Induction of Bv8 expression by granulocyte-colony stimulating factor in CD11b Gr1+ cells: Key role of Stat3 signaling

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Running title: Stat3 regulates G-CSF induced Bv8 expression

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Key words: myeloid cells, Bv8, prokineticin, G-CSF, Stat3

Background: Bv8 expression is strongly induced by G-CSF, but the mechanisms remain unknown. Results: Stat3 activation is required for G-CSF induced Bv8 up-regulation. Conclusion: Stat3 plays a key role in regulating G-CSF induced Bv8 expression. Significance: Elucidating the signaling pathways implicated in Bv8 regulation is crucial to understand the role of this molecule in pathophysiological circumstances.

SUMMARY

Bv8, also known as prokineticin 2, has been characterized as an important mediator of myeloid cell mobilization and myeloid cell-dependent tumor angiogenesis. Bv8 expression is dramatically enhanced by G-CSF, both in vitro and in vivo. The mechanisms involved in such up-regulation are unknown. Using pharmacological inhibitors that interfere with multiple signaling pathways known to be activated by G-CSF, we show that signal transducer and activator of transcription 3 (Stat3) activation is required for Bv8 up-regulation in mouse bone marrow cells, while other Stat family members and extra-cellular signal regulated kinase (ERK) activation are not involved. We further identified CD11b Gr1+ myeloid cells as the primary cell population in which Stat3 signaling is activated by G-CSF. G-CSF-induced Bv8 expression in bone marrow cells was also significantly reduced by siRNA mediated Stat3 knock-down. Moreover, chromatin immuno-precipitation studies indicate that G-CSF significantly induces binding of phospho-Stat3 to the Bv8 promoter, which was abolished by pretreatment with Stat3 inhibitor WP1066. Luciferase assay confirmed the phospho-Stat3 binding site is a functional enhancer of the Bv8 promoter. The key role of Stat3 signaling in regulating G-CSF induced Bv8 expression was further confirmed by in vivo studies. Furthermore, we showed the regulation of Bv8 expression in human bone marrow cells is also Stat3 signaling dependent. Stat3 is recognized as a key regulator of inflammation-dependent tumorigenesis. We propose that such a role of Stat3 reflects at least in part its ability to regulate Bv8 expression.

Endocrine gland-derived VEGF (EG-VEGF) and Bv8, known also as prokineticin-1 and -2, respectively, are two highly related secreted proteins that belong to a larger class of peptides defined by a five disulfide bridge motif called a colipase fold (1-3). Bv8 and EG-VEGF bind two related G-protein coupled receptors (GPCRs), EG-VEGF receptor 1/PKR1 and EG-VEGF receptor 2/PKR2 (4,5). Bv8 was initially identified from the skin of the frog Bombina variegata (3). Later on, the human orthologue of this highly conserved protein was shown to have the same activities, including regulation of neuronal survival (6), gastrointestinal motility (1,7), and circadian locomotor rhythm (7).

Bv8 and EG-VEGF have been previously shown to promote tissue-specific angiogenesis and hematopoietic cell mobilization (2,8,9). Unlike EG-VEGF, Bv8 is mainly expressed by peripheral
blood and bone marrow cells (8). Recently, Bv8 has been shown to be up-regulated in inflammatory granulocytes and to modulate inflammation-associated pain (10). Bv8 expression is up-regulated in CD11b+ Gr1+ cells after implantation of tumor cells (11). Analysis of several xenografts (11) as well as of a transgenic cancer model (RIP-Tag) (12) suggested that Bv8 promotes tumor angiogenesis through increased peripheral mobilization of CD11b+ Gr1+ cells from the bone marrows and local stimulation of angiogenesis. Our previous studies identified granulocyte colony-stimulating factor (G-CSF) as a strong inducer of Bv8 expression in vitro and in vivo (11). G-CSF is a principle regulator of granulopoiesis and neutrophil mobilization from the bone marrow (13) and is widely used in cancer therapy to reduce chemotherapy-associated neutropenia (14). However, some recent observations suggest that G-CSF may, in some circumstances, facilitate tumor angiogenesis (15-17) and can be involved in the development of refractoriness to anti-VEGF agent (18). Thus, G-CSF and Bv8 may represent new therapeutic targets. Elucidation of signal transduction events involved Bv8 upregulation should enhance our understanding of the role of this molecule in tumorigenesis.

Stat3 is a member of the signal transducer and activator of transcription (STAT) family of protein and has been recently implicated as a major regulator of inflammation-associated tumorigenesis (19-22). In the present study, we report that Stat3 signaling plays an essential role in the up-regulation of Bv8 expression by G-CSF, both in mouse and human bone marrow cells.

EXPERIMENTAL PROCEDURES

Reagents - The Stat3 inhibitor WP1066, MEK1/2 inhibitor PD98059, and Stat5 inhibitor N’-((4-Oxo-4H-chromen-chromen-3-yl)methylen) nicotinohydrazide, were from Calbiochem (EMD4 Biosciences, Gibbstown, NJ). MEK1/2 inhibitor GDC-0973/XL518 was from Genentech (South San Francisco, CA). Recombinant mouse IL6, IL10, G-CSF, stem cell factor (SCF), Flt3 ligand, and recombinant human G-CSF, thrombopoietin (TPO) were from R&D Systems (Minneapolis, MN). Phospho-Stat1(Tyr701), phopho-Stat3(Tyr705), phospho-Stat5(Tyr694), phospho-p42/44, total Stat3, total p42/44, and β-actin antibodies were from Cell Signaling (Danvers, MA).

Isolation of mouse bone marrow cells - 6-8-week-old Balb/c mice were purchased from Charles River and were maintained under the guidelines of the Genentech animal care facility. Mouse bone marrow cells were flushed from femurs and tibias of Balb/c mice with DMEM containing 10% FBS. Then cells were washed and re-suspended in HBSS containing 0.2% BSA (Serologicals Corp. Atlanta, GA) for further studies.

In vitro studies - For in vitro inhibition studies, two-million freshly isolated mouse bone marrow cells in HBSS containing 0.2% BSA were pretreated with various inhibitors at several concentrations for 1 hour, except fludarabine for 4 hours, in 24-well plates, then were incubated with 10ng/ml recombinant mouse G-CSF for another 4 hours at 37°C in a 5% CO2 incubator. All inhibitors were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, St Louis, MO) as stock solution and serially diluted to the desired concentration with culture medium. The final concentration of DMSO in cell culture systems was 0.02%. mBv8 expression from each treatment was analyzed by Taqman or ELISA as previously described (11,23). Taqman data were normalized against RPL19 expression and cell viability was evaluated in parallel. The bone marrow cell lysates were subjected to western blot analysis.

To investigate Stat3 phosphorylation following G-CSF, IL6 or IL10 treatment in different cell populations, red blood cells were first lysed using ACK buffer (Lonza, Walkersville, MD), followed by staining with FITC conjugated anti-mouse Gr1 and APC-conjugated rat anti-mouse CD11b antibodies (BD Biosciences Pharmingen, San Diego, CA). CD11b+ Gr1+ and CD11b- Gr1- subsets were then obtained using FACS. Stat3 activation in different subsets was assessed by western blot. The G-CSF receptor (G-CSFR) expression levels in different cell subsets were analyzed by Taqman after incubation with 10ng/ml G-CSF for 4 hours at 37°C in a 5% CO2 incubator. The Taqman primers and probes for G-CSFR were from Applied Biosystems (Foster City, CA), and the assay ID number is Mm00432735_m1.
siRNA experiments - Freshly isolated bone marrow cells were first cultured overnight in DMEM supplemented with 15% FBS, 100ng/ml mouse SCF, 100ng/ml human TPO, and 100ng/ml mouse Flt3 ligand. After being washed, four million cells were re-spurred in 2 ml Accell siRNA delivery media (Dharmacon, Lafayette, CO) in 6-well plates supplemented with 100ng/ml mouse SCF, 100ng/ml human TPO, and 100ng/ml mouse Flt3 ligand, and were incubated with 0.5µM Accell SMART pool of siRNA for mouse Stat3 (a mixture of the four different Stat3 on-target siRNA oligonucleotides: CCAGUAUGCU UGUGCUGUUG, UCAUGUUCUUAAGUUAUA, GGCUGAUCAUCAUAAUAAA, and CUGGA AAAACUGGAUAACUU), or mouse Stat5 (a mixture of the four different Stat5 on-target siRNA oligonucleotides: GAUUCAUCCUUCUU GCUUU, CGUUGAAGACCUUUACGC, UGA ACUACCUCUAAGCU, UUGUUGUCACG AAAUCGC), or 0.5 µM Accell green nontargeting SiRNA (Dharmacon) for 48 hours. After being washed and re-suspended in HBSS containing 0.2% BSA, equivalent numbers of cells from each transfection were incubated with 10ng/ml recombinant mouse G-CSF or with PBS in a 24-well plates for 4 hours at 37°C in a 5% CO2 incubator. Bv8 expression was assessed by Taqman. The knockdown levels of Stat3 and Stat5 were analyzed by western blot and quantified by densitometry.

5’ rapid amplification of CDNA end (5’RACE)- To identify the transcription start site of mouse Bv8, mRNA was purified from total RNA prepared from mouse bone marrow cells using NucleoTrap mRNA mini Kit (Clontech, Mountain View, CA). Then RACE – ready cDNA was generated and 5’RACE PCR was performed using SMARTer™ RACE CDNA amplification kit (Clontech). The sequence of the Bv8 specific primer used for 5’RACE is: 5’- GCAGCGGTAGCACAGAAGTAGCG- 3’. The resulting PCR product was cloned, sequenced and mapped to mouse Bv8 genomic sequence.

Chromatin immunoprecipitation (ChIP) – ChIP was performed using digested chromatin from bone marrow cells (4X10⁶ cells in 1X HBSS media containing 0.2% BSA) treated with 10ng/ml mouse G-CSF or PBS for 30 minutes, and either 10µl of Phospho-Stat3 (Tyr705) antibody or normal rabbit IgG using SimpleChIP™ Enzymatic Chromatin IP kit (Magnetic Beads) from Cell Signaling (Danvers, MA). Bv8 promoter fragment pulled down by phospho-Stat3 (Tyr705) antibody or control rabbit IgG were quantified by quantitative Real-time PCR using Bv8 specific primers from its promoter region: Forward primer is 5’-TTCGTTGCGATGGAGACTGGAAG-3’, and reverse primer is 5’-GAGTTCAAGAACAT CCTGAGACC-3’. For pharmacological inhibition experiments, bone marrow cells were pretreated with either 5µM WP1066 or with the same volume of DMSO for 1 hour, followed by treatment with 10ng/ml G-CSF for 30 minutes. Chip assay was then performed as mentioned above.

Bv8 luciferase reporter assays- A fragment of Bv8 promoter region from -1762 ~ +90, containing ATG start codon and Stat3 ISRE binding site, was obtained by amplification of mouse genomic DNA and was cloned into pGL4.23(luc2/minP) luciferase vector (Promega, Madison, WI) to replace the minimal promoter of luc2 gene. To investigate whether the phospho-Stat3 ISRE binding site within this fragment is a functional enhancer of the Bv8 promoter, the consensus sequence of the ISRE binding site, GAAAGGAAACT, was mutated to TCCACCAGTCT, or deleted using QuickChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The sequences of mutagenic primers for introducing mutations are: forward: 5’-GGCATGGGAGACTGTCCACCAGTCT-3’, reverse: 5’-GACAGACTGGTGGAAGAGTCTCC ATGCC-3’, and the sequences of primers for introducing deletions are: forward: 5’-GACAGACTGGTGGAAGAGTCTCC ATGCC-3’, and the sequences of primers for introducing deletions are: forward: 5’-GCTTCTCG TTGCGTGGAGACTGCGCGAGACTTGCTCC ACCAGTCTG-3’, reverse: 5’-GACAGACTGGTGGAAGAGTCTCC ATGCC-3’. The pGL4.23-Bv8 construct, containing either WT ISRE, mutated ISRE or deleted ISRE binding site, was co-transfected into HEK293T cells with plasmid expressing mouse Stat3 ORF (GeneCopoeia, Rockville, MD) or its control vector, using the pRL-TK Renilla reporter vector (Promega) for a transfection control. The pGL4.23-Bv8 construct was used as negative control. 48 hours post-transfection the cells were lysed with the Passive lysis buffer (Promega) and luminescence was measured using the Dual-luciferase Assay System (Promega). Luminescence (relative light units) was normalized to the Renilla luciferase expression.
Stat3 regulates G-CSF induced Bv8 expression

In vivo studies – Eight week-old Balb/c mice (n=5) were injected subcutaneously with 10µg human G-CSF (Neupogen, Amgen) in 100µl PBS, together with 10mg/kg or 20mg/kg WP1066 in 100µl vehicle, which were administered intraperitoneally (IP). All drugs were administered daily for three consecutive days. Control mice were given PBS and vehicle (mixture of 20 parts DMSO and 80 parts polyethylene glycol 300 (Sigma-Aldrich, St Louis, MO). At the end of the study, bone marrow cells were isolated and peripheral blood was collected for Bv8 expression analysis by Taqman and/or ELISA.

Regulation of Bv8 expression in human bone marrow cells - Fresh human bone marrow samples were obtained from ALLCELLS (Emeryville, CA). First, red blood cells were lysed by adding 4 volumes of 1X buffered ammonium chloride solution (Stem Cell Technology, Vancouver, Canada) and incubated on ice for 10 minutes. The isolated cells were then washed and re-suspended in HBSS media containing 0.2% BSA. To investigate the role of Stat3 in regulating Bv8 expression, 2 million cells in 24 well plates were first pre-treated with Stat3 inhibitor WP1066 at various concentrations for 1 hour, then incubated with 10ng/ml human G-CSF for 4 hours at 37°C in a 5% CO2 incubator. hBv8 expression was assed by Taqman as previously described.

RESULTS

Stat3 activation is required for G-CSF mediated Bv8 expression, while ERK activation is not involved

We previously identified G-CSF as a major positive regulator of Bv8 expression in vivo and in vitro. To elucidate the signal transduction pathways involved in such Bv8 up-regulation, we pretreated the cells with various pharmacological inhibitors of multiple G-CSF-activated signaling pathways, including Jak-Stat and MEK/ERK pathways (24,25). Pretreatment of bone marrow cells with the MEK1/2 inhibitor PD98059 at all concentrations tested (up to 20µM) did not significantly reduce Bv8 expression (Fig. 1A), a finding that was further confirmed with another potent and selective MEK1/2 inhibitor, GDC-0973/XL518 (hereafter GDC-0973) (Belvin et al., US patent #: 20110086837)(26,27) (Fig. 1B). In contrast, both PD98059 and GDC-0973 at all tested concentrations completely inhibited G-CSF induced ERK pathway activation (Fig.1C). Pretreatment with Stat3 inhibitor WP1066, a cell permeable AG490 tyrphostin analog, inhibited G-CSF induced Bv8 expression in a dose-dependent manner with complete inhibition at 5µM WP1066 (Fig. 3A). However, neither inhibition of Stat1 signaling using a potent Stat1 inhibitor, fludarabine (Fig.2A), nor inhibition of Stat5 using N’-((4-Oxo-4H-chromen-3-yl) methylene) nicotinohydrazide (Fig.2B), significantly reduced the Bv8 up-regulation, whereas 50 µM fludarabine (Fig.2D) or 50µM nicotinohydrazide (Fig.2E) completely inhibited G-CSF induced Stat1 and Stat5 activation. None of the inhibitors had a significant effect on the viability of bone marrow cells at the tested concentrations (data not shown).

CD11b+Gr1+ cells are the primary G-CSF responsive cell type expressing Bv8

To confirm that the Stat3 pathway can be activated by G-CSF in mouse bone marrow cells, we assessed Stat3 phosphorylation at Tyr705. We observed a rapid phosphorylation after addition of 10ng/ml mouse G-CSF, starting at 2 minutes and peaking at 10 minutes. After 30 minutes, Stat3 phosphorylation decreased but was still above the non-stimulated background for at least 60 minutes (Fig. 3B). We further investigated the effect of WP1066 on G-CSF-activated Stat3 phosphorylation. Pretreatment with WP1066 blocked G-CSF induced Stat3 phosphorylation at 5µM WP1066 (Fig. 3C).

Stat3 can be strongly activated by G-CSF, IL6 and IL10. However, previous studies indicated that, among these cytokines, only G-CSF significantly up-regulates Bv8 expression (11). Based on our previous finding that CD11b+Gr1+ cells are the major subset of bone marrow cells that produce Bv8 (11), we hypothesized that Stat3 mediated Bv8 expression might be cell-type dependent. To test this hypothesis, we analyzed Stat3 phosphorylation in unsorted, CD11b+Gr1− cells and CD11b−Gr1+ cells following treatment with IL6, IL10 or G-CSF at a maximal effective concentration 10ng/ml. In unsorted bone marrow cells, all three factors were able to activate Stat3 signaling in a time-dependent manner. G-CSF activation of Stat3 was most prominent in CD11b+...
Stat3 regulates G-CSF induced Bv8 expression in mouse bone marrow cells

We previously reported that G-CSF administration results in time- and dose-dependent increases in the levels of Bv8 protein in the bone marrow and in the serum of mice(11). We sought to determine whether such Bv8 increases could be also reduced by simultaneous treatment with WP1066 in vivo. In agreement with previous reports, Bv8 levels in the bone marrow cells increased ~60 fold above background levels following G-CSF administration for three consecutive days (Fig. 6B, Fig. 6C). Serum levels were also markedly increased (Fig. 6A). Vehicle

Stat3 siRNA inhibited G-CSF induced Bv8 expression in mouse bone marrow cells

WP1066 has been shown to inhibit Stat3 activity and to have potent anti-tumor effects both in vitro and in vivo(28). However, WP1066 has been also reported to inhibit JAK-2 and Stat5 activities at least in human glioma cells(28). To confirm whether WP1066 effects on G-CSF-induced Bv8 up-regulation are truly mediated by inhibition of Stat3 signaling, we knock-downed Stat3 using Accell SMART pool of siRNA for mouse Stat3. Densitometric analysis showed that Stat3 siRNA results in 51% decrease in Stat3 expression compared to control siRNA treatment (Fig.4A). Accordingly, siRNA-mediated Stat3 knock-down resulted in more than 60% Bv8 reduction (Fig.4C). In contrast, although Stat5 siRNA almost completely knocked down Stat5 expression (Fig.4B), the Bv8 expression levels remained similar to those in control siRNA treated bone marrow cells (Fig.4C).

Bv8 is a direct transcriptional target of Stat3

To determine whether Stat3-dependent Bv8 up-regulation occurs via direct binding to the Bv8 promoter region, we first performed 5'RACE using mRNA from mouse bone marrow cells to identify the Bv8 transcription start site. A single band with an estimated size of 190 bp was obtained using Bv8 specific primers, which maps 25 nucleotides downstream of ATG translational start codon, and the universal primer A mix provided in the SMARTer™ 5’RACE kit (Fig. 5A). After the PCR product was cloned and sequenced, the transcription start site was mapped to 87 nucleotides upstream of the ATG translation start codon of mouse Bv8 (Fig. 5B), which was designated as the +1 position throughout this study. A typical TATA box was identified 25 bp upstream of the Bv8 transcription start site (Fig. 5B).

We then searched for putative Stat3 binding sites in the Bv8 promoter region. A typical Stat3 interferon-sensitive response element (ISRE) binding site was identified at -1433 ~ -1423 (Fig.5C). To assess if phospho-Stat3 could bind to this region, we performed CHIP experiments using a phospho-Stat3 antibody or rabbit IgG isotype control. As shown in Fig. 5D, binding of phospho-Stat3 to the identified site was significantly increased in G-CSF- but not in PBS-treated cells. Pretreatment of bone marrow cells with 5µM WP1066 completely inhibited the G-CSF-induced binding of phospho-Stat3 to the Bv8 promoter region (Fig.5E).

To further investigate the functional role of this phospho-Stat3 binding site in regulating Bv8 promoter activity, we compared dual luciferase activity driven by the Bv8 promoter region from -1762 ~ +90, or by the same region with the phospho-stat3 ISRE consensus sequence mutated or deleted after HEK 293T cells were co-transfected with plasmid expressing mouse Stat3 ORF or its control vector for 48 hours (Fig. 5F, Fig. 5G). As shown in Fig. 5G, when the cells were co-transfected with Stat3 control vector, both the parental and the mutated/deleted 1.85kb Bv8 promoter region, which containing the TATA box, were able to drive luciferase activity at comparable level. In contrast, when cells were co-transfected with plasmid expressing mouse Stat3 ORF, only the parental 1.85 kb Bv8 promoter region demonstrated significant increase in luciferase activity, whereas, the luciferase activity driven by the mutated or deleted Bv8 promoter region remained at a similar level as the cells co-transfected with the Stat3 control vector.

Up-regulation of Bv8 expression induced in vivo by G-CSF is reduced by Stat3 inhibitor

We previously reported that G-CSF administration results in time- and dose-dependent increases in the levels of Bv8 protein in the bone marrow and in the serum of mice(11). We sought to determine whether such Bv8 increases could be also reduced by simultaneous treatment with WP1066 in vivo. In agreement with previous reports, Bv8 levels in the bone marrow cells increased ~60 fold above background levels following G-CSF administration for three consecutive days (Fig. 6B, Fig. 6C). Serum levels were also markedly increased (Fig. 6A). Vehicle
had no significant effect on \( B\nu 8 \) levels in serum and bone marrow cells (data not shown). ELISA analysis demonstrated a significant dose-dependent reduction in \( B\nu 8 \) levels, both in the plasma and in the bone marrow of mice treated with WP1066 compared to vehicle-treated mice. In the presence of 20mg/kg WP1066, \( B\nu 8 \) levels in the plasma of G-CSF treated mice were comparable to those in PBS-treated mice (Fig. 6A). ELISA (Fig.6B) and Taqman data (Fig.6C) indicated that 20mg/kg WP1066 resulted in more than 60% \( B\nu 8 \) reduction in bone marrow cells.

Stat3 inhibitor suppresses G-CSF induced \( B\nu 8 \) expression in human bone marrow cells

We previously reported that G-CSF induces \( B\nu 8 \) expression in human bone marrow cells (23). To determine whether Stat3 plays a role in regulating G-CSF induced \( B\nu 8 \) expression, human bone marrow cells were pretreated with WP1066 at various concentrations. In agreement with the aforementioned studies (23), 10ng/ml G-CSF resulted in over 10-fold induction of \( B\nu 8 \) expression within 4 hours (Fig 7). WP1066 reduced such G-CSF stimulated \( B\nu 8 \) expression in a dose-dependent manner, with complete inhibition at 5uM WP1066 (Fig 7).

DISCUSSION

Angiogenesis plays an important role in tumor progression and metastasis. Inhibiting angiogenesis represents a clinically validated anticancer strategy (29-32). Although tumor cells have been traditionally thought to be the major source of angiogenic factors (33), tumor stromal cells, such as fibroblast, mesenchymal stem cells, immune cells, and different subpopulations of myeloid cells, are being increasingly recognized as sources of various pro-angiogenic factors (34-38). CD11b\(^+\) Gr1\(^+\) cells, a subpopulations of myeloid cells which include neutrophils, macrophages and myeloid-derived suppressor cells (39), have been shown not only to contribute to tumor angiogenesis (11,12,15,40,41), but also to mediate refractoriness to anti-VEGF therapy (18,41) as well as stimulate the formation of pre-metastatic “niches” that facilitate the metastatic process (42).

In evaluating the mechanism of angiogenesis mediated by CD11b\(^+\) Gr1\(^+\), recent studies identified \( B\nu 8 \) as a critical mediator, and G-CSF was identified as a major inducer of \( B\nu 8 \) expression in vitro and in vivo (11). The observation that neutralizing anti-G-CSF antibodies abrogates the increase in \( B\nu 8 \) expression in CD11b\(^+\) Gr1\(^+\) cells following tumor implantation and also reduced tumor growth suggested a communication between the up-regulation of \( B\nu 8 \) expression, CD11b\(^+\) Gr1\(^+\) cells and tumor growth. Most recently, we identified G-CSF as a key initiator and regulator of lung metastasis, which depends on \( B\nu 8 \)-expressing Ly6G\(^-\) Ly6C\(^+\) cells. Neutralization of G-CSF, \( B\nu 8 \) or Ly6G\(^-\) Ly6C\(^+\) cells reduced metastasis (42). These data suggest that G-CSF promotes tumor angiogenesis and metastasis at least in part through a \( B\nu 8 \)-dependent pathway. Therefore, to better understand the role of G-CSF and \( B\nu 8 \) in tumorigenesis, it is important to define the molecular mechanism responsible for G-CSF-induced \( B\nu 8 \) up-regulation.

The major signaling pathways regulated by G-CSF include MEK/ERK and Jak/Stat pathways (24,25). Using pharmaceutical inhibitors that interfere with those pathways, we ruled out the involvement of the MEK/ERK signaling in G-CSF induced \( B\nu 8 \) expression since two independent MEK-1 inhibitors, PD98059 and GDC-0973, did not significantly reduce G-CSF stimulated \( B\nu 8 \) expression. However, Stat3 inhibition by the pharmacological inhibitor WP1066 or by siRNA significantly reduce \( B\nu 8 \) levels in bone marrow cells. In contrast, the induction of Stat1 and Stat5 phosphorylation was much weaker (Fig. 2C), and blocking Stat1 or Stat5 activity had no significant effect on G-CSF induced \( B\nu 8 \) expression. Our observation is consistent with previous studies reporting redundancy among the Jak kinases in G-CSF signaling (25), while Stats play an essential and non-redundant role (43-46). In bone marrow cells, G-CSF stimulation mainly activates Stat3, and to a lesser extent Stat1 and Stat5 (43,45,47-49). ChIPs assay confirmed that Phospho-Stat3 can directly bind to the \( B\nu 8 \) promoter. Dual luciferase assays further confirmed that the phospho-Stat3 binding site is a functional enhancer of the \( B\nu 8 \) promoter and suggest that \( B\nu 8 \) is a direct transcriptional target of Stat3.

We identified CD11b\(^+\) Gr1\(^+\) cells as the primary cell population in which Stat3 signaling is activated by G-CSF. The finding that IL6 and IL10, two cytokines known to activate Stat3...
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signaling in bone marrow cells but having no
effect on Bv8 expression, induce Stat3
phosphorylation mainly in CD11b-Gr1- cells
supports the hypothesis that Stat3 mediated Bv8
expression is cell-type dependent. Our
experiments provide evidence that such cell–type
dependent G-CSF response is likely due to the
distinct expression levels of the G-CSF receptor in
CD11b-Gr1+ versus CD11b-Gr1- cells. The
finding that Bv8 expression in human bone
marrow cells is also Stat3-dependent suggests that
such signaling pathway may also play a critical
role in regulating Bv8 expression in human bone
marrow-derived cells.

As already pointed out, Stat3 has been
implicated as a central regulator of inflammation-
associated tumorigenesis, including myeloid cell-
dependent tumor promotion (19). We propose that
such a role of Stat3 reflects, at least in part, its
ability to regulate the G-CSF-Bv8 signaling axis,
as reported in the present manuscript. Additional
studies are required to further define the role of G-
CSF-Stat3-Bv8 signaling in various tumor types as
well as other pathophysiological conditions.

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Acknowledgements - We thank the Bioinformatics Department, the Flow Cytometry laboratory and the Animal Care facility for their help. We also thank A. Chung for reading the manuscript, X. Wu for help with in vivo studies and M. Tan for Bv8 ELISA.

FIGURE LEGENDS

FIGURE 1. ERK is not involved in G-CSF induced Bv8 up-regulation. Taqman analysis, as described in the experimental procedure, to evaluate Bv8 expression in MEK-1 inhibitors PD98059 (A) and GDC-0973 (B) treated bone marrow cells. Western blot analysis to detect phospho-Stat3 and total Stat3 after bone marrow cells had been treated with 10ng/ml mouse G-CSF for the indicated time, or after pretreatment with PD98059 or GDC-0973 at the indicated concentrations for 1 hour, followed by incubation with 10ng/ml mouse G-CSF for another 10 min (C). Error bars represent SEM. Data shown are representative of three independent experiments.

FIGURE 2. Stat1 and Stat5 signaling events are not involved in G-CSF induced Bv8 expression. Taqman analysis to evaluate Bv8 expression in Stat1 inhibitor, Fludarabine(A) or Stat5 inhibitor (B) treated bone marrow cells. Western blot analysis to detect phospho-Stat1, phosphol-Stat5, and B-actin after bone
marrow cells had been treated with 10ng/ml mouse G-CSF for the indicated time (C) or to detect phospho-Stat1 after pretreated with fludarabine or 4 hours (D), or to detect phospho-Stat5 after pretreated with Stat5 inhibitor for 1 hour (E) at indicated concentrations, followed by incubation with 10ng/ml mouse G-CSF for another 10 min. Error bars represent SEM. Data shown are representative of three independent experiments.

**FIGURE 3.** Activation of Stat3 signaling is required for G-CSF induced Bv8 expression in mouse bone marrow cells. Taqman analysis to evaluate Bv8 expression in WP1066 treated bone marrow cells. Error bars represent SEM. Single asterisk indicates significant difference in WP1066-treated compared to DMSO-treated groups (P<0.05) (A). Western blot analysis to detect phospho-Stat3 after bone marrow cells had been treated with 10ng/ml mouse G-CSF for the indicated time (B), or after the bone marrow cells had been pretreated with 1, 2, or 5 µM WP1066 for 1 hour, followed by incubation with 10ng/ml mouse G-CSF for another 10 min (C), or in unsorted, CD11b+ Gr1−, and CD11b− Gr1− bone marrow cells after being treated with 10ng/ml of G-CSF, IL6 and IL10 for the indicated time (D). Taqman analysis to evaluate G-CSFR expression in CD11b+ Gr1−, and CD11b− Gr1− cells after being incubated with 10ng/ml G-CSF or PBS for 4 hours. Error bars represent SEM. Single asterisk indicates significant difference in G-CSFR expression between the two cell subsets (P<0.05). Data shown are representative of three independent experiments.

**FIGURE 4.** Stat3 siRNA inhibits G-CSF induced Bv8 expression in mouse bone marrow cells. Bone marrow cells were transfected with Accell SMART pool of Stat3 siRNA, Stat5 siRNA or Accell green none-targeting siRNA. Western blot analysis to detect the protein knock down level of Stat3 (A), or Stat5 (B), and Bv8 expression was analyzed by Taqman 48 hours after siRNA transfection (C). Error bars represent SEM. Single asterisk indicates significant difference in Stat3 siRNA treated compared to control siRNA treated groups (P<0.05). Data shown are representative of three independent experiments.

**FIGURE 5.** Bv8 is a direct transcriptional target of Stat3. (A) Agarose gel electrophoresis of the PCR product obtained from the 5’RACE procedure. Molecular size marker is indicated on the left. (B) Sequence of the mouse Bv8 gene around the translation start codon region. The vertical arrow above the sequence indicates the transcription start site identified by sequencing of the 5’RACE PCR product. The horizontal arrow below the sequence indicates the mouse Bv8 specific primer used for 5’RACE. The translation start codon ATG and TATA box are boxed. (C) Schematic diagram of Stat3 binding site within the proximal promoter region of the Bv8 gene. The bidirectional arrows mark the target regions for ChIP assays. (D) G-CSF significantly induces the binding of phospho-Stat3 to the putative Stat3 binding site in the Bv8 promoter region. ChIPs were performed as described in the experimental procedures. Error bars represent SEM. Single asterisk indicates significant difference in G-CSF treated compared to PBS treated groups (P<0.05). (E) The binding of phospho-Stat3 to the Bv8 promoter region following G-CSF treatment was blocked by pretreatment of the bone marrow cells with 5µM WP1066. Error bars represent SEM. Single asterisk indicates significant difference in WP1066 treated compared to DMSO treated groups (P<0.05). The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, which is equivalent to one. Error bars represent SEM. Data shown are representative of three independent experiments. (F) Schematic diagrams of the pGL4.23-Bv8 constructs, containing WT ISRE (a), mutated ISRE (b) and deleted ISRE (c) binding site. (G) Dual-luciferase assay to investigate the role of phospho-Stat3 ISRE site in regulating mouse Bv8 promoter activity. Error bars represent SEM. Single asterisk indicates significant difference between Stat3 ORF and control vector transfected groups (P<0.05).

**FIGURE 6.** WP1066 reduces Bv8 increases in peripheral blood and bone marrow cells induced by in vivo administration of G-CSF. Bv8 levels in plasma were measured by ELISA (A), and in the bone marrow cells by ELISA (B) and Taqman (C). For ELISA, data were normalized by total protein concentration. Taqman data were normalized against RPL19 expression. Error bars represent SEM.
Single asterisk indicate significant difference in WP1066 treated compared to vehicle treated groups (P<0.05).

**FIGURE 7.** Activation of Stat3 signaling is required for G-CSF induced Bv8 expression in human bone marrow cells. Taqman analysis to evaluate hBv8 expression in pooled human bone marrow cells from 5 different healthy donors without any medication after pretreatment with 0, 1, 2, or 5 µM WP1066 for 1 hour and followed by incubation with 10ng/ml human G-CSF for another 4 hours. Taqman data were normalized against *RPL19* expression. Error bars represent SEM. Single asterisk indicates significant difference in WP1066 treated compared to DMSO treated groups (P<0.05).
Figure 1. Stat3 regulates G-CSF induced Bv8 expression.
Figure 2. Stat3 regulates G-CSF induced Bv8 expression.
Stat3 regulates G-CSF induced Bv8 expression

Figure 3.

A

![Graph showing relative transcript level (Bv8/RPL19)]

- **Black bar**: PBS
- **Gray bar**: G-CSF 10ng/ml

B

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Phospho-Stat3 Y705
Total Stat3

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Phospho-Stat3 Y705
Total Stat3

E

![Graph showing relative transcript level (G-CSF/GAPDH)]

- **Black bar**: PBS
- **Gray bar**: G-CSF 10ng/ml

CD11b+Gr1+  CD11b-Gr1-
Figure 4. Stat3 regulates G-CSF induced Bv8 expression
Stat3 regulates G-CSF induced Bv8 expression

A

B

C

D

E

F

G

Figure 5.
Figure 6.

Stat3 regulates G-CSF induced Bv8 expression

A

**Bv8 level in the plasma (pg/ml)**

- **PBS**
- **G-CSF 10ng/ml**

Vehicle | 10mg/kg | 20mg/kg
---|---|---
0 | 350 | 400

B

**Bv8 level in bone marrow cells (ng per mg of total protein)**

- **PBS**
- **G-CSF 10ng/ml**

Vehicle | 10mg/kg | 20mg/kg
---|---|---
0 | 50 | 40

C

**Relative transcript level (Bv8/RPL19)**

- **PBS**
- **G-CSF 10ng/ml**

Vehicle | 10mg/kg | 20mg/kg
---|---|---
0 | 0.25 | 0.20
Figure 7. Stat3 regulates G-CSF induced Bv8 expression

A

![Graph showing relative transcript level (h-Bv8/RPL19) for different treatments.]

- **PBS**
- **G-CSF 10ng/ml**

DMSO 1µM 2µM 5µM WP1066
Induction of Bv8 expression by granulocyte-colony stimulating factor in CD11b+Gr1+ cells: Key role of Stat3 signaling
Xueping Qu, Guanglei Zhuang, Lanlan Yu, Gloria Meng and Napoleone Ferrara

J. Biol. Chem. published online April 23, 2012

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