expression of NE in a dominant negative manner. Furthermore, G-CSFR d715 suppressed unfolded protein response and apoptosis induced by an SCN-derived NE mutant, which was associated with sustained activation of AKT and STAT5, and augmented expression of BCL-XL. Thus, the truncated G-CSFRs associated with SCN/AML may protect myeloid precursor cells from apoptosis induced by the NE mutants. We propose that acquisition of CSF3R mutations may represent a mechanism by which myeloid precursor cells carrying the ELANE mutations evade the proapoptotic activity of the NE mutants in SCN patients.

Severe congenital neutropenia (SCN) is a group of heterogeneous disorders characterized by severe absolute neutropenia occurring in early life and a maturation arrest of bone marrow myeloid precursors at the promyelocyte stage. Inherited or spontaneous point mutations in ELANE, which encodes neutrophil elastase (NE), have been detected in ∼50–80% of patients with SCN (1–3). Ectopic expression of some NE mutants has been shown to cause premature apoptosis in human leukemic HL-60 cells and promyelocytes (4–6), but the mechanisms of apoptosis induction by the NE mutants are still not fully understood. It has been proposed that ELANE mutations may affect NE intracellular trafficking, resulting in increased membrane and nuclear localization (2, 7). It has also been shown that the ELANE mutations cause cytoplasmic accumulation of nonfunctional NE proteins and subsequent activation of the unfolded protein response (UPR) (6, 8–11).

Patients with SCN are at increased risk of developing myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). The cumulative incidence for MDS and AML in SCN patients is 21% over a period of 10 years (12). During the course of disease progression to MDS/AML, −80% of patients acquired somatic CSF3R mutations in the bone marrow myeloid cells that introduce premature stop codons or cause reading frameshift, leading to truncation of the G-CSFR C terminus (4, 13–17). The truncated G-CSFRs mediate enhanced cell proliferation and survival, which are associated with prolonged activation of STAT5 and AKT, but are impaired in mediating granulocytic differentiation (13, 18–24). Transgenic mice carrying the equivalent CSF3R mutations display a selective expansion of G-CSF-responsive myeloid cells in the bone marrow (20, 25, 26). The truncated G-CSFR also conferred a strong clonal advantage to hematopoietic stem cells in mice (27). Significantly, myeloid cells harboring the CSF3R mutations appeared to undergo clonal expansion during leukemic evolution in SCN patients as the CSF3R mutations were detected only in small percentages of myeloid cells prior to AML conversion but were present in essentially all leukemic cells (14, 17). Together, these studies indicate that the CSF3R mutations contribute to leukemogenesis in SCN patients.

Compelling evidence indicates that ELANE mutations occur prior to acquisition of CSF3R mutations. Significantly, the CSF3R mutations are detected in up to 40% of patients with SCN, and some patients even carry two or more different CSF3R mutations (14, 17). However, except in patients with chronic neutrophilic leukemia and atypical chronic myeloid...
leukemia (28), such mutations are rare in other myeloid disorders including primary AML, aplastic anemia, and other subgroups of chronic neutropenia (29–32). The reason for the prevalence of the CSF3R mutations in SCN patients is unknown. Efforts to address the correlation between the ELANE mutations and the CSF3R mutations have been hampered by a lack of appropriate cell line and mouse models. Although HL-60 cells ectopically transfected with the NE mutants undergo premature apoptosis when induced to differentiate with DMSO (4, 5), they are leukemic cells that do not differentiate in response to G-CSF. Ectopic expression of human NE mutants in mouse hematopoietic cell lines has failed to induce apoptosis (33). Transgenic mice carrying targeted mutations in Elane, which recapitulate mutations found in SCN, are not neutropenic (9, 34).

Here we show that ectopic expression of an SCN-associated NE mutant, G185R, resulted in the activation of UPR and premature apoptosis in mouse non-neutrophil myeloid 32D and multipoint FDCP-mix A4 cells when the cells were induced with G-CSF to undergo terminal granulocytic differentiation. Our data further indicate that a truncated G-CSFR, δ715, derived from an SCN patient suppressed UPR and apoptosis induced by NE G185R, which was associated with strong activation of AKT and STAT5, and augmented expression of BCL-XL. These data provide a potential explanation for the prevalence of the CSF3R mutations in patients with SCN/AML.

Results

Expression of NE G185R Inhibits G-CSF-dependent Survival in 32D/GR Cells—Although the CSF3R mutations are common in patients with SCN/AML, the effect of the mutations on apoptosis induced by the NE mutants has never been addressed due to a lack of appropriate cell line and mouse models. We assessed whether the SCN-associated NE G185R induced apoptosis in murine myeloid 32D cells expressing the wild type (WT) G-CSFR (32D/GR), which proliferated transiently and terminally differentiated into mature granulocytes after culture in G-CSF for 8–10 days (13, 35). 32D/GR cells were stably transfected with the expression constructs for NE or NE G185R and examined for expression of NE proteins by Western blotting analysis using an antibody that recognized only the human NE protein. As shown in Fig. 1A, NE appeared as a triplet, presumably corresponding to the unprocessed, partially processed, and fully processed proteins (2). Unlike NE, NE G185R appeared as two fast migrating bands, which seemed to be consistent with the observation that NE G185R was rapidly processed in myeloid cells (5).

The expression of NE or NE G185R had no significant effect on the proliferation and survival of 32D/GR cells cultured in interleukin-3 (IL-3) (data not shown). Upon treatment with G-CSF, 32D/GR cells transfected with the empty vector (32D/Ctr) or NE (32D/NE) proliferated transiently and gradually lost viability (Fig. 1, B and C). The proliferation and survival of 32D/Ctr and 32D/NE cells were comparable. In contrast, expression of NE G185R in 32D/GR cells led to reduced cell numbers and viabilities, which became apparent after culture in G-CSF for 4–5 days. Consistent with reduced cell survival, considerably more 32D/GR cells expressing NE G185R (32D/G185R) were stained by annexin V as compared with 32D/Ctr and 32D/NE cells after G-CSF treatment for 4 days (Fig. 1D). Morphological examination revealed that the different 32D clones remained largely immature after culture in G-CSF for 5 days (Fig. 1E). Notably, the expression of neutrophil differentiation marker Gr-1 was moderately induced by G-CSF in 32D/NE cells but only weakly induced in 32D/G185R cells (Fig. 1F), presumably because relatively more mature cells died by apoptosis as NE mutants have been shown to cause premature apoptosis in differentiating myeloid cells (4–6).

The G185R Mutant Is Localized in the Cytoplasm—We examined the subcellular localization of NE G185R by immunofluorescence staining with a monoclonal antibody to human NE. As expected, NE was localized in the cytoplasm (Fig. 2A). Unexpectedly, however, NE G185R protein was not detected in 32D/G185R cells. Comparable results were obtained with a polyclonal antibody against human NE (data not shown). Additionally, the G185R mutant expressed in murine pro-B Ba/F3 and human myeloid K562 cells was also undetectable by immunofluorescence staining with the monoclonal and polyclonal antibodies but was easily detected by Western blotting analysis (data not shown). To determine the subcellular localization of NE G185R, we prepared the membrane and cytoplasmic extracts from 32D/GR cells expressing the different NE proteins. As shown in Fig. 2B, both NE and NE G185R were detected only in the cytoplasmic fraction.

The C Terminus of G-CSFR Is Required for Elane Activation in Response to G-CSF—we next assessed the effect of C-terminal truncation of G-CSFR on the expression of endogenous NE in 32D cells expressing the truncated receptor mutant δ715 (32D/δ715; Fig. 3A), which was derived from a patient with SCN (18). The levels of G-CSFR proteins expressed on 32D/GR and 32D/δ715 cells were comparable (35). Cells were cultured in G-CSF for different days, and total RNA was extracted from the cells for evaluation of NE expression by Northern blotting analysis (Fig. 3B). The expression of NE mRNA in 32D/GR cells increased gradually upon G-CSF treatment, reaching a peak by day 4 and declining thereafter. Notably, NE expression was not induced by G-CSF in 32D/d715 cells. Because myeloid cells from SCN patients express both WT and the truncated G-CSFRs, we also examined NE expression in 32D cells expressing both receptor forms (32D/GR/d715). No induction of NE expression was observed following culture of the cells in G-CSF.

To address whether the C-terminal region of G-CSFR was required for activation of the Elane promoter, we examined G-CSF-stimulated activation of a luciferase reporter construct containing the 1.8-kb fragment of the murine Elane promoter (36). Treatment with G-CSF, but not with IL-3, activated the Elane promoter by ~5-fold in 32D/GR cells (Fig. 3C). The Elane promoter exhibited a very low activity in 32D/d715 cells and was only weakly stimulated by G-CSF in 32D/GR/d715 cells. In contrast, G-CSF-stimulated activation of the luciferase reporter construct containing three repeats of the interferon-γ activated site in the interferon regulatory factor-1 (IRF-1) gene, which is dependent on the STAT signaling pathway, was stronger in 32D/d715 and 32D/GR/d715 cells than in 32D/GR cells, consistent with previous studies.
These results indicated that G-CSFR d715 exerted a dominant negative effect on Elane activation mediated by the WT G-CSFR.

C-terminal Truncation of G-CSFR Abolishes the Up-regulation of NE G185R Expressed from the Elane Promoter—We explored the possibility that G-CSFR d715 may abolish the pro-apoptotic effect of NE G185R by suppressing its induction. An expression construct was constructed in which the NE G185R cDNA was under the control of the 1.8-kb Elane promoter fragment and stably transfected into 32D/GR and 32D/d715 cells. The levels of NE G185R expressed from the Elane promoter were considerably lower than that expressed from the pBabe-puro vector when the cells were cultured in IL-3 (Fig. 4A). NE G185R expression was markedly up-regulated in 32D/GR cells upon culture in G-CSF (Fig. 4B). Induction of NE G185R by G-CSF occurred earlier than induction of endogenous NE (Fig. 3B), suggesting that the 1.8-kb Elane promoter fragment lacked a negative regulatory element(s) that restricted NE expression at early stages of G-CSF induction. Notably, G-CSF-induced expression of NE G185R was associated with reduced cell numbers and survival (Fig. 4, C and D). In 32D/d715 cells, however, NE G185R was not up-regulated by G-CSF in the first 2 days and became barely detectable thereafter. Accordingly, NE G185R expressed from the Elane promoter showed no apparent effect on the proliferation and survival of 32D/d715 cells.

G-CSFR d715 Antagonizes the Proapoptotic Activity of NE G185R—Although G-CSFR d715 was defective in inducing NE expression in myeloid 32D cells, it is possible that other cytokines such as GM-CSF and IL-6 may induce the expression of NE mutants in the myeloid precursor cells of SCN patients. As
the truncated G-CSFR mutants have been shown to mediate enhanced cell proliferation and survival (13, 18–24), we investigated whether G-CSFR d715 counteracted the proapoptotic activity of NE G185R. 32D/d715 cells were stably transfected with the pBabe-puro-derived expression construct for NE G185R, and two independent clones expressing NE G185R were examined for proliferation and survival in response to G-CSF (Fig. 5A). The expression of NE G185R had no significant effect on G-CSF-dependent proliferation and survival in 32D/d715 cells (Fig. 5B and data not shown). We previously reported that 32D/d715 cells were impaired in granulocytic differentiation (18, 23, 37). Consistent with this, no significant induction of Gr-1 expression was observed in 32D/d715 cells cultured in G-CSF for 5 days (Fig. 5C).

**G-CSFR d715 Blocks NE G185R-induced Apoptosis in FDCP-mix A4 Cells**—We recently showed that murine multipotent FDCP-mix A4 transfected with human G-CSFR (FDCP/GR) underwent terminal granulocytic differentiation in response to G-CSF (38). We addressed whether NE G185R induced apoptosis in FDCP/GR cells. As in 32D/GR cells, expression of NE G185R resulted in premature apoptosis of FDCP/GR cells induced to differentiate with G-CSF (Fig. 6). To address whether G-CSFR d715 abolished apoptosis induced by NE G185R, we generated FDCP-mix A4 cells expressing G-CSFR d715 (FDCP/d715; Fig. 6A) that were then transfected with NE

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### FIGURE 2. Subcellular localization of NE and NE G185R in 32D/GR cells.

A, NE proteins in 32D/Ctr, 32D/NE, and 32D/G185R cells were examined by immunofluorescence staining using the monoclonal NE antibody. Essentially identical staining patterns were seen using the polyclonal anti-NE antibody. 8, membrane (mem) and cytosolic (cyto) extracts were prepared from cells as indicated and examined for NE proteins by Western blotting. Aliquots of extracts were also examined for β-actin and G-CSFR.

### FIGURE 3. The C-terminal region of G-CSFR is required for induction of NE expression.

A, schematic diagram of WT and d715 forms of G-CSFR. Boxes 1, 2, and 3 denote cytoplasmic regions conserved among the members of the cytokine receptor superfamily. TM, transmembrane domain; aa, amino acids. B, cells as indicated were cultured in G-CSF for different days. Total RNA was extracted and examined for NE transcript by Northern blotting analysis. Sample loadings were determined by ethidium bromide staining of 18S ribosomal RNA. C, differential activation of the *Elane* and *IRF-1* promoters by the WT and d715 forms of G-CSFR. 32D/GR and 32D/d715 cells were transfected with the *Elane* or *IRF-1* promoter-luciferase reporter construct along with the pCMV-β-galactosidase plasmid. Cells were left untreated or treated with G-CSF for 6 h. Luciferase activity was measured and normalized for β-galactosidase activity. Error bars represent S.D. ** denotes *p* < 0.01.
Unlike FDCP/GR cells, expression of NE G185R had no noticeable effect on the survival of FDCP/d715 cells upon transfer to G-CSF-containing medium. Thus, G-CSFR d715 inhibited apoptosis induced by NE G185R in both 32D and FDCP-mix A4 cells.

**FIGURE 4. The effect of NE G185R expression driven by Elane promoter on G-CSF-dependent proliferation and survival.**

A, 32D/GR and 32D/d715 cells were transfected with pcDNA3.1-derived expression construct for NE G185R in which the expressed of NE G185R was under the control of the Elane promoter (ElaP) or the pBabe-puro-derived NE G185R expression vector. The expression of NE G185R in cells cultured in IL-3 was examined by Western blotting analysis. B, 32D/GR and 32D/d715 cells expressing NE G185R from ElaP were cultured in G-CSF and examined for expression of NE G185R. C and D, 32D/GR and 32D/d715 cells transfected with the empty vector (Ctr) or the ElaP-driven NE G185R were cultured in G-CSF. Cell numbers and viabilities were determined on different days. Error bars represent S.D. ** denotes p < 0.01.

**FIGURE 5. G-CSFR d715 suppresses apoptosis induced by NE G185R in 32D cells.**

32D/d715 cells were transfected with the pBabe-puro-derived NE G185R expression construct. A, the expression of NE G185R protein was examined by Western blotting analysis. B, two independent 32D/d715 clones expressing NE G185R were cultured in G-CSF, and the numbers of viable cells were determined on different days. C, surface expression of Gr-1 in one 32D/d715 clone was examined by flow cytometry after culture in G-CSF for 5 days.

G185R (Fig. 6B). Unlike FDCP/GR cells, expression of NE G185R had no noticeable effect on the survival of FDCP/d715 cells upon transfer to G-CSF-containing medium. Thus, G-CSFR d715 inhibited apoptosis induced by NE G185R in both 32D and FDCP-mix A4 cells.

**G-CSFR d715 Inhibits the Induction of Endoplasmic Reticulum (ER) Chaperone Grp78 by NE G185R—** The SCN-associated NE mutants, including NE G185R, have been shown to activate the UPR in myeloid cells, which induces the transcription of the ER stress marker Grp78 (also known as Hspa5 and Bip), a member of the HSP70 family of chaperones (6, 8–11). Because activation of UPR by the NE mutants has been associated with cellular apoptosis and impaired granulopoiesis, we explored the possibility that G-CSFR d715 might suppress UPR induced by NE G185R. In agreement with the previous studies, Grp78 expression was induced by NE G185R in 32D/GR and FDCP/GR cells cultured in G-CSF (Fig. 7). Interestingly, Grp78 induction by NE G185R was blocked in 32D/d715 cells and significantly attenuated in FDCP/d715 cells. These data demonstrated that G-CSFR d715 inhibited Grp78 induction by NE G185R in myeloid cells.

**G-CSFR d715 Mediates Steady Activation of the Prosurvival STAT5 and AKT Pathways—** We and others have previously shown that G-CSFR d715 mediates prolonged activation of the prosurvival STAT5 and AKT pathways (19–24). Consistent with previous studies, G-CSF-stimulated activation of STAT5 and AKT was considerably longer in NE G185R-expressing 32D/d715 and FDCP/d715 cells than in NE G185R-expressing 32D/GR and FDCP/GR cells (Fig. 8A). Because G-CSFR d715, but not WT G-CSFR, suppressed apoptosis induced by NE G185R, we further examined the activation status of STAT5 and AKT in the 32D and FDCP-mix A4 cells that were cultured in G-CSF for up to 3 days. STAT5 was poorly activated in the 32D/GR and FDCP/GR cells,
being visible only after prolonged exposure (data not shown), but strongly activated in 32D/d715 and FDCP/d715 cells (Fig. 8B). AKT activation was weak in 32D/GR cells and barely detectable in FDCP/GR cells but was steadily activated in 32D/d715 and FDCP/d715 cells. In contrast, no significant differences in the activation status of STAT5 and AKT were observed when the cells were cultured in IL-3. Thus, prolonged activation of STAT5 and AKT mediated by G-CSFR d715 led to their steady activation in cells continuously cultured in G-CSF.

**G-CSFR d715 Is More Effective than WT G-CSFR in Inducing the Expression of BCL-XL**—The AKT and STAT5 pathways have been shown to stimulate the expression of the prosurvival BCL-2 family members BCL-2, BCL-XL, and MCL-1 in hematopoietic cells (39–41). As G-CSFR d715 mediated sustained activation of AKT and STAT5, we examined the expression of the three BCL-2 family members in NE G185R-expressing 32D and FDCP-mix A4 cells treated with G-CSF for up to 4 days. As shown in Fig. 9, the expression of BCL-XL was consistently higher in 32D/d715 and FDCP/d715 cells than in 32D/GR and FDCP/GR cells. BCL-2 expression was increased in 32D/d715 cells but not in FDCP/d715 cells, whereas MCL-1 expression was not augmented in 32D/d715 and FDCP/d715 cells. Thus, G-CSFR d715 up-regulated BCL-XL expression in NE G185R-expressing 32D and FDCP-mix A4 cells.

**Discussion**

Patients with SCN show an increased propensity to develop AML, and disease progression from SCN to AML is accompa-
CSF3R and ELANE Mutations in SCN/AML

FIGURE 7. G-CSFR d715 suppresses Grp78 induction by NE G185R. Control (Ctr) and NE G185R-expressing 32D and FDCP-mix A4 cells transfected with the WT or d715 form of G-CSFR were cultured in G-CSF for 24 h. The expression of murine Grp78 mRNA was assessed by qRT-PCR. Error bars represent S.D. * denotes p < 0.05; ** denotes p < 0.01.

FIGURE 8. G-CSF-stimulated activation of STAT5 and AKT in NE G185R-expressing 32D and FDCP-mix A4 cells transfected with the WT or d715 form of G-CSFR. A, cells as indicated were starved of IL-3 for 2 h prior to stimulation with G-CSF for the indicated minutes. B, cells were cultured in IL-3 (day 0) or G-CSF for up to 3 days. The phosphorylation (p) and expression of STAT5 and AKT were examined by Western blotting analysis.

graculocytic precursor cells, it is likely that acquisition of the CSF3R mutations may represent the most effective means of counteracting the proapoptotic effect of the NE mutants, which may provide a potential explanation for the prevalence of the CSF3R mutations in SCN/AML patients.

If the proapoptotic effect of the ELANE mutations facilitates the CSF3R mutations in SCN/AML patients, one would expect that the CSF3R mutations should be more common in patients with the ELANE mutations that exhibit a strong cytotoxic effect and therefore are associated with the more severe forms of SCN. This indeed appears to be the case (1, 12, 42). Notably, the G185R ELANE mutation, which correlates with severe expression of neutropenia, is frequently associated with the CSF3R mutations and AML conversion (4, 42, 43). Conversely, ELANE mutations seen in less severe forms of neutropenia, including cyclic neutropenia, are not or only weakly associated with CSF3R mutations and leukemic conversion (44). In addition, one would expect that other types of SCN-associated mutations such as those in HAX1 and WAS that result in increased apoptosis of granulocytic precursors may also generate selection pressure for acquiring the CSF3R mutations. In line with this, CSF3R mutations have been reported in SCN/AML patients with HAX1 and WAS mutations (45, 46). However, it is possible that myeloid cells in certain SCN patients may use other mechanisms to evade apoptosis. Interestingly, the CSF3R mutations and activating RAS mutations appear to be mutually exclusive in SCN/AML patients (43), suggesting that the two types of mutations may act on shared signaling pathways to inhibit apoptosis.

It has been shown that the SCN-derived truncated G-CSFRs mediate prolonged activation of STAT5 and AKT (19–24). Our data further indicate that prolonged activation of STAT5 and AKT mediated by G-CSFR d715 leads to their steady activation in cells continuously cultured in G-CSF, which is associated with augmented expression of BCL-XL. These results may explain why G-CSFR d715, but not the WT G-CSFR, is capable of protecting myeloid cells from apoptosis induced by NE G185R. Notably, it has been shown that forced expression of a constitutively active AKT rescued apoptosis induced by SCN-associated NE mutants in induced pluripotent stem cell-derived myeloid precursor cells (11). It is also of note that STAT5 proteins, including STAT5A and STAT5B, have been shown to be associated with ER and that siRNA-mediated knockdown of STAT5A/B resulted in increased accumulation of GRP78 in ER (47). As G-CSFR d715 suppresses NE G185R-induced Grp78 expression, it would be interesting to investigate whether strong activation of STAT5 mediated by G-CSFR d715 is responsible for repression of Grp78 expression.

Finally, our results indicate that NE G185R protein is localized in the cytoplasm, not in the plasma membrane as reported previously (5). Unexpectedly, we were unable to detect the G185R mutant by immunofluorescence staining with the monoclonal and polyclonal anti-NE antibodies in 32D, Ba/F3, and K562 cells. Notably, the G185R mutant expressed in RBL-1 cells, a rat basophilic leukemia line, also could not be detected by immunofluorescence staining with a different polyclonal anti-NE antibody (33). It is possible that NE G185R protein may be masked by associating with chaperone proteins or folds very differently from the WT NE protein such that it cannot be rec-
ognized in its native conformation by the NE antibodies. Alternatively, the G185R protein might be diffusely spread throughout the cytoplasm rather than concentrated in primary granules and therefore escapes detection by immunofluorescence staining.

**Experimental Procedures**

**Cells**—Murine 32D cells stably transfected with the human WT and/or the d715 receptors have been described (13, 23). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% WEHI-3B cell-conditioned medium as a crude source of IL-3, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Murine multipotential FDCP-mix A4 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 15% horse serum and 10% WEHI-3B cell-conditioned medium.

**Reagents**—Mouse anti-human G-CSFR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat polyclonal antibody to human NE was purchased from Bio-Rad. Fluorescein isothiocyanate (FITC)-conjugated anti-Gr-1 antibody was purchased from eBioscience (San Diego, CA). Mouse anti-human G-CSFR antibody and subsequently transfected with RV-GFP-G185R construct along with pBabe-puro vector. Following selection in puromycin (1 µg/ml), GFP-positive cells were sorted by FACS, and expression of NE protein was examined by Western blotting analysis.

**Northern Blotting Analysis and Real Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)—** Total RNA was extracted using TRizol reagent (Invitrogen). Northern blotting analysis was performed as described (35). cDNA was synthesized using the GoScript™ Reverse Transcription System and oligo(dT)15 primer (Promega, Madison, WI). qRT-PCR was performed using the SuperScript™ One-Step qRT-PCR System and oligo(dT)15 primer (Invitrogen). The following primer sets were used:

- mouse Grp78 forward primer, 5’-ctgaggcgtatttgggaaag-3’; mouse Grp78 reverse primer, 5’-ctcatgacattcagtccagca-3’; mouse Gapdh forward primer, 5’-tcatgacattcagtccagca-3’; mouse Gapdh reverse primer, 5’-ggctgagctgttgctgta-3’. The relative mRNA levels of Grp78 were normalized to Gapdh mRNA expression.

**Luciferase Reporter Assay**—The 1.8-kb proximal promoter fragment of murine Elane gene was inserted into the Smal site of pGL3-basic vector to generate pGL3-Elae2p luciferase reporter construct. 32D cells expressing the WT and/or the d715 receptors were transfected by electroporation with the reporter construct. 32D cells expressing the WT and/or the d715 receptors were transfected by electroporation with the reporter construct. 32D cells expressing the WT and/or the d715 receptors were transfected by electroporation with the reporter construct.

**Preparation of Whole Cell Extracts, Subcellular Fractionation, and Western Blotting Analysis—** Whole cell, membrane, and cytosolic extracts were prepared as described (49). Cell extracts were boiled in sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-PAGE prior to transfer to Immobilon membranes. The membranes were incubated with the appropriate antibodies, and the reactive proteins were visualized by enhanced chemiluminescence.

**Transfection**—32D cells expressing the human G-CSFRs were transfected with the expression constructs for NE or the G185R mutant and selected in medium containing puromycin (1 µg/ml) or hygromycin (1 mg/ml). Individual clones were obtained by limiting dilution and examined for expression of NE proteins by Western blotting analysis. Unless otherwise stated, three independent pooled clones were used in subsequent experiments.
pGL3-Ela2P plasmid or a luciferase reporter plasmid containing three repeats of a GAS element derived from IRF-1 gene promoter (50) along with a CMV-β-gal plasmid. After culture for 16 h, cells were washed and left untreated or stimulated with G-CSF for 6 h. Luciferase activity was measured and normalized on the basis of the β-galactosidase activity.

**Apoptosis Assay**—Apoptosis was examined using the Annexin V-phycocerythrin apoptosis detection kit (BD Biosciences). Briefly, 0.3 × 10⁶ cells were collected and incubated with Annexin V-phycocerythrin and 7-aminoactinomycin. Cells were analyzed by two-color flow cytometry on an LSR Fortessa (BD Biosciences) using FACSDiva and analyzed with FlowJo (Tree Star).

**Flow Cytometry**—Cells were washed in PBS with 2% horse serum and blocked with Fc block (eBioscience) for 15 min. Cells were then incubated with isotype control FITC-conjugated anti-mouse IgG, anti-Gr-1, or anti-G-CSFR antibody for 30 min prior to washing in PBS with 2% horse serum. Flow cytometry was performed as briefly described above.

**Statistics**—All data are presented as mean ± S.D. Significant differences were determined using Student’s t test. A p value <0.05 was considered significant and is shown as *. ** denotes p < 0.01.

**Author Contributions**—Y. Q., Y. Z., N. H., and F. D. performed experiments and analyzed results. F. D. designed the research and wrote the paper. All authors approved the final version of the manuscript.

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