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Interleukin 32 promotes hematopoietic progenitor expansion and attenuates bone marrow cytotoxicity

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The identification of soluble factors involved in stem cell renewal is a major goal in the assessment of the BM niche. We have previously shown that human endothelial cell (EC) supernatants can induce the proliferation of hematopoietic progenitor cells (HPCs), especially after stimulation with IL-1β. To identify new potential growth factors, we compared the expression profile of IL-1β-stimulated ECs over 4, 8 and 16 h with non-stimulated ECs using oligonucleotide microarrays covering more than 46 000 transcripts. Most significant changes were detected after 4 h. Utilization of Gene Ontology annotation for the stimulated EC transcriptome indicated multiple upregulated genes encoding extracellular proteins with a cell–cell signaling function. Using flow cytometry, delta, colony and cobblestone assays, we assessed the proliferative capacities of 11 gene products, i.e. IL-8, IL-32, FGF-18, osteoprotegerin, Gro 1–3, ENA78, GCP-2, CCL2 and CCL20, which are not known to induce HPC expansion. Notably, IL-32 and to a lesser degree osteoprotegerin and Gro 3 significantly induced the proliferation of HPCs. Furthermore, IL-32 attenuated chemotherapy-related BM cytotoxicities by increasing the number of HPCs in mice. Our findings confirm that the combination of microarrays and gene annotation helps to identify new hematopoietic growth factors.

Keywords: Endothelial cells · Hematopoietic stem cells · Molecular genetics

Supporting Information available online

Introduction

Endothelial cells (ECs) have been shown to support the proliferation of hematopoietic $CD34^+$ progenitor cells by the constitutive production of cytokines [1, 2]. In previous studies, we demonstrated that ECs stimulated by TNF-α induced the generation of dendritic cells from $CD34^+$ cells for more than 6 wk [3]. ILs, on the other hand, can also induce the proliferation of hematopoietic and myeloid progenitors [4].

So far, GM-CSF and G-CSF are known to be secreted by IL-stimulated ECs [5]. Other endothelial factors propagating progenitor expansion include stem cell factor (SCF) [6], leukemia

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inhibitory factor (LIF) [7] and IL-6 [8, 9]. Beyond the known cytokine scenario, ECs synthesize multiple other proteins [10], i.e. chemokines of the C-X-C, C-C and TNF receptor superfamily; however, whether these factors can also support hematopoietic progenitor cell (HPC) expansion remains unknown.

Notably, microarray technologies monitoring expression changes for thousands of genes have been the basis for several systematic studies of immune and stem cells and their involvement in a variety of processes [11–15]. For example, microarrays of ECs helped to reveal unknown signaling pathways in the endothelial immune cascades [16], specify the role of inflammatory stimuli in neutrophil transmigration [17] and identify the effects of biochemical forces [18]. Microarrays of cultured HPCs also defined detrimental components of engineered extracellular matrices [19]. To use microarrays of feeder cells for the identification of new hematopoietic growth factors is another aspect. Choong et al., for example, discovered proliferin-2 after microarray analyses of several supportive stroma cell lines [20]. Chute et al. used a similar approach when they discovered the hematopoietic activity of adrenomedullin expressed by human brain ECs [21].

Besides brain ECs, IL-stimulated human umbilical cord ECs can also support hematopoiesis [4]. In order to identify new expansion factors, we performed oligonucleotide microarray analyses on IL-1β-stimulated ECs in combination with analyses of the hematopoietic properties of candidate factors using delta and colony assays in combination with flow cytometry.

Results

IL-1β-upregulated endothelial gene clusters are associated with the immune response and cytokine activity

Time course oligonucleotide microarrays were performed in order to elucidate endothelial factors involved in HPC proliferation and differentiation. Measurements were taken for IL-1β-stimulated EC samples after 4, 8 and 16 h, and for control ECs without IL-1β (0 and 16 h). A hierarchical cluster analysis of expression profiles revealed two clusters. While the gene signals from the IL-1β-stimulated EC samples at different time points were clustered together, the control ECs without IL-1β (0 and 16 h) were assigned to the other cluster, suggesting that the expression changes caused by IL-1β dominate over expression changes over time (Fig. 1A). A pair-wise display of logged (base 2) expression values indicates a strong overall correlation between the EC samples, i.e. only a subset of genes is differentially expressed (Fig. 1B). The larger scattering of expression values between the treated and control EC groups compared with the scattering within these groups confirms the results of the clustering analysis. A total of 198 genes significantly changed (false discovery rate <0.2) with 165 being upregulated. Especially after 4 h of IL-1β stimulation, many differentially expressed genes were detected (Fig. 1C and D). To identify temporal expression patterns, we clustered genes based on their corresponding microarray signals. The subsequent assessment of the functional composition of detected gene clusters demonstrated that the majority of upregulated genes are involved in immune responses and cytokine activity (Fig. 1E). The discovered clusters indicate several distinct, increased temporal expression responses to IL-1β stimulation. Most expression increases occurred when the endothelium had been subjected to IL-1β for 4 h (cluster 1, 3, 4, 5, 7 and 8); gene signal intensities remained high throughout the observed time span in four clusters (1, 5, 7 and 8).

Identification of novel candidate factors inducing HPC expansion

The set of differentially expressed genes provided numerous candidates for novel factors of HPC proliferation. However, the large number of differentially regulated genes would pose considerable challenges in their individual validation. For a more efficient identification of potential HPC expansion factors, we utilized additional annotations provided by gene ontology (GO). Here, we focused on gene products associated with cytokine activity, receptor binding and extracellular region/space. Remarkably, the integration of gene annotation and expression data enabled us to rapidly assemble a concise list of promising candidate genes for further validation. A total of 20 significantly upregulated genes belonged to the GO category "receptor binding" and the majority of these were also associated with the extracellular space/region (Table 1). Out of these 20, three factors were present in the subcategory cytokine activity in cluster 1 (IL-32, epithelial cell-derived neutrophil-activating peptide (ENA)-78, granulocyte chemotactic protein (GCP)-2), seven in cluster 5 (G-CSF, GM-CSF, IL-1α, Gro 1, Gro 2, osteoprotegerin (OPG), monocyte chemotactic protein (MCP)-2), and seven in cluster 8 (IL-6, IL-8, LIF, Gro 3, GM-CSF, macrophage inflammatory protein (MIP)-3α, fractalkine). Notably, several signals for the same gene product were repeatedly presented within one cluster, implying a high level of consistency in our analysis. The other components listed in Table 1 are not subcategorized among cytokine activity, though the hematopoietic growth properties of one, namely Jagged 1, has been demonstrated in the past [22]. Fibroblast growth factor (FGF) 18 was significantly upregulated in cluster 4 under receptor binding; it was the only gene that was significantly upregulated after 4 h of IL-1β stimulation and returned to baseline levels within the observed time span of 16 h. RT-PCR of four upregulated genes confirmed the microarray results (Table 1).

Gro 3, OPG and IL-32 induce HPC expansion

The hematopoietic properties of the selected candidate genes were assessed using three different functional assays in ex vivo cell cultures. Gro 3, OPG and IL-32 were found to significantly enhance the expansion of isolated $CD34^+$ cells (Fig. 2). Other

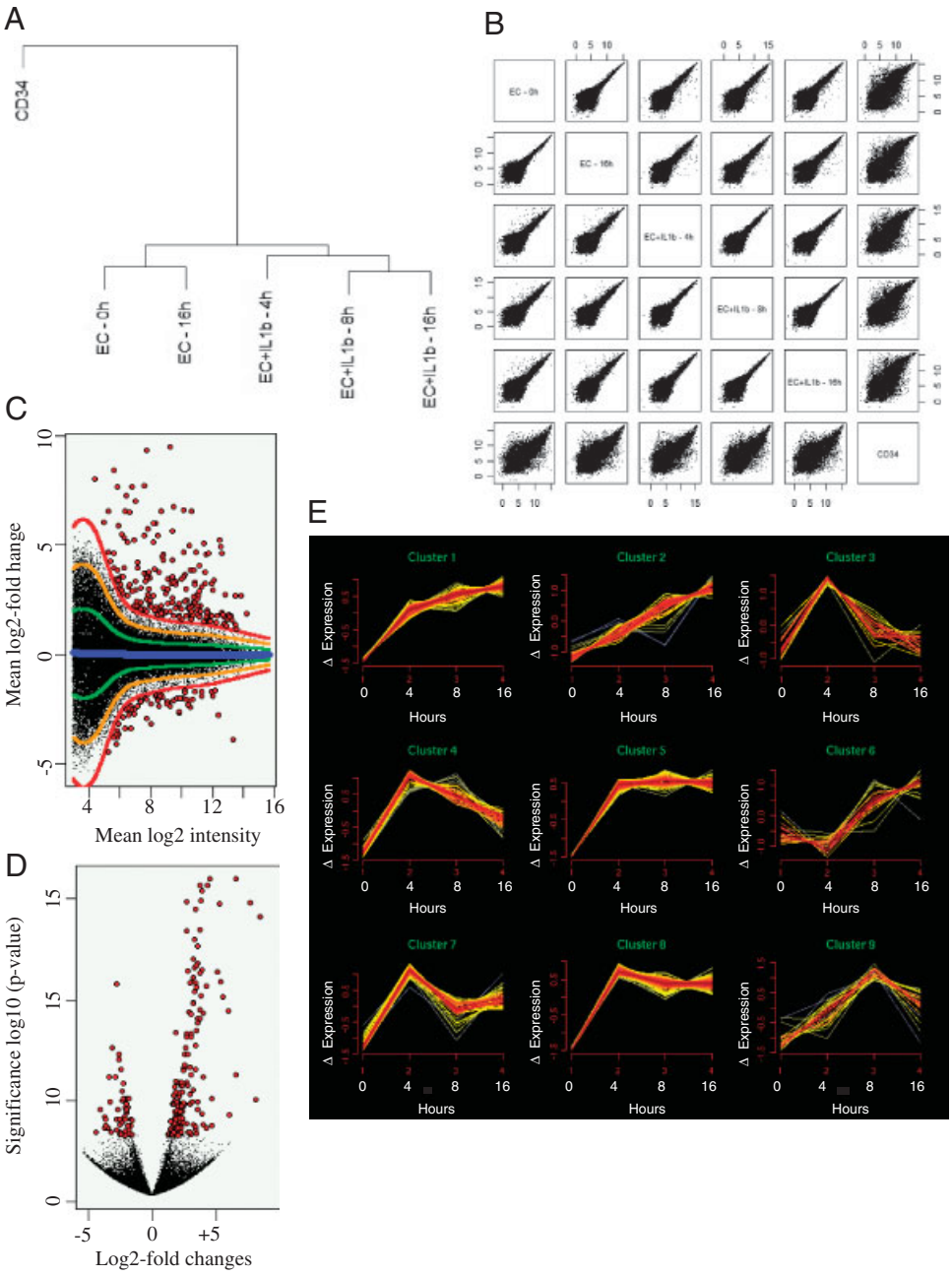

Figure 1. Microarray analyses. (A) Cluster dendrogram. Hierarchical clustering of samples from IL-1β-treated and non-treated HUVEC from one isolation, cultured for two passages until confluency, was performed. Gene expression patterns of non-stimulated controls at baseline (EC-0 h) and after 16 h in medium alone (EC-16 h) were clustered together as well as of IL-1β-stimulated ECs after 4, 8 and 16 h (EC+IL-1b-4 h, EC+IL-1b-8 h and EC+IL-1b-16 h). For comparison, microarray data of pooled hematopoietic $CD34^+$ cells isolated from cord blood ($n = 6$) were included. (B) Scatter plots: Pair-wise display of logged expression values indicates a strong overall correlation between the EC samples. (C) Significance of differential expression. Differences in expression between stimulated ECs after 4 h and non-stimulated controls at baseline were displayed as the log2-fold change of probe sets versus the corresponding mean log2-intensity in both arrays. Green, orange and red lines represent 1, 2 and 3 standard deviations. (D) Vulcano plot displaying the derived significance versus observed log2-fold changes. Red spots represent probe sets with a false discovery rate less than 0.2. As low intensity spots interfered with the assessment of significance, a threshold for the average logged intensity of 3 was set. (E) Cluster analysis. Genes showing differential expression were clustered over time. Clusters were subsequently examined for the significant enrichment in genes associated with particular GO categories of molecular function (MF), biological process (BP) and cellular component (CC). The following representative GO categories were statistically significant: Cluster 1 containing $n = 67$ genes: MF in cytokine activity with six genes (FDR = 0.006), BP in immune response with 17 genes (FDR = 4.5×10^{-8}) and CC extracellular region with 14 genes (FDR = <0.0006); cluster 2 ($n = 47$): BP response to biotic stimuli (eight genes, FDR = 0.046); cluster 3 ($n = 23$): MF protein binding (12 genes, FDR = 0.073); cluster 4 ($n = 40$): BP negative regulation of cellular physiological processes (six genes, FDR = 0.07), cluster 5 ($n = 69$): MF cytokine activity (seven genes, FDR = 0.002), BP response to stimuli (17 genes, FDR = 9.87×10^{-4}), cell proliferation (six genes, FDR = 4.78×10^{-2}) and CC extracellular space (seven genes, FDR = 0.076); cluster 6 ($n = 21$): MF protein binding (eight genes, FDR = 0.0921); cluster 7 ($n = 51$): MF protein binding (14 genes, FDR = 0.049), BP immune response (eight genes, FDR = 0.0087); cluster 8 ($n = 95$): MF cytokine activity (seven genes, FDR = 0.0037), BP immune response (17 genes, FDR = 6.64×10^{-6}); cluster 9 ($n = 21$): no GO categories were significantly enriched.

Table 1. Upregulated genes ($\geq 2^2$-fold) categorized in receptor binding and log2 expression levels dependent on IL-1β stimulation time[a)]

ID	Sequence description	0 h	4 h	8 h	16 h
147760	IL-1α	5.75	10.33	10.37	11.19
147620	IL-6	9.28	15.02	13.79	13.85
146930	IL-8	12.49	15.43	15.23	15.19
606001	IL-32	6.55 (0.82±0.57)	9.86 (2.1±0.11)	10.93 (2.4±0.24)	11.12 (2.9±0.14)
138970	G-CSF	13.74	10.74	10.79	11.47
138960	GM-CSF	6.87	13.02	12.86	13.46
155730	CXCL1	10.08	15.33	15.08	14.99
139110	CXCL2	6.3	9.26	8.23	8.12
139111	CXCL3	7.36 (0.12±0.17)	14.75 (7.5±0.25)	14.01 (6±0.2)	13.89 (5.7±0.32)
600324	CXCL5	5.43	10.01	11.28	11.98
138965	CXCL6	6.06	11.48	12.5	12.83
601880	CX3CL1	7.36	14.75	14.01	13.89
158105	CCL2	9.82	15.27	15.18	15.02
601960	CCL20	3.07	13.7	11.96	12.39
605717	Inducible T-cell costimulatory ligand	6.49	8.79	8.79	9
601920	Jagged 1	9.55	11.77	11.92	11.71
602643	TNFR superfamily member 11 (OPG)	2.93 (0.24±0.2)	10.86 (9.2±0.27)	10.43 (7.8±0.11)	10.6 (9.4±0.44)
600978	Lymphotoxin b	6.62	7.53	8.12	9.07
159540	LIF	5.58	9.48	8.17	8.49
603726	FGF-18	4.69 (0.4±0.56)	7.71 (4.1±0.23)	5.94 (2.5±0.39)	5.07 (2±0.19)

[a)] Genes categorized in receptor binding and in extracellular space/region are underlined. G-CSF, GM-CSF, Jagged-1, LIF and CXCL1 are known to have growth factor activities. CXCL1: melanoma growth stimulating activity α (Gro 1); CXCL2: MIP-2α (Gro 2); CXCL3: MIP-2β (Gro 3); CXCL5: neutrophil-activating peptide ENA-78; CXCL6: GCP-2; CCL2: monocyte chemotactic protein 1; CCL20: MIP-3α; TNFR: TNF receptor. Kit ligand or stem cell factor, annotated in growth factor activity, appeared in cluster 4 under biological process, subcategory organogenesis. Since its signal increase was below 2^2-fold, stem cell factor is not listed. In brackets: log2 mean expression values±SEM determined by quantitative real-time RT-PCR of one representative experiment in triplicate.

factors tested, i.e. GCP-2, IL-8, ENA-78, CCL2, CCL 20 and FGF-18, did not induce any significant cell expansion. IL-8 significantly inhibited an SCF-dependent proliferation, which stands in line with a previous report [23].

OPG increased the number of $CD34^+$ cells at the lowest concentration of 1 ng/mL (2.9±1.2 versus 0.96±0.13, $p = 0.002$) and seemed to support an SCF-based increase. Without SCF, 12.7±2.3% of the expanded cells were positive for CD34 and negative for CD45. After 3 wk in culture, less than 1.5% of the cells expressed the CD34 antigen.

Gro 3 at all concentrations (1, 10 and 100 ng/mL) resulted in more HPCs than medium alone (2.6±1.1 versus 0.96±0.13, $p = 0.047$). With Gro 3, the highest number of $CD34^+45^-$ cells were determined after 1 wk in culture (21.3±7.8%). After 3 wk in culture, this value decreased to 5.3±1.5%. In combination with SCF, Gro 3 did not enhance hematopoietic cell expansion (43.1±7 in SCF alone versus 31.4±4.4 in SCF plus 100 ng/mL Gro 3; $p = 0.4$). The highest cumulative cell counts were seen after culture with IL-32 compared with all other tested factors (8.2±2.4 at 10 ng/mL, $p = 0.014$).

IL-32 promotes HPC and myeloid progenitor expansion

When we looked closer into IL-32, the cultured cells also maintained a stem cell-like morphology with a round nucleus and minimal cytoplasm (Fig. 3A). At 1 and 100 ng/mL of IL-32, no differences compared with cells in medium alone were detected, whereas significant cell expansion at 10 ng/mL were determined starting from the first week (Fig. 3B). This was inhibited by monoclonal antibodies against IL-32, which reduced the IL-32 expansion rate by one-third (Fig. 3C). In combination with SCF, however, IL-32 inhibited an SCF-induced cell expansion in a dose-dependent manner (Fig. 3D).

After 4 wk, three to five times more $CD34^+$ cells were present in those cultures using IL-32 than in control samples ($p<0.018$, Table 2). These differences were in part accompanied by a higher number of 2-wk cobblestones formed by cells cultured in IL-32 plus SCF ($p<0.015$) than those formed by cells cultured in SCF alone. The highest numbers of 5-wk cobblestones, an indicator for more primitive HPCs, were achieved in cultures supplemented with 100 ng/mL IL-32 (compared with intra-assay control $p = 0.014$).

After 2 wk in culture, the frequency of $CD34^+$ cells ranged from 5 to 39%. The IL-32 expanded cells continued to be positive for CD34 until the end of the culture period; they also increasingly expressed CD45, indicating leukocyte differentiation (Fig. 4A and B).

The cells' colony-forming capacity, especially the total number of burst-forming unit erythrocyte and the plating efficiency were significantly better than in control cultures consisting of medium only (Fig. 4C). The total numbers of colonies of cells cultured with IL-32 were equivalent to those cultured in SCF alone, while they led to a significantly higher plating efficiency (11±1.3% versus 4.9±0.43%, $p<0.001$). The other potential growth factors

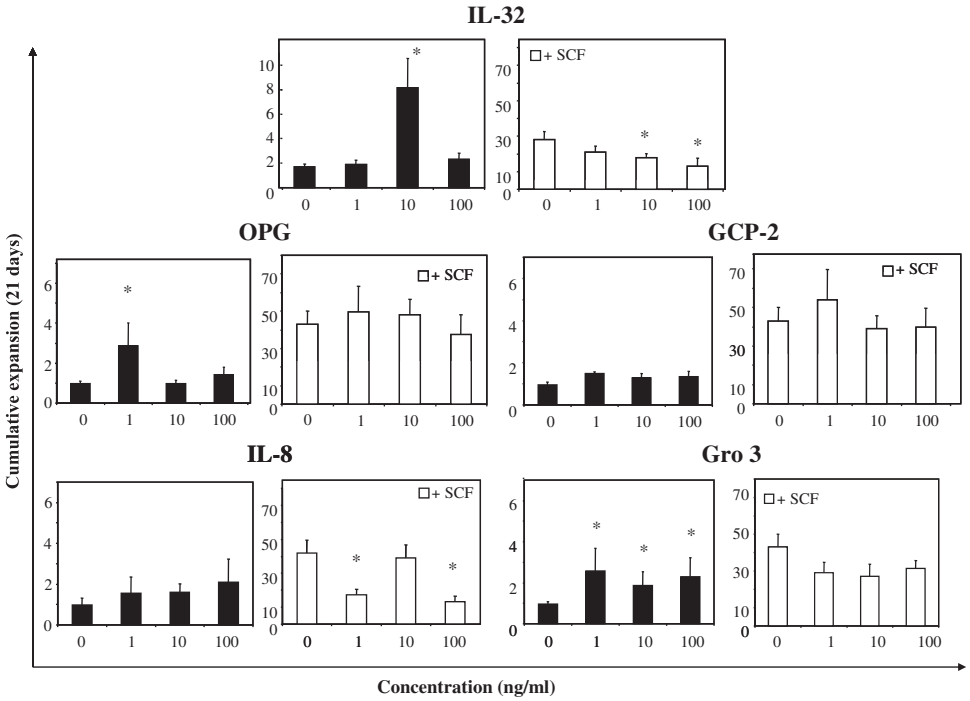

Figure 2. Identification of hematopoietic growth factors expressed by IL-1β-stimulated ECs. $CD34^+$ cells (4×10^4) isolated from cord blood were cultured in potential hematopoietic growth factors alone (black columns) or in combination with SCF (white columns) at concentrations of 1, 10 and 100 ng/mL. Every wk, 4×10^4 were replated anew in 100 μL of fresh medium supplemented with the tested component ± SCF. Shown are the means of cumulative expansion rates after 21 days + SEM from eight independent experiments. *$p<0.05$ by Student's t-test for paired samples.

we tested led to significantly fewer numbers of colonies than SCF (Supporting Information Fig.).

IL-32 attenuates BM cytotoxicities in a murine chemotherapy model

Injections of 5-fluorouracil (FU) produce profound myelosuppression in Balb/c mice within 7 days, and regeneration usually begins around day 10 [24]. In our study, myelosuppression was attenuated when human recombinant IL-32 was applied after 5-FU treatment. Both white blood cell (WBC) and platelet counts were significantly higher in mice treated with IL-32 on day 7 (Fig. 5A and B). On day 4, WBC counts were 30% higher, if 5 μg IL-32 had been administered ($97.5 \pm 15 \times 10^8$/L versus normal saline $68.6 \pm 5.5 \times 10^8$/L, $p<0.03$). On day 7, the difference was even more prominent ($53 \pm 6.6 \times 10^8$/L versus normal saline $33.6 \pm 3.1 \times 10^8$/L, $p = 0.011$), which paralleled significantly higher monocyte counts ($191.2 \pm 41.8 \times 10^6$ versus normal saline $34.5 \pm 10.1 \times 10^6$, $p = 0.002$). On this day, platelet counts of mice treated with 5 μg IL-32 were also significantly higher than in the control group ($169.4 \pm 11 \times 10^9$/L versus normal saline $130.2 \pm 10.3 \times 10^9$/L, $p = 0.013$), and they were surpassed by platelet counts in mice, which had received the high dosage of 50 μg IL-32 ($216.9 \pm 22.4 \times 10^9$/L, $p = 0.038$). Though the number of thrombocytes seemed to be higher in IL-32 treated mice on days 10 and 14, differences discontinued to be significant ($p > 0.1$). On day 14, twice the number of granulocytes was present in mice treated with 50 μg IL-32 compared with the normal saline group ($1315.6 \pm 344 \times 10^6$ versus $670.3 \pm 290.8 \times 10^6$, $p = 0.04$). No differences between the three different treatment groups were found in the hemoglobin contents, hematocrits, lymphocyte and red blood cell counts.

In the BMs we detected significantly higher numbers of $c\text{-}kit^+$, $SCA\text{-}1^+$ and $CD45^+$cells (Fig. 5C) of IL-32-treated mice than in placebo controls on day 10 after 5-FU injection. This paralleled a higher marrow cellularity in bone sections (Fig. 5C) with twice the number of cells in 5 μg IL-32-treated mice (Table 3, $p = 0.046$) and three times the numbers of colony-forming cells ($p = 3.3 \times 10^{-5}$). The higher number of BM cells paralleled a higher frequency of $SCA\text{-}1^+c\text{-}kit^+$ cells, which was comparable with non-treated controls (Table 3). Mice that had received 50 μg IL-32 had twice the BM cell count of untreated specimens on day 14 ($64.4 \pm 10.9 \times 10^5$ cells versus normal saline $32 \pm 8.2 \times 10^5$ cells, $p = 0.024$), whereas the values of those treated with 5 μg were between those of the normal saline and the 50 μg IL-32 groups ($46.9 \pm 8.3 \times 10^5$). Two weeks after 5-FU and IL-32 treatment, the number of total colonies rose to $3.8 \pm 1.2 \times 10^3$ in the normal saline-treated control; that was still surpassed by the results in 5 μg IL-32-treated mice ($9.5 \pm 1.6 \times 10^3$, $p = 0.006$) and in 50 μg IL-32-treated mice ($6.4 \pm 0.87 \times 10^3$).

Discussion

As we demonstrated, endothelial gene signals of several cytokines were significantly upregulated upon stimulation with IL-1β for

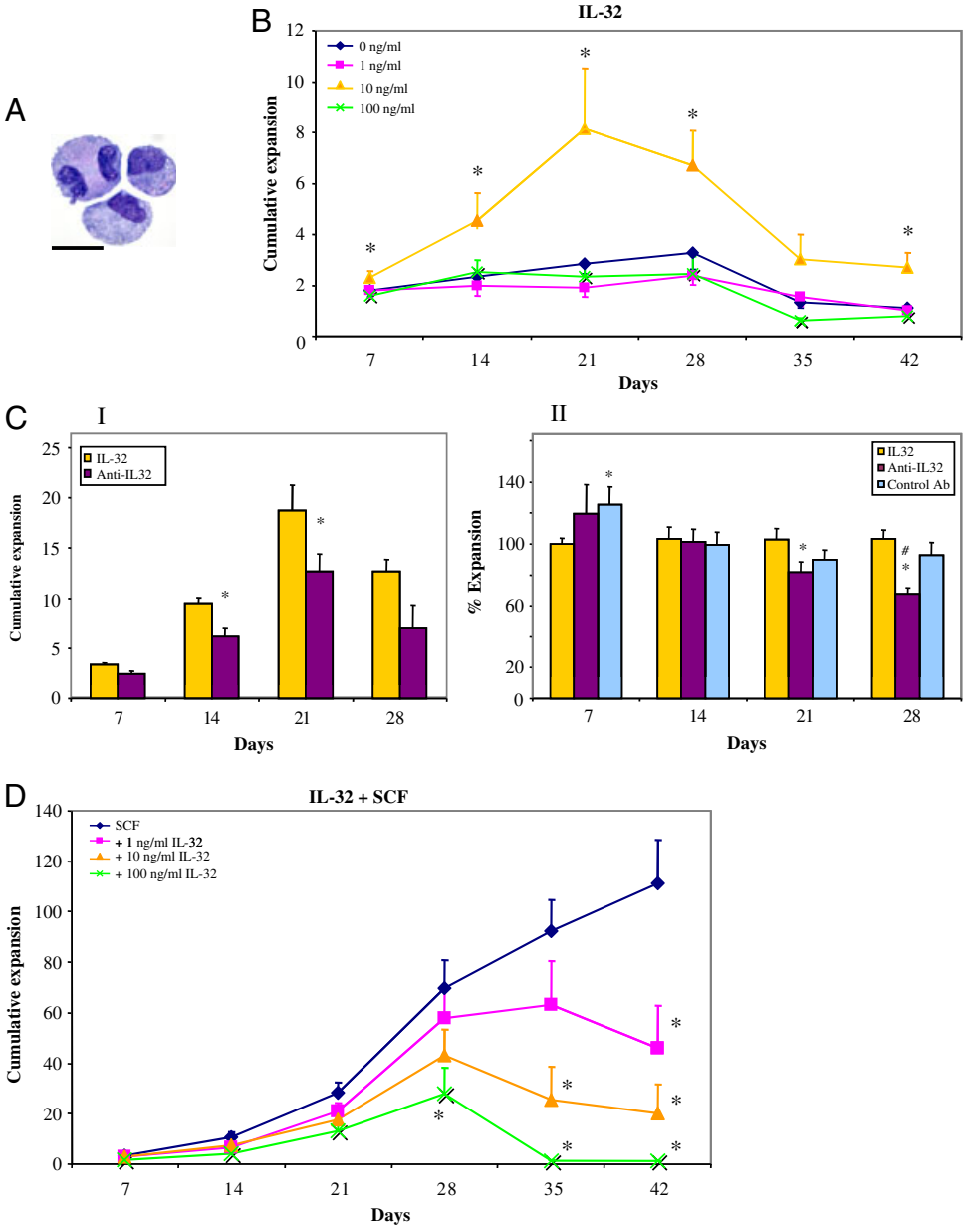

Figure 3. IL-32 induces expansion of isolated $CD34^+$ HPC cells. (A) After 2 wk in culture with IL-32, cell morphology was assessed by cytospin and light microscopy. Size bar: 1 μm, 400× magnification. (B) HPCs were cultured without or with IL-32 at 1, 10 and 100 ng/mL for the times indicated and cell expansion analyzed by manual counts. (C) (I) For inhibition experiments ($n = 4$), anti-IL-32 (1 μg/mL) was added in combination with IL-32 (10 ng/mL) to the HPC cultures and cumulative expansion rates were determined on a weekly basis. (II) In a new set of experiments ($n = 3$), commercially available IL-32, anti-IL-32 plus IL-32 or control antibodies plus IL-32 were added. Expansion rates were determined by manual counts and compared with expansion rates with IL-32 alone. (D) HPCs cultured in a combination of IL-32 (1, 10 and 100 ng/mL) and SCFs (20 ng/mL) were counted and replated on a weekly basis. Here, IL-32 significantly inhibited the SCF-induced expansion. Mean results of at least eight independent experiments+SEM are shown, if not indicated otherwise. *: significant differences to controls without IL-32 and between IL-32 and anti-IL-32 or between control antibody (Control Ab) and IL-32 ($p<0.033$). ♯: significant difference between anti-IL-32 and Control Ab ($p<0.047$) as tested by paired Student's *t*-test.

4 h. These included IL-8, IL-32, FGF-18, OPG, CXCL1 to 6, CCL2 to 6 and CCL20. Using a complex experimental design, we evaluated the HPC expansion potentials of 11 gene products: FGF-18, IL-8, Gro proteins 1, 2 and 3 (also called CXCL1 to 3), OPG, IL-32, ENA-78 (also called CXCL5), GCP-2 (also called CXCL6) and the chemoattractants CCL2 and CCL20.

Although none of these are known to affect HPC expansion, some of them can induce the proliferation of other cell types. FGF-18, for example, stimulates the proliferation of hepatocellular carcinoma cells and induces hepatocellular proliferation in vivo [25]. As an inflammatory cytokine, IL-8, also called GCP-1, induces the proliferation of cancer cells [26] and ECs in an

Table 2. Frequency and number of $CD34^+$ cells and cobblestones of IL-32 expanded $CD34^+$ cells after 28 days

IL-32 (ng/mL)	%$CD34^+$	Number of $CD34^+$ cells ($\times 10^4$)	2-Wk cobblestones	5-Wk cobblestones
0 (+SCF)	12.6±0.7 (23.2±5.2)	0.48±0.11 (2.6±0.66)	1.4±0.36 (1.6±0.55)	12.8±2.6 (14.3±2.8)
1 (+SCF)	31.1±4.8* (23.7±5.3)	2.3±0.46* (2.9±0.57)	2.6±1.2 (4±0.48*)	13.7±1.5 (18.3±7.7)
10 (+SCF)	25.7±1.4* (23.6±5.3)	1.4±0.31* (5.3±0.58)	3±0.52 (2.4±0.6)	8.7±4.6 (6.2±1.4)
100 (+SCF)	26.8±1.7* (14±0.3)	1.5±0.41* (1.3±0.01)	1.7±0.55 (5±0.48*)	21±3.4* (16±2.9)

*Significant difference ($p<0.05$) to controls with or without SCF. Values in brackets: conditions plus SCF (20 ng/mL). No differences between different IL-32 concentrations were detected. Mean results±SEM of seven independent experiments in triplicate.

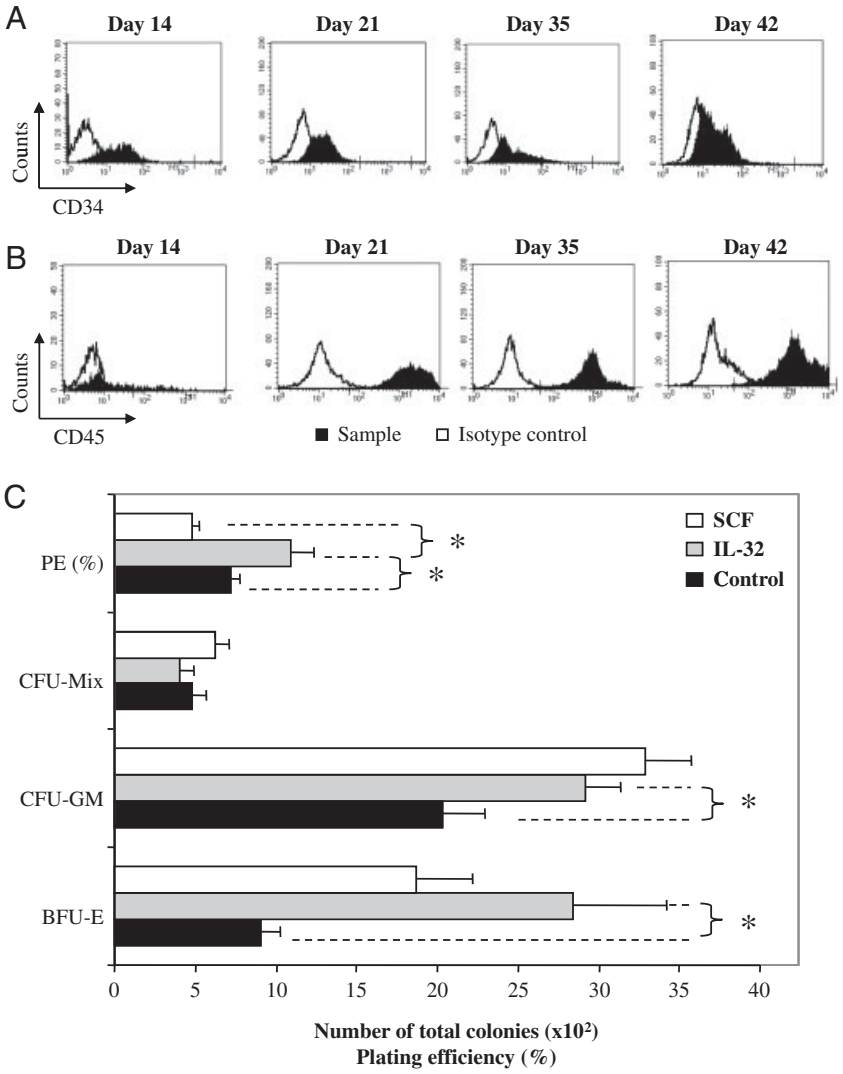

Figure 4. HPCs cultured in IL-32 remain $CD34^+$ and have a higher plating efficiency than those cultured in SCF. (A and B) Flow cytometry profile. After 14, 21, 35 and 42 days in culture with IL-32, HPC cells were analyzed for the presence of (A) CD34 and (B) CD45 on a dual platform. Representative results of one out of eight independent experiments. (C) Colony formation. Cells cultured for 1 wk in IL-32, SCF or medium alone were plated in methylcellulose using a four-cytokine combination and scored for their colony-forming capacities after 2 wk. Mean results+SEM of six (IL-32) and ten (SCF and control) independent experiments in triplicate; *$p<0.05$ by Student's *t*-test for paired and non-paired samples. BFU-E: burst-forming unit erythrocyte; CFU: colony forming unit Mix: mixed; PE: plating efficiency.

autocrine fashion [27]. Other granulocytic chemoattractants like ENA-78 and GCP-2 induce hepatocellular [28] and carcinoma cell [29] proliferation. IL-32, another proinflammatory cytokine, is produced by natural killer cells upon stimulation with IL-2. IL-32 can induce the differentiation of monocytes into macrophages, but reverses GM-CSF-induced macrophage differentiation [30].

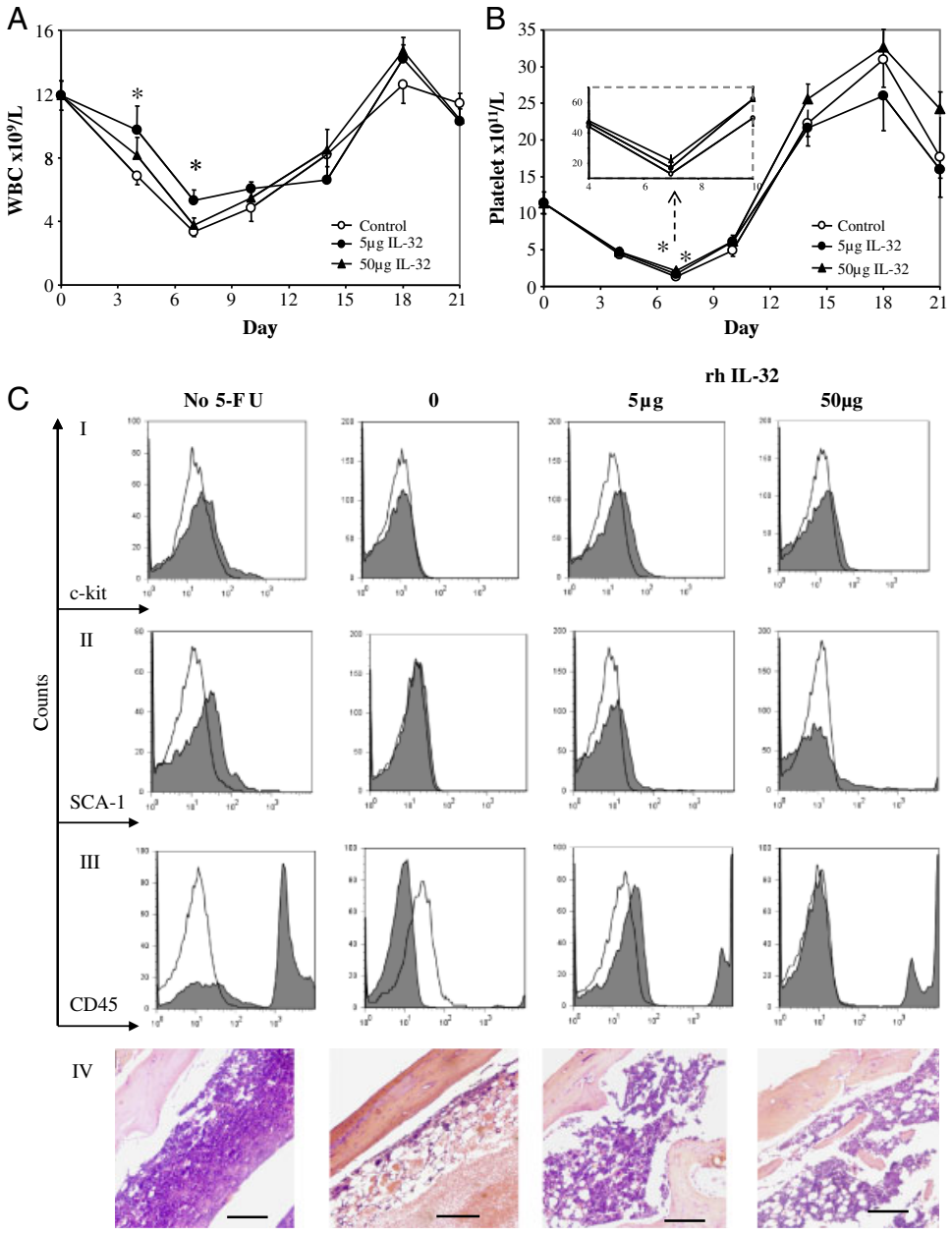

Figure 5. Recombinant human IL-32 attenuates chemotherapy-induced BM toxicity. (A) Peripheral WBC and (B) platelet counts were determined from venipunctures in 5-FU-treated mice twice a wk after injection of rhIL-32 (5 or 50 µg/kg) or normal saline (control). Shown are mean results ± SEM of five independent experiments using four and six mice per group ($n = 18$). The arrow and additional diagram within (B) highlight the significant differences ($p = 0.011$ and 0.013, paired Student's *t*-test) between the IL-32-treated mice and the control group. (C) Flushed BM of IL-32-treated (5 µg and 50 µg/kg), saline-treated (0) and healthy control (no 5-FU) mice were analyzed by flow cytometry for the presence of (I) c-kit$^+$, (II) SCA$^+$ and (III) CD45$^+$ cells white histograms: Ig G control. (IV) The opposite femurs were microscopically assessed in HE-stained bone sections. Magnification × 20, Size bar: 100 µm. Shown is one representative result out of five independent experiments.

To our knowledge, this is the first time that the hematopoietic growth factor properties of OPG, Gro 3, and especially IL-32 are demonstrated. In previous studies, several CXC chemokines, such as IL-8, ENA-78 and MIP-2, have been tested in vitro for their BM suppressiveness. That was determined according to a reduced colony-forming capacity of cytokine-treated myeloid progenitors, in which each chemokine was added to a standard cytokine combination in colony assays [31, 32]. We chose instead to apply the candidate factors directly to isolated HPCs and assess the cultured cells' hematopoietic qualities by flow cytometry, colony and cobblestone assays. From the 11 potential growth factors we tested, OPG, Gro 3 and IL-32 induced a significant expansion of CD34$^+$ HPCs isolated from cord blood.

OPG is secreted by osteoblasts within the stem cell niche [33] and inhibits the differentiation of osteoclasts [34]. Induction of cell proliferation does not belong to its known qualities. The CXC

Table 3. BM cell count, frequency of SCA1$^+$ and c-kit$^+$ cells and number of colony-forming units in Balb/c mice treated with IL-32 or normal saline (No IL-32) after 5-FU injection on day of nadir

Condition	BM cell count ($\times 10^5$) per femur	% SCA-1	% SCA-1$^+$c-kit$^+$	% c-kit$^+$	CFC ($\times 10^3$) per femur
No 5-FU	209.5±12.5	8.4±2.7	5.8±1.5	6±0.88	21.6±2.8
No IL-32	9.7±2.3	2.2±1.2	0.32±0.17	1±0.65	0.7±0.25
5 μg IL-32	19±3.3*	7.1±1.15*♯	4.9±1.6*♯	1.9±0.79	7.4±0.87*
50 μg IL-32	14.6±2.7	2.3±1.4	1.2±0.34*	1±0.53	4.1±0.63*

*Significant difference compared with normal saline-treated mice (= No IL-32); ♯: no significant difference compared with normal mice (= No 5-FU).

chemokines have well-documented neutrophil chemotactic, angiogenic and mitogenic properties. Among these, the Gro proteins comprise a family of melanoma growth stimulatory factors. They can support tumor genesis (Gro 1, [35]), angiogenesis and malignant cell proliferation (Gro 2 and 3 [36], also termed MIP-2α and 2β). The *GRO* genes were originally isolated from transformed fibroblasts. They belong to a superfamily of genes comprising, amongst others, platelet factor 4 and IL-8 [37]. In the past, none of the Gro proteins suppressed myeloid progenitor formation or synergized with other suppressive chemokines [31]; Gro 1 and 2 instead blocked suppressive effects caused by members of the same superfamily. In our assays, Gro 3 caused a significant proliferation of CD34$^+$ cells, whereas Gro 1 and Gro 2 had no effect. Cell expansion rates of Gro 3 were only topped by those of IL-32.

IL-32 was first identified as an inducer of TNF-α [38] with an important role in inflammatory diseases [39] and viral [40, 41] and bacterial infections [42]. Our data suggest that IL-32 alone can induce the expansion of HPCs leading to a ten-fold higher cumulative cell number after 3 wk in culture and a two-fold higher cell number after 1 wk; the expanded cells retained the CD34 antigen and a stem cell-like morphology. Furthermore, their plating efficiency was 1.5 times higher than that of HPCs cultured in SCF, while the total numbers of CFU-GM colonies were equal in both groups.

The presence of IL-32 in vascular ECs was confirmed recently [43, 44], though controversial opinions exist as to whether it is a secreted protein or not [45, 46]. We, too, share the opinion that IL-32 might not be secreted or produced to detectable levels by naive ECs, as the signal intensities in our microarray analysis and mRNA in non-stimulated ECs were rather low. Upon treatment with IL-1β, however, IL-32 can be detected in the supernatant at unprecedented high amounts [43]. It is very unlikely that this amount should come solely from apoptotic ECs, though this has been proposed [45]. As IL-32 was found to be secreted by lymphocytes [47] and is listed within the GO category "extracellular space", stimulated ECs could secrete it as well. In synergism with the nucleotide oligomerization domains (NOD) 1 and 2, IL-32 initiates caspase 3 and induces the expression of IL-1β and IL-6 [48]. Both domains were most recently identified on BM-derived HPCs [49]. This also explains why monoclonal antibodies against IL-32 did not completely inhibit its expansive effect: the complex of IL-32/αIL-32 could still activate nucleotide oligomerization domains and promote HPC expansion. As IL-32 can do both, i.e. induce the development of macrophages from monocytes as well as prevent a GM-CSF/IL-4-dependent differentiation of dendritic cells [30], it could very well function as a hematopoietic growth factor. This dual role was also seen in our results on HPC expansion: when used alone, IL-32 led to twice the number of HPCs, whereas in combination with SCF, IL-32 significantly reduced cell expansion induced by SCF.

Apart from its in vitro effects, IL-32 also increased the number of HPCs in vivo in a model of chemotherapy-induced BM suppression, thereby alleviating BM regeneration. The fact that, as with IL-1β [50], one injection of IL-32 sufficed, speaks in favor of the activation of secondary mechanisms. Interestingly, a rodent form of IL-32 has not yet been identified [44]; the human homolog can, however, activate murine macrophages to secrete TNF-α [46]. TNF-α has a detrimental effect on HPC renewal [51]. Therefore, other bystander effects, in combination with the expansion potential of IL-32, are most likely responsible for a sustained stem cell renewal in a well-established mouse model [24].

In conclusion, the combination of unbiased microarray analyses of IL-1β-stimulated ECs with a hypothesis-driven filtering by gene annotation allowed the targeted identification of cytokines with previously unknown hematopoietic growth factor potential. The most outstanding discovery was that IL-32 induced the expansion of functional HPCs in vitro and in vivo, thus attenuating chemotherapy-related BM cytotoxicity; on the other hand, IL-32 reduced an SCF-dependent cell expansion. Future in vitro and in vivo studies will help to further define the role of IL-32 within hematopoiesis.

Materials and methods

Cord blood and HPC selection

Cord blood specimens were collected from full-term deliveries, after informed consent was obtained from the mothers, and HPCs were immunomagnetically isolated as previously described [52]. This study was approved by the ethical review board of the Charité.

Culturing of ECs, RNA isolation

Human umbilical cord ECs were harvested and cultured as described previously [3]. Confluent ECs of passages two to four were stimulated with IL-1β for 4, 8 and 16 h, and cells were harvested by collagenase (0.1% in PBS). $CD34^+$ HPCs were used post isolation. Cell pellets were dissolved in RNA lysis buffer (Qiagen, Hilden, Germany) supplemented with β-mercaptoethanol (10 μg/mL) and stored at −80°C.

Lysed cells were mixed with 0.2 mL of chloroform for 3 min at room temperature and then centrifuged at 11 500 rpm for 15 min at 4°C. The upper aqueous phase was collected in RNAse-free Eppendorf tubes and mixed with 0.5 mL isopropanol for 10 min. Supernatants were aspirated after recentrifugation, pellets were resuspended in 75% ethanol in DEPC-H_20 air-dried and the RNA quantity was measured by spectrophotometry.

Samples were run through an RNeasy column (Qiagen) and precipitated with ethanol. Total RNA was analyzed by Affymetrix 133 plus 2.0 arrays (Affymetrix, Santa Clara, CA) as previously described [53].

Bioinformatic assessment

Signal intensities for probe sets were derived using Affymetrix's Microarray Suite version 5. Significance of differential expression was based on the calculation of an intensity-dependent, locally pooled error estimate and the corresponding z-scores (also called standard errors) [54], describing log2-fold changes as a number of local standard deviations. Subsequently, *p*-values were derived from the z-scores and adjusted for multiple testing using the Benjamini–Hochberg procedure [55].

To detect transient expression patterns, noise robust soft clustering was applied after excluding genes non-differentially or poorly expressed in all samples, i.e. genes with a corresponding z-score >3 in all time points [56]. Detected gene clusters were examined for enrichment of functional categories based on GO annotation. Statistical significance was assessed using Fisher's exact test and converted to the false discovery rates using the Benjamini–Hochberg procedure [55].

To obtain an optimal number of clusters, we assessed the functional enrichment of detected clusters, varying the number of clusters [57]. The cluster number was set to 9, as it maximized the total number of significantly enriched GO categories. Detailed microarray data can be accessed at the NCBI GEO database under the accession number GSE19420 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE19240).

Quantitative real-time RT PCR

Non-redundant transcripts that were consistently overexpressed ($>$2-fold, false discovery rate $<$0.01) were analyzed by quantitative RT-PCR using the iCyclerIQ real-time PCR detection system (Biorad, Hercules, CA, USA). Technical triplicate real-time PCR were performed using the optimized TaqMan assays-on-demand (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using the housekeeping gene β-actin as standard reference.

Functional analyses of potential hematopoietic growth factors secreted by IL-1β-stimulated ECs

Potential growth factors were analyzed by sequential dilution expansion (delta assays) for three and 4 wk as previously described [58] with minor modifications. In brief, 2×10^4 $CD34^+$ cells isolated from cord blood were cultured in 200 μL of stem cell medium [4]. Potential growth factors (all R&D, www.rndsystems.com) were added at 1, 10 or 100 ng/mL concentrations, alone or in combination with 20 ng/mL stem cell factor (Peprotech, www.peprotech.com). IL-32 and anti-IL-32 were kindly provided by Charles Dinarello. Human IL-32 used in half of the animal experiments was purchased from Abnova, Taiwan. Additional cell expansions were performed using commercially available IL-32, anti-IL-32 (AF3040, R&D), and control antibodies (goat anti-rabbit, Jackson Immuno Research, Newmarket, UK). Control expansion samples were cultured in medium only or in SCF. Cell counts were determined on a weekly basis, and expanded cells were re-cultured at the initial input concentration. The morphology of the cells was assessed after Diffquik staining. Excessive cells were analyzed for the presence of CD34 and CD45 by flow cytometry [52], for their clonogenic efficiency by methylcellulose colony assays [59] and for their BM reconstitution capacity by cobblestone assays on the murine stroma cell line MS-5 [4].

In vivo assessment of BM suppression

Female, 8-wk old Balb/c mice ($n = 54$) were treated with intravenous injections of 5-FU at a concentration of 200 mg/kg [60]. Subsequently, we administered one dose of either normal saline or recombinant human IL-32 at 5 and 50 μg/kg through one of the tail veins. Blood counts from venipunctures were determined on an automated blood cell counter (Celltec alpha, Nihon Kohden) twice a week; differentials were confirmed by manual counts of blood smears. On days 7, 10, 14 and 21, subsets of mice were killed and BMs were extracted from one femur for colony assays and flow cytometry. IgG isotype controls, anti-murine SCA-1, c-kit, CD45, CD11b and CD3-fluorescence conjugated antibodies were purchased from eBioscience (Shanghai, China). The opposite femurs were fixed in 4% paraformaldehyde, before they were decalcified by nitric acid, anhydrated in increased ethanol concentrations, incubated with xylene and embedded in paraffin. Bone sections were performed, the paraffin was melted, dried and finally removed by reverse xylene and graded ethanol concentrations. Samples were stained by hematoxylin/eosine as previously described [61]. Non-chemotherapy-treated mice served as normal controls. Bone histology specimens were photographed on an Olympus IX 71 microscope using a

DP70 camera and the DP-controller software, version 3.1.1.267 (both Olympus, Shanghai, China). The review committee on animal care of the Jiaotong-University Shanghai had approved animal studies.

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Abbreviations: **EC:** endothelial cell · **ENA:** epithelial cell-derived neutrophil-activating peptide · **FU:** fluorouracil · **GCP:** granulocyte chemotactic protein · **GO:** gene ontology · **HPC:** hematopoietic progenitor cell · **LIF:** leukemia inhibitory factor · **MIP:** macrophage inflammatory protein · **OPG:** osteoprotegerin · **SCF:** stem cell factor **WBC:** white blood cell

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